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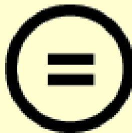

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A Thesis

For the Degree of Master of Science in Medicine

Effect of fermented fish oil against particulate matter 2.5-induced skin aging

Graduate School

Jeju National University

Department of medicine

Yu Jae Hyun

February, 2019

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초미세먼지(PM2.5)로 유도된 피부노화에 발표어유의 효과

지도교수 현 진 원

지도교수 강 희 경

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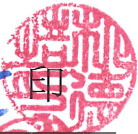

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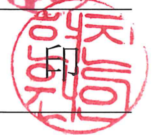

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Effect of fermented fish oil against particulate matter 2.5-induced skin aging

Yu Jae Hyun

(Supervised by professor Jin-Won Hyun and Hee-Kyoung Kang)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science in Medicine

2018. 12.

This thesis has been examined and approved.

Deokbae Park

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ABSTRACT

Skin is exposed to harmful environmental such as air pollution which including various types of particulate matters (PMs). These atmospheric PMs have harmful effect on human through increase of the reactive oxygen species (ROS). It has been reported that ROS induced skin aging via generation of matrix metalloproteinases (MMPs) which causes skin aging through degradation of collagen. This study investigated the effect of fermented fish oil (FFO), which derived from mackerel, in $PM_{2.5}$ (particulate with a dimeter of < 2.5 μm)-induced skin aging in human keratinocyte. FFO inhibited $PM_{2.5}$-induced intracellular ROS and MMPs including MMP-1, MMP-2, and MMP-9. In addition, FFO significantly abrogated the intracellular Ca^{2+} level in $PM_{2.5}$-treated cells. Furthermore, FFO blocks $PM_{2.5}$-induced MAPKs/AP-1 pathway. In conclusion, FFO has anti-aging effect on $PM_{2.5}$-induced skin aging on human keratinocyte.

Keyword: Particulate matters 2.5, Matrix metalloproteinases, Fermented fish oil, Oxidative stress, Skin aging

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1. Introduction

Since reactive oxygen species (ROS) have unpaired electrons and unstable bounds, it is able to lead cellular damage (de Jager et al. 2017; Ryu et al. 2018) and to regulate transcription factors such as activator protein 1 (AP-1), and nuclear factor kB (NF-kB) (Ranneh et al. 2017). The cellular ROS can be accumulated by exogenous sources like air pollutions (Ranneh et al. 2017).

Skin is the largest organ in body and acts as the first defense barrier against harmful stimuli such as ultraviolet (UV) and air pollution including particulate matters (PMs). PMs can be classified as ultrafine PM (particulate with a dimeter of <0.1 μm, $PM_{0.1}$), fine PM (particulate with a dimeter of <2.5 μm, $PM_{2.5}$), and coarse PM (particulate with a dimeter of <10 μm, PM_{10}) which depending on the particle size (Kim et al. 2015). PMs lead to the development of various skin diseases such as skin aging, alopecia and skin cancer through inducing oxidative stress (Kim et al. 2016). In addition, PMs induced oxidative stress via production of ROS and increase matrix metalloproteinases (MMPs) (Kim et al. 2016; Seok et al. 2018). MMPs including MMP-1, MMP-2, and MMP-9 caused skin aging through the degradation of collagen (Chaiprasongsuk et al. 2017; Kim et al. 2017).

ROS generations have been reported to affect skin aging by increasing the expression of MMP-1 in keratinocyte (Leiros et al. 2017). Several studies were reported that UVB-induced ROS caused skin photoaging via generation of MMP-1 in human keratinocytes and dermal fibroblasts (Kim et al. 2018; Xuan et al. 2017). Therefore, it is important to find an effective antioxidant to prevent skin aging.

Oxidative stress stimulates mitogen-activated protein kinases (MAPKs) signaling pathway, which affect the regulation of transcription factor AP-1 activity (Pittayapruek et al. 2016).

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Activation (phosphorylation) c-Jun and c-Fos can be comprised of homodimer or heterodimer to bind to AP-1 binding sites in the promoter region of target genes to promote gene transcription (Lu et al. 2016) such as MMPs transcription (Kim et al. 2017).

Previous study was reported that fermented fish oil (FFO) has antioxidant effects and protective effect against UVB-induced oxidative damage (Park et al. 2018). But the effect of FFO in $PM_{2.5}$-induced skin aging is poorly understood. Therefore, this study demonstrates the effect of FFO against $PM_{2.5}$-induced skin aging.

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2. Materials and methods

2-1. Cell culture and treatment

The HaCaT human keratinocyte (CLS Cell Lines Service GmbH, Eppelheim, Germany) were cultured in DMEM medium (Gibco, Life Technologies Co., Grand Island, NY, USA) supplemented with 10% fetal bovine serum and antibiotics (100 units/mL penicillin, 100 μg/mL streptomycin and 0.25 μg/mL amphotericin B) (Gibco, Life Technologies Co.) at 37℃ in an incubator with a humidified atmosphere of 5% CO_2. Cell were treated to 50 μg/mL of diesel particulate matter NIST 1650b ($PM_{2.5}$) (Sigma-Aldrich Chemical Company, St. Louis, MO, USA) and 20 μg/mL of FFO. Preparation of $PM_{2.5}$ was described previous study (Piao et al. 2018) and preparation of FFO was described previous study (Park et al. 2018).

2-2. Detection of intracellular ROS

To detect intracellular ROS in HaCaT cells, cells were seeded in plates at a density of 1.0×10^5 cells/well, cultured for 16 h, and treated 20 μg/mL of FFO, 50 μg/mL of $PM_{2.5}$. After 30 min later, 50 μM 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA, Molecular Proves, Eugene, OR, USA) solution was added. DCF fluorescence was measured using a BD LSRFortessa flow cytometry (PerkinElmer, Waltham, MA, USA) and images were collected by using a FV1200 laser scanning confocal microscope (Olympus, Tokyo, Japan).

2-3. Detection of β-galactosidase activity

To detect the cell senescence, cells were seeded in plates at a density of 1.0×10^5 cells/ml. After 16 h of incubation period at 37°C, cells were treated 20 μg/mL of FFO, 50 μg/mL of $PM_{2.5}$. After 24 h later, 2 μM SPiDER-βGal solution (Dojindo Molecular Technologies, Inc., Rockville, MD, USA) was added. After 15 min at 37°C, Cells were mounted in mounting

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medium containing DAPI to label nuclei. SPiDER-βGal fluorescence was measured by using a BD LSRFortessa flow cytometry and images were collected using a FV1200 laser scanning confocal microscope (Olympus).

2-4. MMP-1 activity

The MMP-1 activity was measured by using the Fluorokine® E human active MMP-1 fluorescent assay kit (R&D Systems Inc., Minneapolis, MN, USA). HaCaT cells were seeded on a 60 mm culture dish at 1.0×10^5 cells/mL. After 16 h of incubation period at 37°C, cells were treated with 20 μg/mL of FFO and after 1 h, cell were treated with 50 μg/mL of $PM_{2.5}$. Then MMP-1 activity was assessed according to the manufacture's instruction. Fluorescence was measured by using Spectra Max i3x microplate reader (Molecular devices, San Jose, CA, USA).

2-5. Reverse transcription –PCR (RT-PCR)

Cells were seeded at 1.5×10^5 cells/mL and after 16 h later cell were treated with 20 μg/mL of FFO, 50 μg/mL of $PM_{2.5}$. After 24 h, we isolated total RNA from cells using the easy-BLUE™ total RNA extraction kit (iNtRON Biotechnology Inc., Seongnamsi, Korea). And then the cDNA was amplified by using reverse transcription reaction buffer, primers, dNTPs, and Taq DNA polymerase in a final volume of 20 μL. The amplified products were mixed with blue/orange 6X loading dye, resolved by electrophoresis on a 1% agarose gel which stained with RedSafe™ nucleic acid staining solution (iNtRON Biotechnology Inc., Seongnamsi, Korea), and photographed under UV light using Image Quant™ TL analysis software (Amersham Biosciences, Uppsala, Sweden). The PCR conditions were as followers: initial denaturation at 94℃ for 5 min and then followed by 30 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 1 min. The primers were used in this study: human MMP-1, forward (5'-GGAGGAAATCTTGCTCAT-3′) and reverse (5′-CTCAGAAAGAGCAGCATC-3′);

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human GAPDH, forward (5′-TCAAGTGGGGCGATGCTGGC-3′) and reverse (5′-TGCCAGCCCCAGCGTCAAAG-3′).

2-6. Western blot analysis

The protein lysates (30 μg per lane) were electrophoresed on 12% SDS-polyacrylamide gels. Then transferred to a nitrocellulose membrane which was incubated with the primary antibodies and incubated with HRP-conjugated secondary antibodies (Invitrogen, Carlsbad, CA, USA). Next, membranes were exposed to a Western blotting detection kit (GE Healthcare Life Sciences, Little Chalfont, UK) for to detect the protein bands and then exposed to X-ray film. Primary antibodies were used in this study: MMP-1 (Cusabio Tecnology LLC., Houston, TX, USA), MMP-2 (Abcam, Cambridge, UK), MMP-9 (Abcam), phospho-c-Jun (Cell Signaling Technology, Danvers, MA, USA), c-Fos (Cell Signaling Technology), phospho-SEK (Cell Signaling Technology), phospho-MEK (Cell signaling Technology), phospho-ERK (Santa Cruz Biotechnology, Santa Cruz, CA, USA), phospho-JNK (Cell Signaling Technology), Actin (Sigma-Aldrich Chemical Company).

2-7. Measurement of Ca^{2+} level

To detect Ca^{2+} level, cells were seeded in plates at a density of 1.0×10^5 cells/well, cultured for 16 h, and treated 20 μg/mL of FFO, 50 μg/mL of $PM_{2.5}$. After 24 h later, 5 μM Fluo-4-AM (Molocular Probes, Eugene, OR, USA) solution was added. Fluo-4-AM fluorescence was measured by using a BD LSRFortessa flow cytometry and images were collected by using a FV1200 laser scanning confocal microscope (Olympus).

2-8. Chromatin immunoprecipitation (ChIP) assay

The ChIP assay was performed by using the SimpleChIP™ enzymatic chromatin IP kit

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(Cell Signaling Technology). HaCaT cells were seeded at 1.5×10^5 cells/mL and after 16 h, cells were treated with 20 μg/mL of FFO, 50 μg/mL of $PM_{2.5}$. All processes were performed according to the instructions. The following antibody and primers were used in this study: c-Jun antibody (Invitrogen), MMP-1 gene promoter (-67 to +94 of the MMP-1 gene sequence from the transcription starting site, Bionics) were designed as sense 5′-CCTCTTGCTGCTCCAATATC-3′ and antisense 5′-TCTGCTAGGAGTCACCATTTC-3′. The PCR products were separated on 1% agarose gel, DNA bands were photographed under UV light using Image Quant™ TL analysis software (Amersham Biosciences).

2-9. Statistical analysis

All data were performed in triplicate and all values are expressed as the mean ± standard error of the means. This study used Tukey's test analysis to determine the statistical significance of differences between means. $p < 0.05$ were considered statistically significant.

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3. Results

3-1. Effect of FFO on $PM_{2.5}$-induced intracellular ROS

Because ROS has been reported to affect skin aging via generation of MMPs (Leiros et al. 2017), this study measured generation of intracellular ROS by DCF-DA fluorescence dye. As shown by flow cytometry data, $PM_{2.5}$-treated cell induced ROS and pretreatment of FFO reduced $PM_{2.5}$-indeuced ROS in HaCaT cell and which was confirmed by using confocal microscopy (Figure 1A and B).

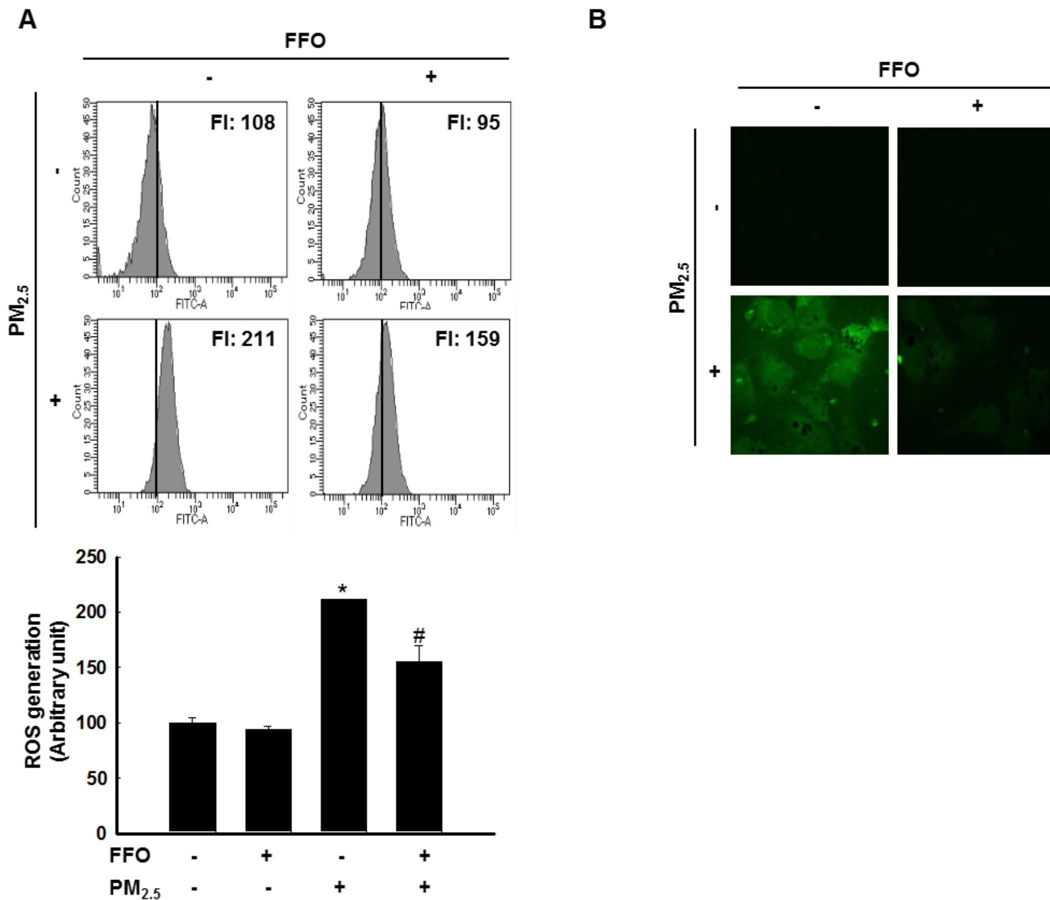

Figure 1. Scavenging effect of FFO on $PM_{2.5}$-induced intracellular ROS. Intracellular ROS was detected by (A) flow cytometry and (B) confocal microscopy after DCF-DA staining. *p

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< 0.05, $^{\#}p < 0.05$ compared to untreated cells and $PM_{2.5}$-treated cells, respectively.

3-2. $PM_{2.5}$-induced keratinocyte senescence

Next, this study measured β-galactosidase activity for to detect of HaCaT cell senescence using flow cytometry and confocal microscopy after SPiDER-βGal staining. $PM_{2.5}$-treated cells increased β-galactosidase activity in the cytosol and FFO-treated cells decreased $PM_{2.5}$-induced β-galactosidase activity (Figure 2A and B).

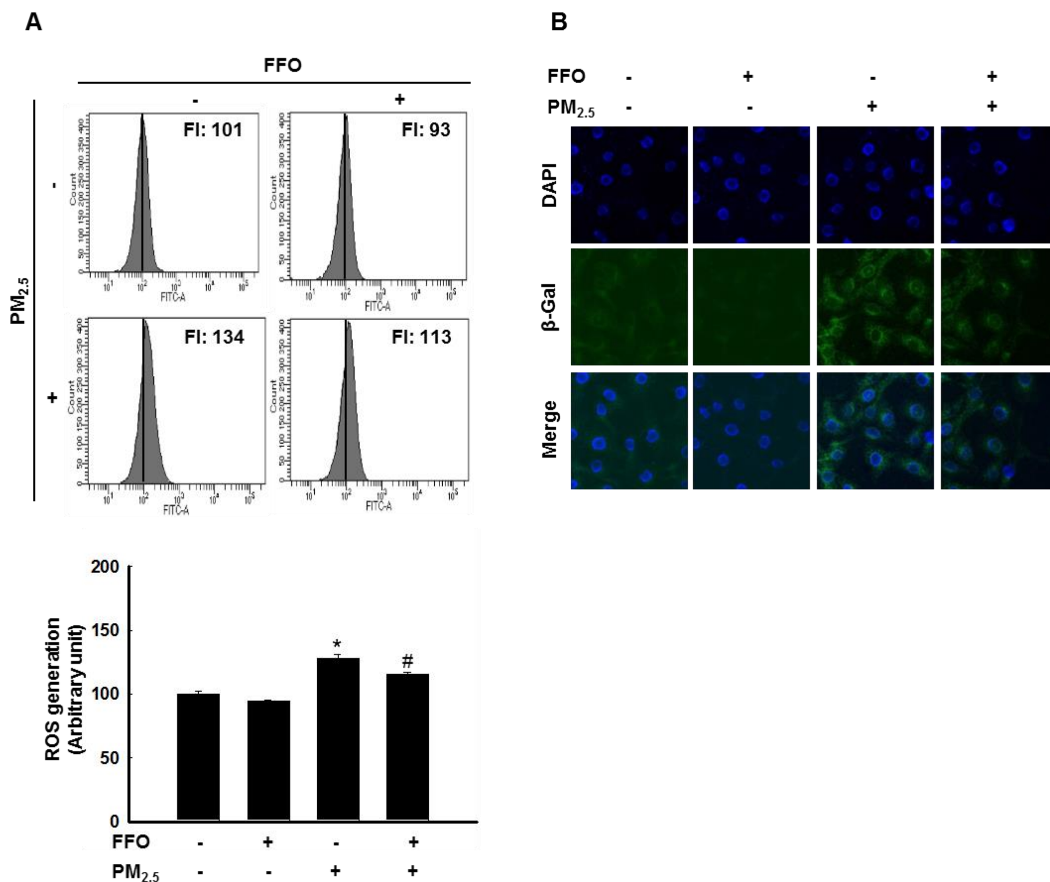

Figure 2. $PM_{2.5}$-induced keratinocyte senescence. (A) The β-galactosidase activity was measured by flow cytometry and (B) confocal microscopy after SPiDER-βGal staining. $*p <$

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0.05, $^{\#}p < 0.05$ compared to untreated cells and $PM_{2.5}$-treated cells, respectively.

3-3. Effect of FFO on $PM_{2.5}$-induced MMP-1 activation and MMPs expression

Treatment of $PM_{2.5}$ significantly increased the activation of MMP-1 in after 6, 12, 24 h (Figure 3A) and FFO pretreatment decreased the $PM_{2.5}$-induced activation of MMP-1 (Figure 3B). Expression of MMP-1 mRNA and protein levels are also increased in $PM_{2.5}$-treatment cells and decreased in FFO-pretreatment cells (Figure 3C and D). Since MMP-2 and MMP-9 are also reported to be involved in skin aging through degradation of collage, I analyzed the expression of MMP-2 and MMP-9 protein levels. MMP-2 and MMP-9 protein level also increased by $PM_{2.5}$ treatment and decreased by FFO pretreatment (Figure 3E).

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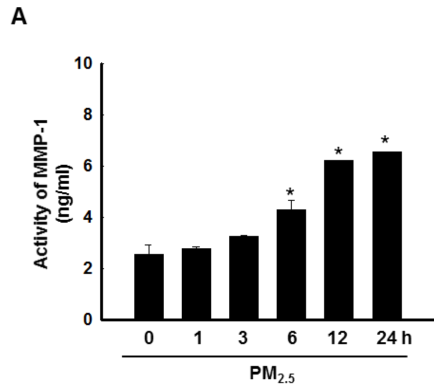

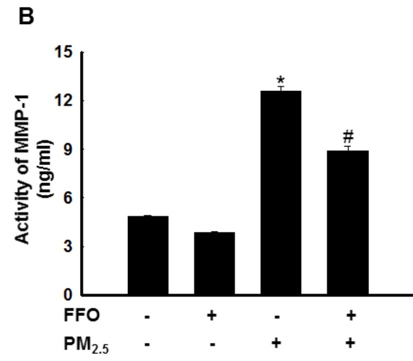

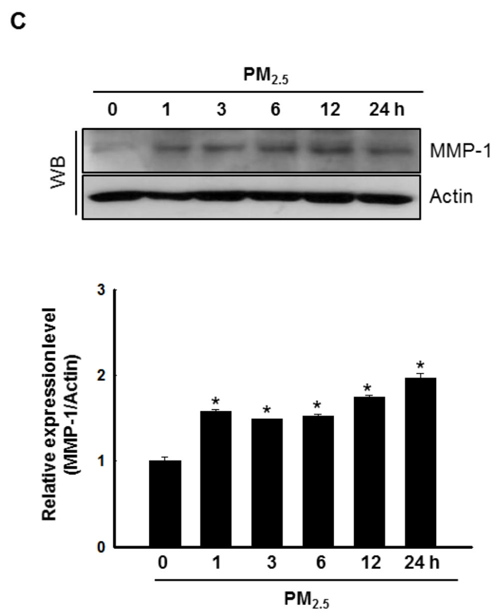

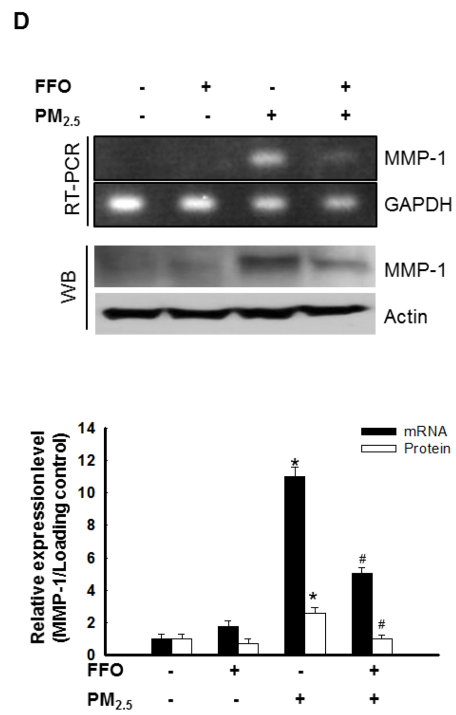

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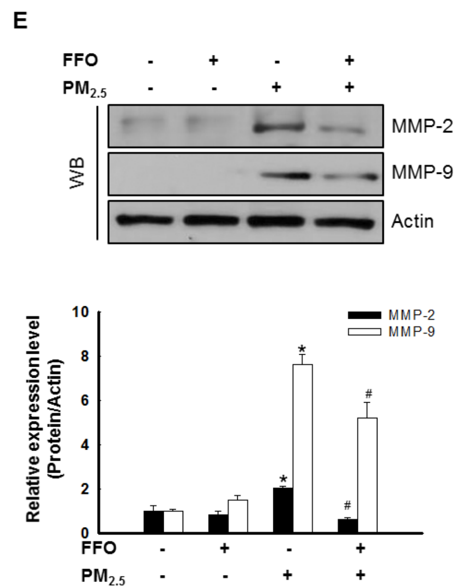

Figure 3. Effect of FFO on $PM_{2.5}$-induced MMP-1 activation and MMPs expression. (A) The MMP-1 activity of $PM_{2.5}$-treated cells at the indicated times and (B) The MMP-1 activity of FFO and $PM_{2.5}$ treated cells was determined using the human active MMP-1 fluorescent assay kit. $^{*}p < 0.05$, $^{\#}p < 0.05$ compared to untreated cells and $PM_{2.5}$-treated cells, respectively. (C) Expression level of MMP-1 was analyzed by western blot. Actin was used to loading control. $^{*}p < 0.05$, compared to untreated cells. (D) The mRNA level of MMP-1 and protein level of MMP-1 were analyzed by RT-PCR and western blot, respectively. GAPDH and actin were used to loading control. $^{*}p < 0.05$, $^{\#}p < 0.05$ compared to untreated cells and $PM_{2.5}$-treated cells, respectively. (E) Expression level of MMP-2 and MMP-9 were analyzed by western blot. Actin was used to loading control. $^{*}p < 0.05$, $^{\#}p < 0.05$ compared to untreated cells and $PM_{2.5}$-treated cells, respectively.

3-4. Effect of FFO on $PM_{2.5}$-induced MAPKs and intracellular Ca^{2+} level

MAPKs, which enhance expression of MMP-1, are activated by an increase in the intracellular Ca^{2+} level (Liu et al. 2010). As shown western blot data, $PM_{2.5}$ treatment induced the activation (phosphorylation) JNK and ERK however, FFO pretreatment

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decreased. Furthermore, $PM_{2.5}$ treatment induced the activation (phosphorylation) MAPK kinase (MEK) 1/2 and SAPK/ERK kinase (SEK) 1 (Figure 4A). In addition, $PM_{2.5}$ significantly increased intracellular Ca^{2+} level and FFO decreased $PM_{2.5}$-induced Ca^{2+} level (Figure 4B and C).

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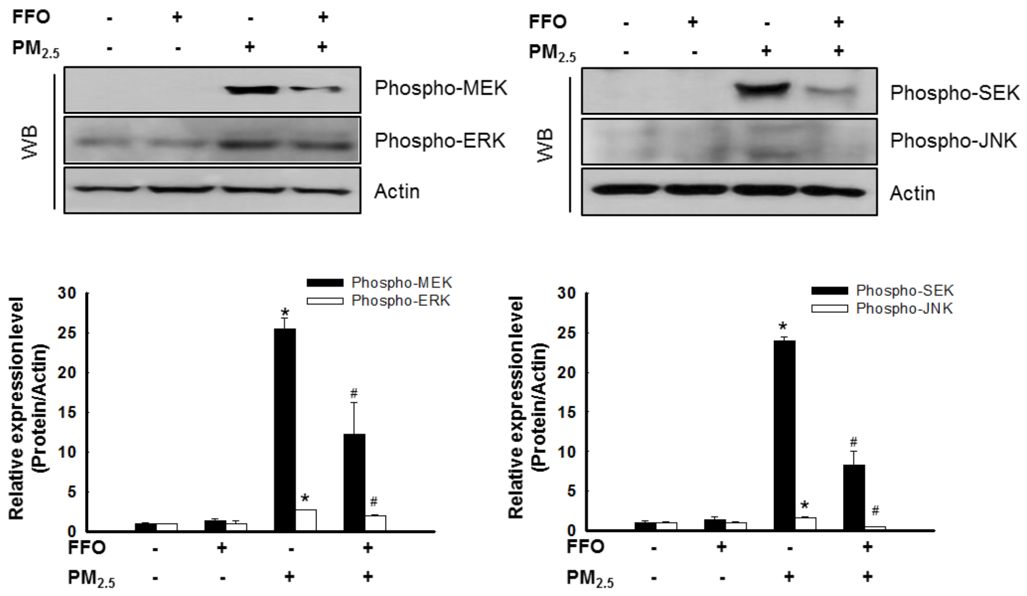

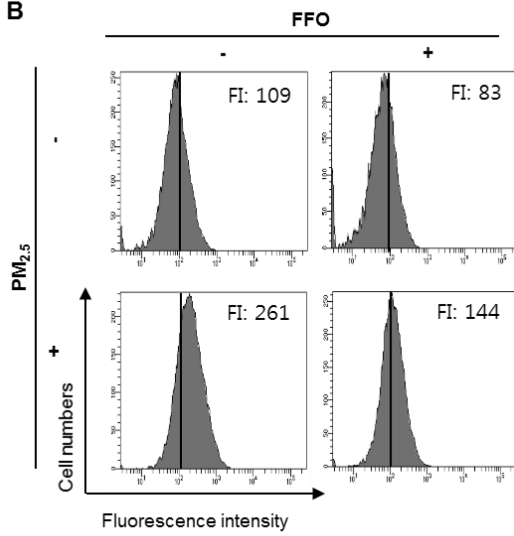

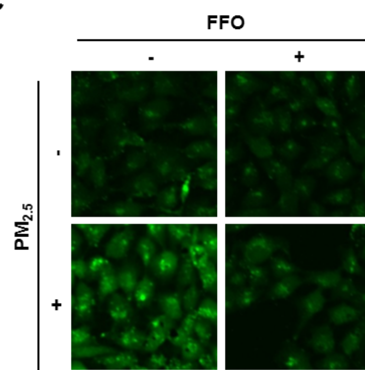

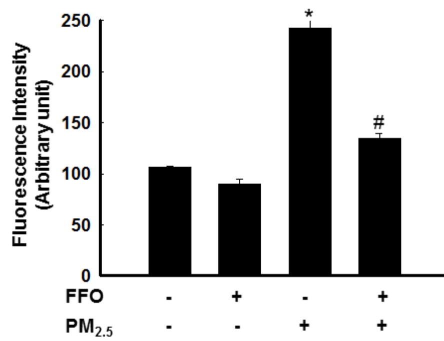

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Figure 4. Effect of FFO on $PM_{2.5}$-induced MAPKs and intracellular Ca^{2+} level. (A) Expression level of p-JNK, p-ERK, p-MEK, and p-SEK by western blot analysis. Actin was used to loading control. $^{*}p < 0.05$, $^{\#}p < 0.05$ compared to untreated cells and $PM_{2.5}$-treated cells, respectively. (B) Intracellular Ca^{2+} level was detected by flow cytometry and (C) confocal microscopy after Flou-4-AM staining. $^{*}p < 0.05$, $^{\#}p < 0.05$ compared to untreated cells and $PM_{2.5}$-treated cells, respectively.

3-5. Effect of FFO on $PM_{2.5}$-induced transcription factor activator protein 1 (AP-1) expression

The nuclear transcription factor AP-1 regulated by MAPKs, which increase MMP expression (Kim et al. 2018). Activation of MAPKs results in the heterodimeratization of c-Jun/c-Fos and the formation of the AP-1 complex (Kim et al. 2017). As shown Figure 4, $PM_{2.5}$ treatment increased MAPKs and intracellular Ca^{2+} level. Next, I determine c-Fos, phopho-c-Jun level using by western blot analysis. FFO significantly decreased $PM_{2.5}$-indeced phopho-c-Jun and c-Fos level (Figure 5A). In addition, FFO reduced the $PM_{2.5}$-induced AP-1 binding to the MMP-1 promoter (Figure 5B).

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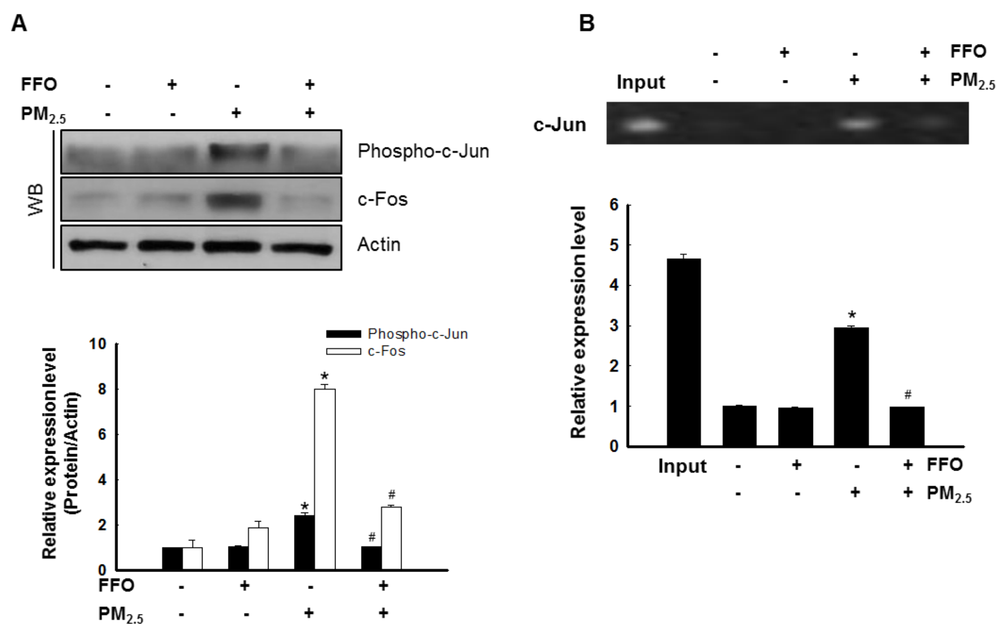

Figure 5. Effect of FFO on $PM_{2.5}$-induced transcription factor activator protein 1 (AP-1) expression. (A) Expression level of phospho-c-Jun and c-Fos by western blot analysis. Actin was used to loading control. *$p < 0.05$, #$p < 0.05$ compared to untreated cells and $PM_{2.5}$-treated cells, respectively. (B) AP-1 binding to the MMP-1 promoter was assessed by ChIP assay. *$p < 0.05$, #$p < 0.05$ compared to untreated cells and $PM_{2.5}$-treated cells, respectively.

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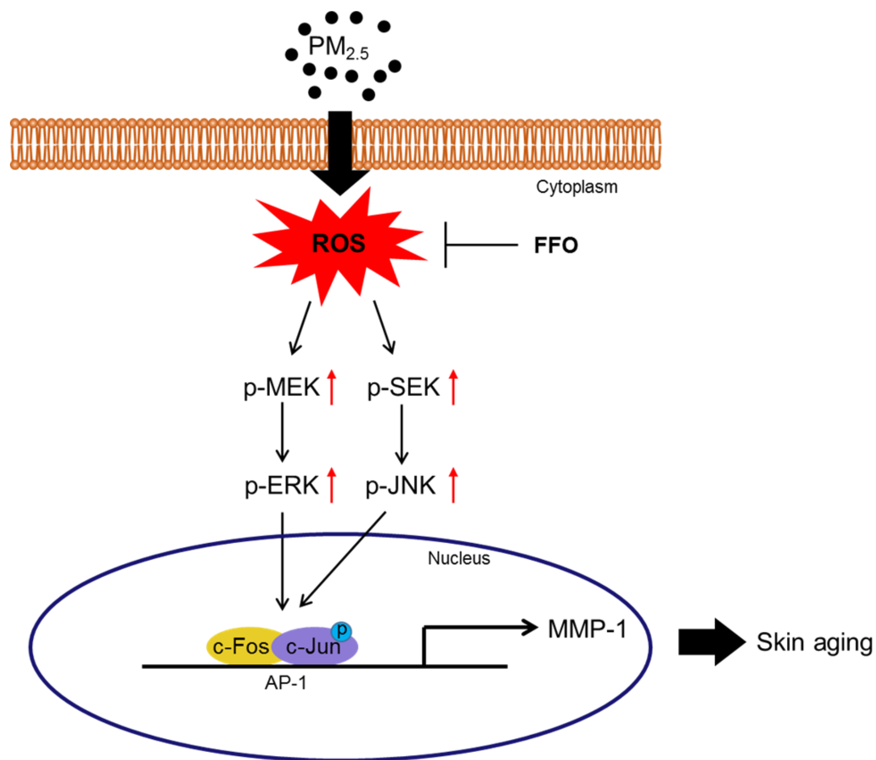

Figure 6. Schematic diagram of effect of FFO on $PM_{2.5}$-induced skin aging. Expose of $PM_{2.5}$ increased intracellular ROS and induced skin aging in HaCaT cells. The mackerel derived FFO has anti-aging effect against $PM_{2.5}$-induced skin aging through reduced intracellular ROS.

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4. Discussion

Skin is the largest organ in body which acts as the first defense barrier against harmful stimuli. Therefore, skin is always exposed harmful environment including PMs. In several studies, $PM_{2.5}$ has been reported to have harmful effects such as inflammatory skin diseases, skin aging, and damage of respiratory system through generation of intracellular ROS (Kim et al. 2016; Romani et al. 2018; Xing et al. 2016).

The accumulation of ROS has been reported that induced skin aging through the expression of MMPs such as MMP-1, MMP-2, and MMP-9 (Subedi et al. 2017). Therefore, it is important to find an effective antioxidant to prevent skin aging.

Previous study demonstrated that mackerel derived FFO has directly ROS scavenging effect and protective effect on UVB-induced oxidative damage (Park et al. 2018). The present study focused on the effect of FFO which derived from mackerel against $PM_{2.5}$-induced skin aging.

First, this study measured generation of intracellular ROS after $PM_{2.5}$ treatment. Induction of intracellular ROS by $PM_{2.5}$ treatment significantly reduced pretreatment of FFO (Figure 1A and B). Since the generation of ROS cause skin aging, this study detected keratinocyte senescence using β-galactosidase (Yoshimoto et al. 2018). $PM_{2.5}$ treatment increased β-galactosidase activity and FFO significantly reduced $PM_{2.5}$-induced β-galactosidase activity (Figure 2A and B). Next, because of MMPs caused skin aging through degradation of collagens (Chaiprasongsuk et al. 2017; Kim et al. 2017), this study detected activity of MMP-1 and expression level of MMP-1, MMP-2, and MMP-9. FFO has effect on $PM_{2.5}$-induced MMPs (Figure 3). These results indicate that $PM_{2.5}$ induced intracellular ROS and skin aging through generation of MMPs. In addition, FFO has anti-aging effect via

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scavenging ROS.

ROS activates the MAPK signaling pathway and activation of MAPKs induced various transcription factors such as AP-1 and NF-κB (Pittayapruek et al. 2016; Sun et al. 2017). As a result of translocation of the activated AP-1, a heterodimer composed of c-Jun and c-Fos, MMPs were synthesized (Hwang et al. 2011; Kim et al. 2013). As shown Figure 4A, ERK and JNK activated by $PM_{2.5}$ treatment and reduced by FFO pretreatment. MEK and SEK, the upstream of ERK and JNK respectively, also increased by $PM_{2.5}$ treatment and decreased by FFO pretreatment (Figure 4A). In addition, intracellular Ca^{2+} level, regulating MAPKs, was significantly increased in $PM_{2.5}$-treated cells. However, FFO was decreased the $PM_{2.5}$-induced Ca^{2+} level (Figure 4B and C). Furthermore, phospho-c-Jun and c-Fos levels were increased in $PM_{2.5}$-treated cells and decreased in FFO-pretreatment cells (Figure 5A). $PM_{2.5}$-induced AP-1 binding to the MMP-1 promoter also reduced by FFO-pretreatment cells (Figure 5B). These results demonstrate that FFO can block $PM_{2.5}$-induced MAPKs/AP-1 pathway in human keratinocyte.

In conclusion, mackerel derived FFO has anti-aging effect against $PM_{2.5}$-induced skin aging through reduced intracellular ROS and expression of MMPs (Figure 6).

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6. Abstract in Korean

피부는 미세먼지(particulate matters, PMs)를 비롯한 대기오염과 같은 유해한 환경에 항상 노출된다. 이러한 미세먼지는 활성 산소 종(reactive oxygen species, ROS)의 증가를 통해 인체에 해로운 영향을 미친다. 세포 내 ROS는 콜라겐을 분해하는 단백질 분해효소(matrix metalloproteinases)인 MMP-1을 생성할 수 있고 그로 인해 피부 노화를 유발 할 수 있다고 알려져 있다. 이 연구는 고등어에서 추출한 발효 어유(FFO)가 인간각질세포에서 초미세먼지($PM_{2.5}$)로 유도되는 피부노화에 미치는 영향을 조사하였다. $PM_{2.5}$를 처리한 세포에서 ROS증가와 노화인자가 증가하는 것을 확인하였고 FFO 전처리 그룹에서 $PM_{2.5}$로부터 유도되는 ROS 증가, 노화인자 증가가 감소되는 것을 확인 할 수 있었다. 또한, 콜라겐분해를 통해 피부노화를 일으킨다고 알려져 있는 MMP-1의 활성도와 mRNA, 단백질 발현 수준 역시 $PM_{2.5}$ 처리로 증가되었지만 FFO전처리로 감소되는 것을 볼 수 있었다. 또한 FFO는 MMP-1의 발현을 조절하는데 영향을 주는 세포 내 Ca^{2+} 수준과 MAPKs/AP-1 경로를 차단 하는 것을 보여준다. 따라서 이러한 결과는 고등어로부터 유래된 FFO는 $PM_{2.5}$로 유도되는 피부노화에 있어서 세포 내 ROS를 소거함으로써 피부노화에 효과를 가진다는 것을 시사한다.

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