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A keratinocyte and integrated fibroblast culture model for studying particulate matter-induced skin lesions and therapeutic intervention of fucosterol

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ABSTRACT

Increased levels of particulate matter (PM) air pollutants in East Asia have resulted in detrimental health impacts increasing morbidity and mortality. Epidemiological studies suggest a possible relation between the cutaneous exposure of PM and increased oxidative stress and inflammation which lead to skin lesions. The present study utilizes an integrated cell culture model of keratinocytes and fibroblasts to mimic viable skin layers and investigate the possible effects of PM exposure after penetration through corneocytes. The skin perfection is upheld by homeostatic functionality of epidermal cells and the integrity of connective tissues. Exposure to xenobiotics could alter the skin cell homeostasis aggravating premature skin aging. Stimulation of HaCaT keratinocytes by PM collected from Beijing, China (CPM) increased the intracellular ROS levels triggering a cascade of events aggravating inflammatory responses and connective tissue degradation. In HDF fibroblasts, treatment with preconditioned keratinocyte culture media augmented inflammatory responses, cellular differentiation, and connective tissue degradation. Above events were marked by the increased intracellular ROS, inflammatory mediators, pro-inflammatory cytokines, matrix metalloproteinases (MMP)-1 and -2 levels, collagenase, and elastase activity. Fucosterol treatment of keratinocytes dose-dependently attenuated the detrimental effects both in keratinocytes and fibroblasts restoring the conditions near to physiological levels. Further evaluations could be advanced on developing fucosterol, in forms such as rejuvenating cosmeceuticals which could attenuate detrimental responses of CPM exposure.

1. Introduction

Recent epidemiological studies have reported that airborne particulate matter (PM) or fine dust is a causative agent of skin damage. The effects are mainly attributed to PM-induced oxidative stress and inflammation [1]. These air pollutants are a heterogeneous mixture of solid particles and chemical agents where the size, composition, and source of origin vary based on its microenvironment [2]. At present, fine dust air pollution has become a major issue in East Asian countries such as China, Korea, and Japan. These emissions are due to both environment factors (sand storms from Mongolian deserts) and anthropogenic activities (industrial and traffic emissions). Beijing, the capital of China is at the center contributing to aggravate these issues due to the large industrial zones [3]. Sometimes the level of particulate matter in Beijing ($PM_{2.5}$) have reported soaring up to 500 $\mu g\, m^{-3}$ which is 20 times higher than the WHO safety limits (25 $\mu g\, m^{-3}$ for $PM_{2.5}$) [4]. Though vigorous actions have been implemented to reduce anthropogenic emissions, the outbreak of various PM associated disease conditions has raised alarms to investigate effective drugs to counter these conditions.

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PM exposure affects the exacerbation of skin diseases via increasing the cellular oxidative stress which in turn aggravate inflammatory responses and increases MMPs resulting in connective tissue degradation [1]. PM-induced chronic inflammatory skin diseases include acne, atopic dermatitis, and psoriasis whereas the degradation of connective tissues causes skin wrinkling. Adverse effects include skin cancer caused by carcinogenic agents such as nitrogen dioxide, sulfur dioxide, carbon black, polycyclic aromatic hydrocarbons (PAHs) and other irritants. It is known that the mitogen-activated protein kinase (MAPK) signaling pathway is activated by PM-induced ROS. This is marked by the activation (phosphorylation) of c-Jun N-terminal kinases (JNK), extracellular signal-regulated kinases (ERK)1/2, and p38 MAPK. Activated MAPK could induce transcription factors, such as AP-1 and nuclear factor kappa B (NF-κB). Inflammatory cytokines and matrix metalloproteinases (MMPs) are synthesized as a result of their nuclear translocation. Cytokines such as interleukin (IL)-1α, IL-6, IL-8, and tumor necrosis factor (TNF)-α closely relate with inflammatory skin conditions and skin aging. Degradation of connective tissues proceeds via the action of MMPs. The degradation of collagen initiates via collagenase (MMP-1) and are further degraded by stromelysins (MMP-3) and gelatinases (MMP-2 and MMP-9), resulting in skin aging. Moreover, ROS impairs the skin durability aggravating the skin aging process [1].

Marine-derived natural products have recently acquired attention as sources of bioactive metabolites with diverse bioactive properties. Fucosterol is one of the most prominent sterols presents in brown algae. The recent review by Ahmed, Joo [5] describes the health beneficial functionalities of fucosterol such as antioxidant, anti-inflammatory, anticancer, antifungal, hepatoprotective, antidiabetic, antihyperlipidemic, antiadipogenic, and blood cholesterol-reducing, activities. Present study objectives were to investigate the effects of CPM on exacerbation of skin aging conditions such as oxidative stress, inflammation, and connective tissue degradation. The keratinocyte–fibroblast integrated culture system presented here closely mimic the cutaneous tissue. The idea being that keratinocytes which make up the external layer of the skin are susceptible to CPM exposure. The exposed cells may produce signaling molecules which would detrimentally affect cells in its microenvironment. Fucosterol which indicated protective effects against CPM exposure during the initial screening was further subjected to investigations.

2. Materials and methods

Urban aerosols (CRM No. 28) were purchased from the National Institute for Environmental Studies, Ibaraki, Japan. Acridine orange, ethidium bromide, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and 2′ 7′-dichlorodihydrofluorescein diacetate (DCFH2-DA) were purchased from Sigma-Aldrich Corp., St. Louis, MO, USA. HaCaT keratinocytes and human dermal fibroblasts (HDF) were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). Dulbecco's Modified Eagle's Medium (DMEM), Ham's F12 nutrient mixture, fetal bovine serum (FBS), and antibiotics (penicillin and streptomycin) were purchased from Thermo Fisher Scientific, Waltham, MA, USA. Antibodies for western blot analysis were purchased from Santa Cruz Biotechnology, Dallas, TX, USA. Cytokine kits were purchased from R&D Systems (Minneapolis, MN, USA), Thermo Fisher Scientific (Waltham, MA, USA), Invitrogen (Carlsbad, CA, USA), and Becton Dickinson & Co. (Franklin Lakes, NJ, USA).

2.1. Characterization of CPM

Details regarding the analysis of composition and size of CPM is reported by Mori, Sun [6]. The scanning electron microscope image of CPM was taken using a JSM-6700F field-emission microscope (JEOL, Tokyo, Japan).

2.2. Purification of fucosterol from Sargassum binderi

The purification and structure characterization of fucosterol is described in our previous study [7]. Briefly, *S. binderi* was collected from the Hikkaduwa area of Sri Lanka during January 2018. The sample powder was extracted with 70% ethanol and the crude extract was obtained by evaporating off ethanol. The crude extract was suspended in water and fractionated into hexane. The hexane fraction was further purified by two consecutive silica open columns and by preparative thin-layer chromatography obtaining fucosterol. The structure of fucosterol was characterized by GC–MS/MS (Shimadzu GCMS-TQ8040 system Kyoto, Japan) [8], and NMR (JNM-ECX400, JEOL, Japan) analysis [9].

2.3. Evaluation of inhibitory effects of fucosterol on elastase and collagenase

Elastase from porcine pancreas and collagenase from *Clostridium histolyticum* were used for the assays. *N*-succinyl-Ala-Ala-Ala-p-nitroanilide and azo dye-impregnated collagen were respectively used as enzyme substrates. The assays were carried out according to the method described by Wang, Lee [10]. Briefly, the mixture containing the specific substrate and different concentrations of fucosterol were incubated with the respective enzymes for a defined period at their optimum temperature. The difference in absorbance measured at the start and after the incubation period was used to calculate the percentage inhibition using the following equation. All data values were compared with the positive control, epigallocatechin gallate (EGCG).

$$\text{Percentage inhibition} = \left\{1 - \left(\frac{\left(\begin{matrix}\text{Sample}\\ \text{absorbance}\end{matrix} - \begin{matrix}\text{Average absorbance}\\ \text{of blank}\end{matrix}\right)}{\left(\begin{matrix}\text{Average absorbance}\\ \text{of control}\end{matrix} - \begin{matrix}\text{Average absorbance}\\ \text{of blank}\end{matrix}\right)}\right)\right\} \times 100$$

2.4. Cell lines

HDF (human dermal fibroblasts) and HaCaT (human keratinocyte) cells were purchased from ATCC (ATCC, Rockville, MD, USA). The cells were maintained in DMEM medium supplemented with 10% FBS, and 1% penicillin, and streptomycin mixture in a humidified atmosphere at 37 °C with 5% CO_2. For HDF cells Ham's F12 nutrient mixture was incorporated to the above DMEM culture media with a ratio of 1/3. The cells were periodically sub-cultured and cells under exponential growth were used for experiments [10].

2.5. The culture of HaCaT and HDF cells

Given culture method (Fig. 1) is a modification of our previous method [7] optimized following dose (CPM and fucosterol) and time response studies. For the treatment fucosterol samples were prepared by dissolving in DMSO (80 mg mL^{-1}), emulsified and diluted using PBS. CPM was prepared by suspending in DMEM medium. Briefly, HaCaT cells were seeded at 1×10^5 cells mL^{-1} concentration and incubated for 24 h. The cells were then treated with different concentrations of fucosterol. Following 1 h, the cells were induced with CPM (125 μg mL^{-1}) and incubated for 30 min. The cells were washed twice with new culture media to remove CPM and replaced by new media. After 24 h of the incubation period, the cell media was collected filtered and stored at −80 °C until further use. Meanwhile, HDF cells were seeded at 1×10^5 cells mL^{-1} 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. Next, the wells were emptied and washed twice with new culture media to

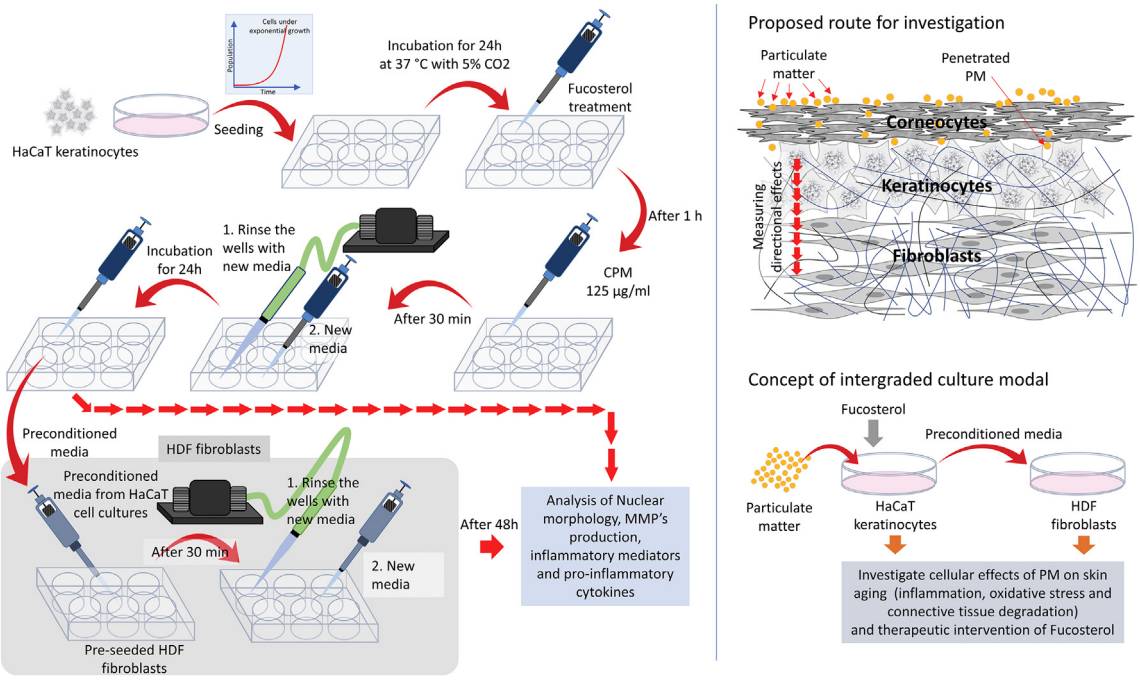

Fig. 1. Molded culture system of keratinocytes and fibroblasts. Keratinocytes were preconditioned by stimulation with CPM with and without fucosterol. Keratinocyte preconditioned media was used to stimulate fibroblasts. Culture media and cells were retrieved after designated incubation periods to analyze the levels of molecular mediators.

remove the remaining preconditioned media and replaced with new culture media. Here the idea is 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.

2.6. Cell viability and ROS

The viability of the cells was evaluated by MTT colorimetric assay and the intracellular ROS levels were evaluated by DCFH-DA assay. The methods were adopted from Fernando, Sanjeewa [11]. Briefly, after the 24 h incubation period following the sample treatment, MTT was added to the wells and incubated for 3 h. After the formazan crystals were dissolved in DMSO and the absorbance was measured at 540 nm. For measuring intracellular ROS, DCFH-DA was added to the wells after the 2 h incubation period following treatment. After 10 min of incubation, the fluorescence was measured at 485 nm of excitation and 530 nm of emission.

2.7. Evaluation of nuclear morphology

Treated and CPM-stimulated cells after the designated post-incubation period were supplemented with a mixture of 10 µg mL^{-1} of acridine orange and ethidium bromide stain. After an incubation period of 10 min, the fluorescence images were taken using a fluorescence microscope equipped with a CoolSNAPPro digital camera [8].

2.8. Western blot analysis

Western blot analysis was carried out to evaluate the levels of some key molecular mediators. The analysis was carried out according to the method described by Fernando, Jayawardena [12]. To analyze the levels of cyclooxygenase (COX-2), the cells were harvested following a 24 h incubation period after the treatment. However, for the analysis of upstream molecular mediators of mitogen-activated protein kinases (MAPK) and the nuclear factor-kappaB (NF-κB) pathways, the cells were harvested 25 min after the stimulation.

2.9. Analysis of cellular collagenase and elastase activity and MMP levels

Harvested cells from each treatment group were washed with PBS, lysed and centrifuged at 40,000 ×*g* for 20 min at 4 °C. The supernatants after quantification and standardizing their protein levels were used as the keratinocyte and fibroblast enzyme solutions [13]. The elastase and collagenase activities were measured using the synthetic substrates azo dye-impregnated collagen and N-succinyl-Ala-Ala-Ala-p-nitroanilide respectivelly [10]. The collected culture media from each treatment group were analyzed for Prostaglandin E2 (PGE_2) inflammatory cytokines (tumor necrosis factor (TNF)-α, Interleukin (IL)-1β, and IL-6) and MMPs. Experiments were carried out using commercial ELISA kits following the manufacturer's instructions. Fold induction was calculated using the following equation.

$$\text{Fold} = \frac{(\text{Sample absorbance} - \text{Average absorbance of blank})}{(\text{Average absorbance of control} - \text{Average absorbance of blank})}$$

2.10. Statistical analysis

Data values are expressed as means ± SD based triplicate determinants. Significant differences between data were obtained with one-way ANOVA using Duncan's multiple range test at $P < 0.05$ "*" and $P < 0.01$ "**". Analyses were carried out using IBM SPSS Statistics v. 20.

3. Results

3.1. Size and composition of CPM

Size and chemical composition of CPM is essential for understanding its health effects. The size and composition details of CPM are provided in our previous publication [7]. Accordingly, a higher proportion of CPM was having a diameter of < 5 µm. The composition indicated the presence of toxic metallic elements such as Pb, As and Cd and also polycyclic aromatic hydrocarbons (PAHs).

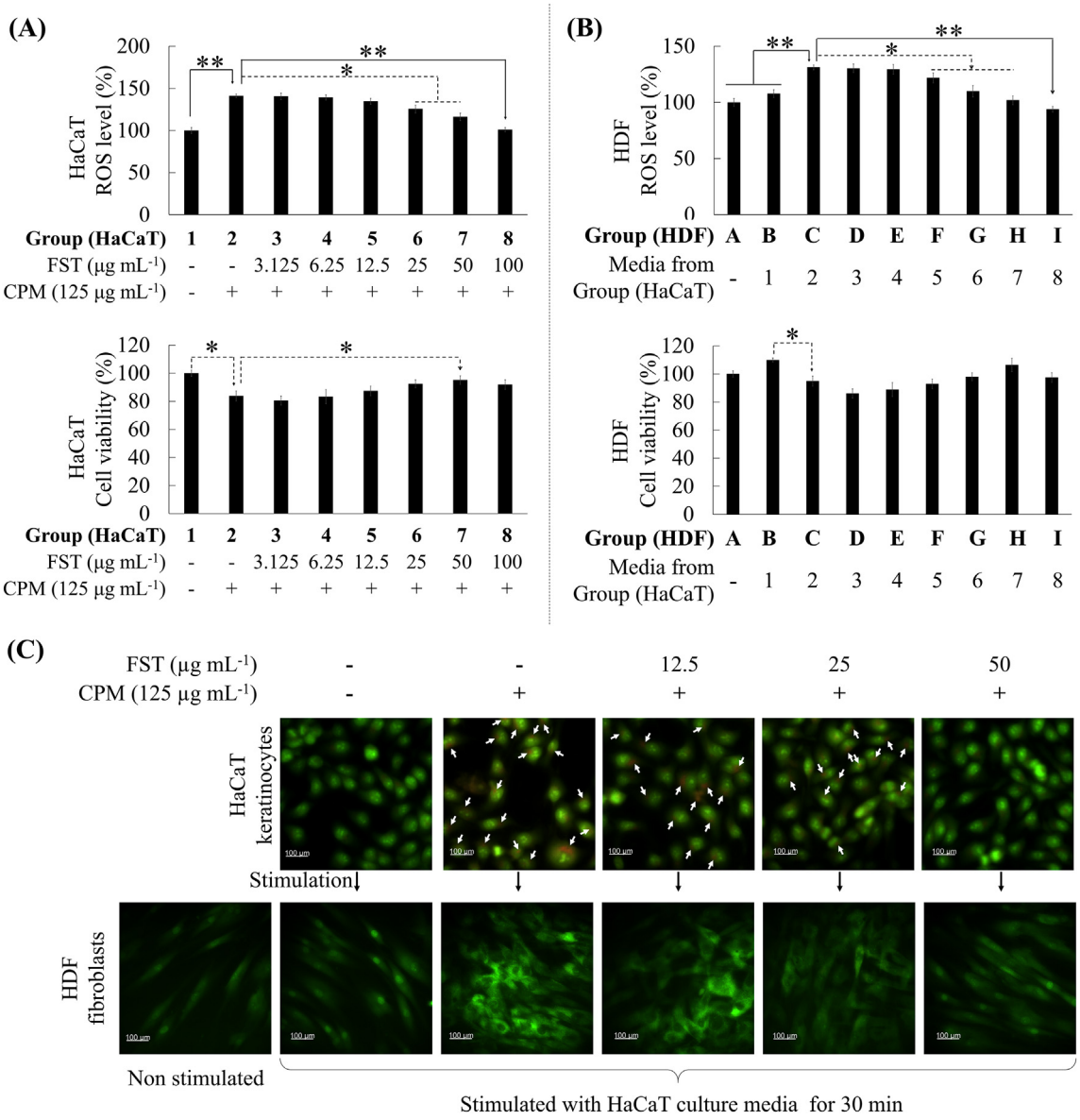

Fig. 2. Effect of fucosterol (FST) on oxidative stress and apoptosis in CPM-stimulated HaCaT cells and preconditioned HaCaT cell media treated HDF cells. (A) Analysis of the effect of fucosterol on CPM-induced intracellular ROS levels and cell viability in HaCaT cells. (B) Effect of preconditioned HaCaT cell media on intracellular ROS levels and cell viability in HDF cells. (C) Evaluation of nuclear morphology of HaCaT cells and preconditioned HaCaT cell media treated HDF cells (arrowheads indicate late apoptosis). Reproducibility of results was confirmed by three independent tests. Graphical illustrations are means ± SD based on three independent determinations (n = 3). *P < 0.05 and **P < 0.01 indicate values which are significantly different from those of the CPM-treated group in each separate experiment.

3.2. Protective effects of fucosterol against CPM-induced ROS production and apoptotic body formation

Though ROS are crucial mediators of cell signaling pathways, excessive production and uncontrolled regulation of ROS lead to detrimental effects. As Fig. 2(A) illustrates, the intracellular ROS levels in HaCaT cells (treatment group 2) went up with CPM stimulation while decreasing the cell viability. Following the dose-dependent treatment of fucosterol, the ROS levels were declined while the cell viability was increased. As Fig. 2(B) indicates, integration of HaCaT cell (non-stimulated) preconditioned media to HDF cultures, augmented the HDF cell viability. Treatment of CPM-stimulated HaCaT preconditioned media (CPMHM) increased the intracellular ROS levels in HDF cells while decreasing the cell viability. Preconditioned media from fucosterol treated CPM-stimulated HaCaT cells (F-CPMHM) decreased the intracellular ROS levels while increasing the HDF cell viability. As assessed via nuclear double staining (Fig. 2(C)), CPM increased apoptotic bodies (early and late apoptosis stages) in HaCaT keratinocytes indicated by fragmented orange and green color nuclei compared to the green nuclei in the control that represent viable cells. Fucosterol dose-dependently reduced the appearance of apoptotic bodies (fragmented orange and green nuclei) in CPM treated HaCaT cells. Compared to the non-stimulated HDF cells, an increase in cell density was observed when preconditioned HaCaT cell media was integrated with HDF cells. A distinct change in the HDF cell morphology was observed when the cells are being treated with the CPMHM. The HDF cell morphology recovered back to normal following the dose-dependent increase in fucosterol treatment at HaCaT cells.

3.3. Protective effects of fucosterol against CPM-induced MMP production

Collagenases and elastases are two groups of proteases that degrade

connective tissues. Downregulating their levels in keratinocytes and fibroblasts is an effective strategy of reducing skin wrinkling. As indicated in Fig. 3(A), fucosterol dose-dependently inhibited collagenase (from *Clostridium histolyticum*) and elastase (from porcine pancreas) compared to the 0% inhibition when treated with the substrate only. However, the inhibitory effects of fucosterol were not stronger compared to the inhibitory effects of the positive control epigallocatechin-3-gallate (EGCG). Next, the activities were evaluated using cell lysates of the treated cells relative to the lysates of the untreated cells. CPM-stimulation increased the collagenase and elastase activities in HaCaT keratinocytes (Fig. 3(B)) whereas the activities were decreased dose-dependently with fucosterol treatment. In CPMHM integrated HDF cultures (Fig. 3(C)), a prompt increase was seen for the collagenase activity followed by the increase of elastase activity (treatment group C). However, both collagenase and elastase activities were comparatively low in HCF cultures incorporated with F-CPMHM (from treatment groups D to F). MMPs are a family of proteases capable of degrading extracellular matrix proteins [10]. As Fig. 3(D) indicates, MMP-1, and MMP-2 levels increased in HaCaT keratinocytes following CPM-stimulation (treatment group C). The levels decreased dose-dependently with fucosterol treatment. Similarly, in the HDF cells treated with CPMHM, an increase was observed for both MMP-1 and MMP-2 levels with the increase of MMP-1 five-folds higher than the untreated HDF cells (Fig. 3(E)). The MMP-1 and 2 levels decreased dose-dependently with the incorporation of F-CPMHM.

3.4. Anti-inflammatory functionalities of fucosterol in HaCaT cells

Skin inflammatory responses are known to aggravate wrinkling via connective tissue degradation [1]. Stimulation with CPM increased COX-2 and PGE_2 levels (Fig. 4(A)) and levels of pro-inflammatory cytokines including TNF-α and IL-6 (Fig. 4(B)) in HaCaT keratinocytes. The increase in TNF-α was eight folds higher compared to the control levels. The levels decreased dose-dependently with fucosterol treatment. CPM increased the nuclear translocation of NF-κB-p65 (Fig. 4(C)) and phosphorylation of p38 MAPK, Erk1/2, and JNK (Fig. 4(D)). Fucosterol treatment dose-dependently reduced NF-κB-p65 nuclear translocation and phosphorylation of p38 MAPK, Erk1/2, and JNK equalizing the levels nearly similar to the levels in the control.

3.5. Effect of preconditioned HaCaT cell culture media on HDF cell inflammatory responses

In skin tissues, signaling molecules produced by stimulated epidermal keratinocytes may affect dermal fibroblast cells in its microenvironment. Treatment of CPMHM increased the PGE_2, COX-2 levels (Fig. 5(A)), levels of pro-inflammatory cytokines including TNF-α, IL-1β and IL-6 (Fig. 5(B)) and increased the nuclear translocation of p65 and p50 (Fig. 5(C)) in HDF fibroblasts. The increase in IL-6 was 30 folds higher compared to the levels in the non-stimulated HDF fibroblasts. With the integration of preconditioned F-CPMHM, the levels of inflammatory markers expressed by HDF fibroblasts were reduced. In fact, the levels reduced dose-dependently with fucosterol treatment at the HaCaT keratinocytes. When considering the MAPK proteins, treatment of preconditioned HaCaT cell media increased the phosphorylation of p38 MAPK Erk1/2 and JNK in HDF cells (Fig. 5(D)). However, similar phosphorylation levels were observed for the p38 MAPK and Erk1/2 when HDF cells were integrated with media from HaCaT cell cultures regardless of the CPM-stimulation or fucosterol treatment. However, the JNK levels in HDF cells were fluctuated depending on the HaCaT cell media integration. Thus, the JNK phosphorylation dose-dependently decreased in HDF cells with the integration of F-CPMHM

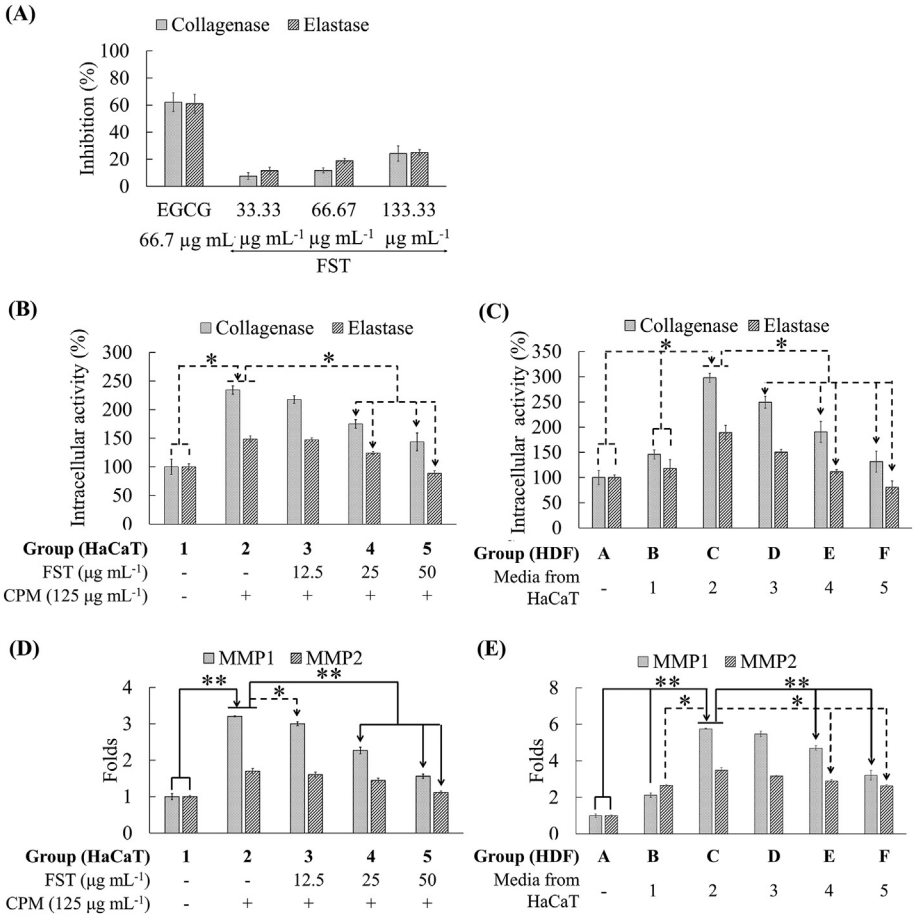

Fig. 3. Effect of fucosterol (FST) on reducing connective tissue degradation in CPM-stimulated HaCaT cells and preconditioned HaCaT cell media treated HDF cells. (A) Analysis of the inhibitory activity of fucosterol on collagenase and elastase enzymes. (B) Effect of fucosterol on intracellular collagenase and elastase activities in CPM-stimulated HaCaT cells and (C) Effect of preconditioned HaCaT cell media on intracellular collagenase and elastase activities in HDF cells. ELISA results for (D) Effect of fucosterol on MMP-1 and MMP-2 levels in CPM-stimulated HaCaT cells and (E) Effect of preconditioned HaCaT cell media on MMP-1 and MMP-2 levels in integrated HDF cells. The ratio of MMP expression in cells belonging to each treatment group with respect to control group is indicated as folds. Graphical illustrations are means ± SD based on three independent determinations (n = 3). *P < 0.05 and **P < 0.01 indicate values which are significantly different from those of the CPM-treated group in each separate experiment.

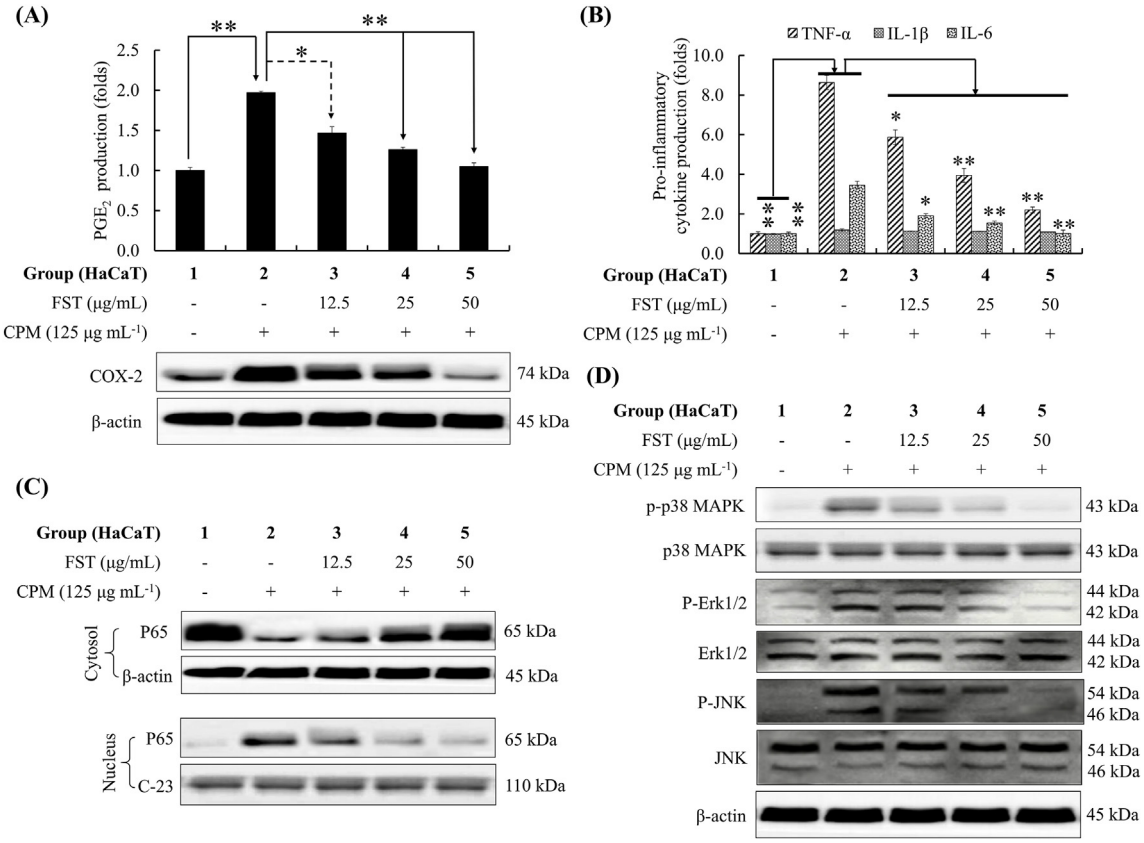

Fig. 4. Effect of fucosterol (FST) on reducing inflammatory responses in CPM-stimulated HaCaT cells and preconditioned HaCaT cell media treated HDF cells. Analysis of fucosterol treated CPM-stimulated HaCaT cells for the levels of (A) PGE_2 and COX-2, (B) pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6, (C) phosphorylation and nuclear translocation of NF-κB-p65, and (D) phosphorylation of p38 MAPK, Erk1/2, and JNK. Reproducibility of results was confirmed by three independent tests. Graphical illustrations are means ± SD based on three independent determinations (n = 3). *P < 0.05 and **P < 0.01 indicate values which are significantly different from those of the CPM-treated group in each separate experiment.

with increased fucosterol concentrations.

4. Discussions

Airborne particulate matter has been identified as a major cause of various skin diseases [1]. A detailed review regarding properties of PM which determine their health effects is presented by Harrison and Yin [14]. The effects are best described by their size and chemical composition. According to our previous publication [7] CPM is composed of particles with diameters between 1 and 14 μm with defined metal and PAH contents. Majority of the dust particles had a diameter < 5 μm. The major metallic elements in CPM are Ca, Al, Fe, Mg, K, and Na. Toxic heavy metals such as Pb, As, and Cd is found in low doses. Majority of epidemiological studies indicate that coarse particles with an aerodynamic diameter of 2.5–10 μm are responsible for various adverse effects. The effects are more prominent in fine particles (< 2.5 μm) and are more toxic than coarse particles that they penetrate skin and lungs. The chemical composition of PM varies based on their source. PAH produced by incomplete combustion or pyrolysis of organic material is a major constituent of PM which causes cytotoxic, endocrine-disrupting, and mutagenic effects. At low doses, PAH may increase oxidative stress and induce inflammation. Trace metals in PM influence tissue inflammation by contributing to the production of free radicals such as hydroxyl. The theory is that metals are redox-active and induce or catalyze chemical reactions leading to free radical production. [14,15].

The keratinocyte–fibroblast integrated culture system presented in this study closely mimic the cutaneous tissue. The idea being that keratinocytes which make up the external layer of the skin are susceptible for the exposure to xenobiotics. The exposed cells may produce signaling molecules which would affect cells in its microenvironment. Keratinocyte–fibroblast co-culture systems are frequently used to evaluate cutaneous tissue repair mechanisms [16]. These co-culture systems provide an environment that contains a synergistic mix of extracellular matrix components, growth factors, and direct cell-cell contacts. However, the integrated culture method described here is much suitable to determine the directional effects of skin exposure (keratinocytes ➔ fibroblasts) rather than the use of a co-culture method (keratinocytes + fibroblasts) which is more suitable for studying cutaneous tissue repair.

Based on current observations treatment of CPM to HaCaT keratinocytes increased the oxidative stress and reduced their viability (Fig. 2(A) treatment group 2). The effects were countered by dose-dependent treatment of fucosterol indicating cytoprotective effects. The treatment of HaCaT cell preconditioned culture media had dissimilar effects on fibroblasts growth, differentiation, oxidative stress, inflammatory responses, and MMPs production. Integration of preconditioned media from HaCaT cells increased the viability of HDF cells (Fig. 2(B) treatment group 2) compared to the control group of HDF cells (treatment group 1). This evidence is supported by the observed increase in cell density (Fig. 2(C)). It has been shown that keratinocytes could induce the synthesis of growth factors and cytokines in fibroblasts [16]. Keratinocyte-derived IL-1 has been identified as the primary inductor of growth factors and cytokines in fibroblasts. In the present results, there is no apparent fluctuation of the IL-1β levels in HaCaT cells under different treatment conditions. However, when preconditioned media from untreated HaCaT cell cultures were integrated with HDF cells the apparent increase in HDF proliferation (Fig. 2(B)

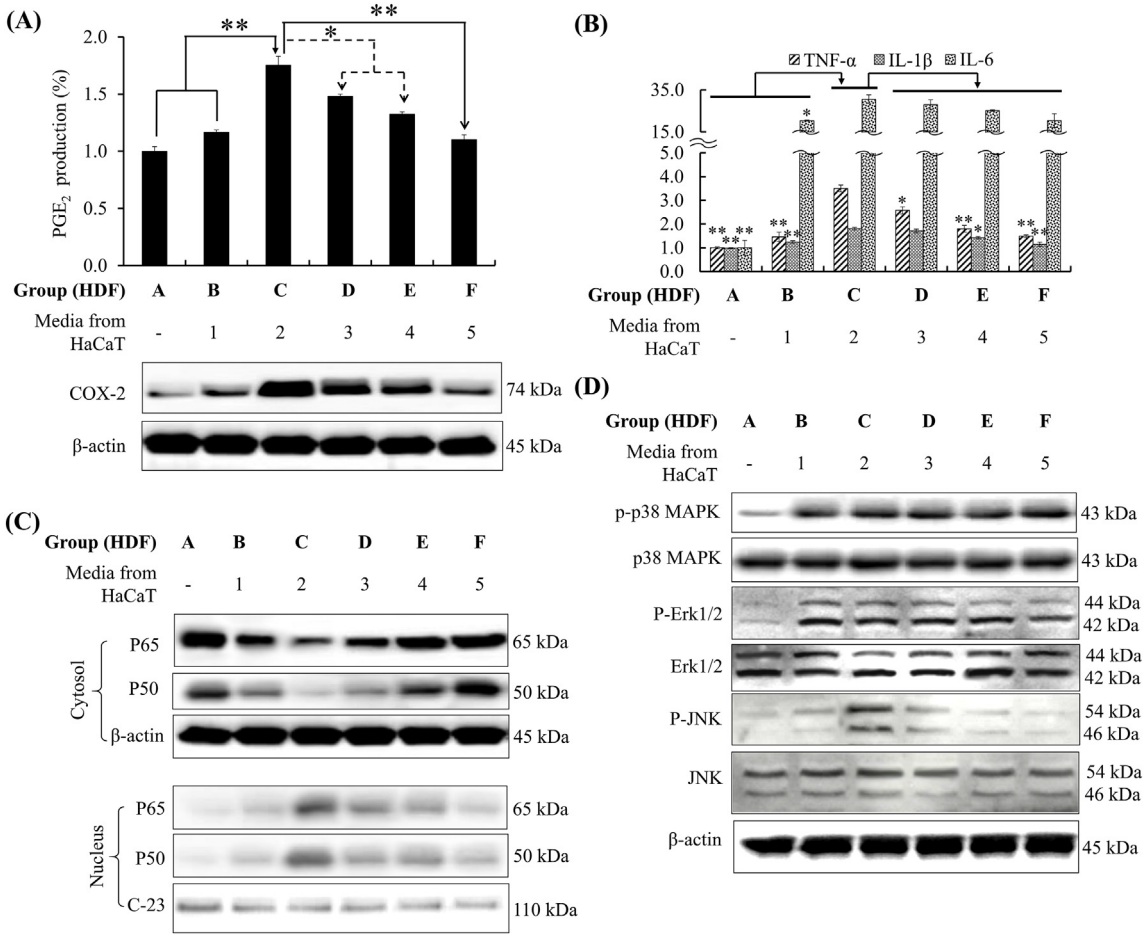

Fig. 5. Effect of fucosterol treatment in preconditioned HaCaT cell media on suppressing inflammatory responses in HDF cells. Analysis of HaCaT cell media integrated HDF cells for the levels of (A) PGE_2 and COX-2 levels, (B) pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6, (C) phosphorylation and nuclear translocation of NF-κB-p65 and p50 and (D) phosphorylation of p38 MAPK, Erk1/2, and JNK. Reproducibility of results was confirmed by three independent tests. Graphical illustrations are means ± SD based on three independent determinations (n = 3). *P < 0.05 and **P < 0.01 indicate values which are significantly different from those of the CPM-treated group in each separate experiment.

treatment group B) in comparison with the control group (treatment group A) may have resulted due to IL-1β in HaCaT cell culture media. As comprehensively described by Werner, Krieg [16], in addition to IL-1, there are examples of keratinocytes being a source of several other growth factors which stimulate the proliferation of fibroblasts and other mesenchymal cells.

Nuclear morphology analysis indicated that CPM could induce the formation of apoptotic bodies in HaCaT cells apparent from the fragmented green and orange color nuclei. However, none of the fibroblasts indicated apoptosis. Alternatively, the HDF proliferation was slightly increased with the treatment of preconditioned HaCaT cell media. As previously described by Funayama, Chodon [17], this might be due to the protective effects exerted by keratinocytes towards fibroblasts under certain stress conditions. The reduction of viability when HDF cells were treated with CPMHM (Fig. 2(B) treatment group C) recovered back to normal with the integration of media from fucosterol treated HaCaT cell cultures. However, the cell morphology abruptly altered when treated with preconditioned CPMHM. This is an indication of cellular differentiation whereas the cells become enlarged, and flattened acquiring increased volume [18]. Such differentiation can take place in fibroblasts due to TGF-β, which is an inducer of myofibroblast differentiation [19]. Endogenous IL-1 is reported to be capable of antagonizing TGF-β-mediated fibroblast differentiation. Further studies are required to evaluate the production of markers associated with fibroblast differentiation. The differentiated HDF have reported reducing the levels of some proteins such as collagen I-1α, collagen III-1α, and elastin while having increased production of enzymes that breakdown collagenase and stromelysin [18].

To study the effects of oxidative stress towards connective tissues degradation, we monitored the levels of MMP-1 and MMP-2 in both keratinocytes and fibroblasts. MMPs degrade extracellular matrix proteins including the once that make up the fibrous connective tissues. MMP inhibitors are widely used to treat inflammation, skin aging, rosacea, acne, to reduce skin discoloration, body, and oral cavity odor, to increase hair growth and for wound healing [20]. Previous studies indicate that fucosterol could inhibit the UVB radiation-induced MMPs expression in HaCaT keratinocytes [21]. CPM-stimulation of HaCaT cells increased MMP-1 and MMP-2 levels as well as intracellular collagenase and elastase activity. Fucosterol treatment dose-dependently suppressed MMP-1 and MMP-2 production and it reduced the collagenase and elastase activity in HaCaT cell lysates (Fig. 3(B) and (D)). Treatment of preconditioned CPMHM promptly increased the MMP-1, MMP-2, collagenase, and elastase activity in HDF fibroblasts (Fig. 3(E) and (C) treatment group C). The activities were reduced when treated with F- CPMHM. Hence, fucosterol proves to counter CPM-induced degradation of extracellular matrix proteins.

Evaluations were further extended to evaluate direct collagenase and elastase inhibitory effects of fucosterol. Inhibitory effects were not prominent being around 20% inhibition at 133.33 μg mL^{-1} concentration (Fig. 3(A)). These observations nevertheless confound with the steep reduction seen in intracellular collagenase and elastase activity at 50 μg mL^{-1}. Possible reasoning would be that fucosterol could suppress

the collagenase and elastase activity by suppressing early events that lead to the transcription of these enzymes.

Recent studies have revealed that inflammatory responses have a major role in inducing MMPs expression [22]. Hence expression levels of several key inflammatory molecular mediators were investigated including COX-2, PGE_2, pro-inflammatory cytokines including TNF-α, IL-1β, and IL-6 and upstream regulatory elements including MAPK and NF-κB pathway proteins which report of having a connection to MMPs expression [22,23]. Inflammatory processes are mediated via several different pathways including NF-κB, MAPK, Janus kinase, and phosphatidylinositol 3-kinases (PI3K)/Akt, signaling pathways [24]. The NF-κB family comprises p50, p52, p65, re*l*B, and c-*re*l proteins. They regulate the transcription of inflammatory mediators and pro-inflammatory cytokines. In unstimulated cells, NF-κB transcription factors including p50 and p65 remain bound to the inhibitor IκB not translocating to the nucleus. Phosphorylation of IκB breaks down the complexes IκB-p50 and IκB-p65 enabling their nuclear translocation. These proteins then bind with target genes inducing the transcription of inflammatory mediators (iNOS, COX-2, and PGE_2) and pro-inflammatory cytokines (TNF-α, IL-6, and IL-1β). Primary human keratinocytes and HaCaT cells have many features in common and certain differences [12]. HaCaT keratinocytes have been used as substitutes for primary human keratinocytes in many studies. CPM-stimulation increased the levels of inflammatory regulators COX-2, PGE_2, pro-inflammatory cytokines including TNF-α and IL-6. The TNF-α expression was prominently higher compared to the up-regulation of other inflammatory markers that it could be taken as a biomarker of CPM-induced inflammation. However, a considerable fluctuation was not observed for the levels of IL-1β. Similar results have been observed in our previous studies whereas CPM stimulation mainly affects the upregulation of TNF-α and IL-6 except for IL-1β [12]. Investigation of the upstream MAPK and NF-κB related signaling molecules revealed that CPM could increase the nuclear translocation of NF-κB p65 proteins indicated by the decreased p65 levels in the cytosol and its increased levels in the nucleus (Fig. 4(C)). CPM-stimulation also increased the phosphorylation of p38 MAPK, Erk1/2, and JNK in HaCaT cells (Fig. 4(D)). The effects were reversed with the fucosterol treatment.

Integration of preconditioned HaCaT cell medium increased the phosphorylation of ERK1/2, JNK, and p38-MAPK in HDF cells (Fig. 5(D)). These results were consistent with previous observations that the synchronous activation of ERK1/2, JNK and p38-MAPK are reported to be essential for the proliferation of dermal fibroblasts [25,26]. The observed increase in HDF cell viability (Fig. 2(B)) and cell density (Fig. 2(C)) upon the treatment of HaCaT cell preconditioned media are further supported by these arguments. Though the treatment of CPMHM increased the JNK levels compared to the JNK level in the untreated HaCaT cell media (Fig. 5(D)), no apparent change was observed in the ERK1/2 and p38-MAPK levels (based on densitometry analysis of western blot bands using ImageJ software which is not shown in the results). Hence, the activation of MMPs in HDF cells may have proceeded via the JNK phosphorylation. Further, the integration of F-CPMHM dose-dependently reduced the JNK levels restoring it lower to the level in the untreated HaCaT cell media. The previously reported evidence is consistent with these observations that JNK is a critical mediator of MMPs expression [22,27]. Further, the increase of pro-inflammatory cytokines, in HDF cells including TNF-α and IL-6 due to the treatment of CPMHM may have resulted from the activation of JNK. Further research using specific inhibitors of ERK1/2, JNK and p38-MAPK could be useful for confirming the mechanistic insights of this pathway.

5. Conclusions

Current observations are evident that CPM-induced oxidative stress and associated inflammatory conditions in keratinocytes may provoke degradation of the connective tissues of its own accord and by stimulating fibroblasts to produce elastase and MMPs including collagenase. This relatively simple model system could be applied to evaluate the effects of xenobiotics on the skin. In conclusion, fucosterol indicated promising cytoprotective effects in keratinocytes and integrated fibroblasts by reducing the inflammatory responses induced by PM which leads to skin aging. Present findings suggest that fucosterol may have beneficial effects in alleviating chronic skin aging due to exposure to air pollutions. With further studies, fucosterol or algal extracts containing fucosterol and other desirable phytochemicals could be considered as candidates for formulating skincare products.

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