*IFSCC 2025 full paper (**IFSCC2025-517**)*

"Eyelash Growth Cosmetics Containing Aleurites moluccanus (AMS) Oil: Hair Growth Mechanism by Antioxidant Nrf2/ARE-AKR1C Family- PGF2α Axis Activity"

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1. Introduction

Hair is a vital organ that protects the eyes and skin from external stimuli and makes a person's appearance attractive. Eyelash care and nurturing are essential because eyelashes have a significant impact on facial attractiveness [1]. Hair loss leads to psychological morbidity because attractiveness has a great impact on self-esteem, not only in healthy people, but also in cancer survivors [2,3]. Therefore, hair and eyelash growth not only give a healthy person an attractive appearance, but also significantly increases cancer survivors' self-esteem.

Many consumers desire organic hair growth cosmetics. *Aleurites moluccana* seed (AMS) oil is extensively used in traditional folk medicine [4]. The hair loss prevention and growth effects of AMS oil have been demonstrated empirically [5]. However, only a few animal studies have shown that AMS oil induces hair growth, and the underlying mechanism has not yet been elucidated [6,7]. In this study, we aimed to reveal the mechanism of hair growth by AMS oil, focusing on the hair growth-inducing substance prostaglandin F2 alpha (PGF2α) and its efficacy in human hair growth by developing an eyelash growth serum containing AMS oil.

2. Materials and Methods

Ethics statement

Experiments using human skin and *in-vivo* experiments were approved by the Ethics Committee of The University of Osaka (Yakuhito2019-28) and Mandom Corp. (No. 107-026), respectively. All studies were performed according to relevant guidelines and regulations. Informed consent was obtained from all study participants.

Treatment of cultured skin cells and hair follicles

To prepare a stock solution of AMS oil, AMS oil (Nikko Chemicals) was diluted with dimethylsulfoxide (DMSO) (Merck) at an oil:DMSO ratio of 1:9 to make the oil water-soluble. Human keratinocyte cell line (HaCaT), and human hair follicles (HF), which were micro dissected individually from skin samples, were cultured and treated with 0.09% DMSO (control treatment), 0.001% or 0.01% AMS oil, or 100 nM bimatoprost (positive control) (Fujifilm) in DMEM containing 10% fetal bovine serum (FBS) for HaCaT cells and William's E Medium, GlutaMAX™ Supplement (Thermo Fisher Scientific) that contained 1% antibiotic-antimycotic, 100 ng/mL hydrocortisone (Merck), and 5 mg/mL insulin (Fujifilm) for HF.

ELISA

The concentration of PGF2α in the supernatant was determined using a PGF2α High Sensitivity ELISA kit (Enzo Life Sciences) according to the manufacturer's protocol.

RT-qPCR

cDNA was generated via RNA extraction. The PCR mixture was prepared with SYBR® Green Realtime PCR Master Mix (Toyobo). Gene expression of Aldo-keto Reductase 1C1 (*AKR1C1*), *AKR1C2*, *AKR1C3*, *NFE2L2* (*NRF2*), Heme Oxygenase 1 (*HMOX1*), and as an internal control, *18S*, was detected using QuantStudio™ 6 Pro (Thermo Fisher Scientific).

Western blotting

Cells were lysed using RIPA buffer (Santa Cruz Biotechnology) containing protease and phosphatase inhibitors, and mixed with sample buffer (Bio-Rad). SDS-PAGE and transfer were performed using a Trans-Blot Turbo System (Bio-Rad). Each membrane was then incubated with the primary antibodies for AKR1C1, AKR1C2, AKR1C3, Ki-67, Cyclin B1, D1, E1, Phospho-Histone H3 (Ser10), Prostaglandin F Receptor (PTGFR), and β-actin. Finally, the blots were detected using an ECL kit (Cytiva) following secondary antibody incubation. β-actin was used as an internal positive control. Images were captured using an Amersham Imager 600 (Cytiva).

RNA interference for gene knockdown

Small interfering RNA knockdown was performed using *AKR1C1*, *AKR1C2*, *AKR1C3*, *NRF2*, and a negative control (Silencer® Select or Silencer®, Thermo Fisher Scientific). Cells were transfected using RNAi at a final concentration of 10 nM and Lipofectamine™ RNAiMax (Thermo Fisher Scientific) according to the manufacturer's instructions in an Opti-MEM™ Reduced Serum Medium (Thermo Fisher Scientific) and harvested after 72 hours of the treatment following medium change.

Luciferase assay

HaCaT cells were co-transfected with 0.5 μg NanoLuc® luciferase reporter (pNL[NlucP/ARE/Hygro] Vector or pNL[NlucP/minP/Hygro] Vector (Promega) and 0.05 μg

firefly loading control reporter (pGL4.54[luc2/TK] Vector; (Promega) with PEI MAX® (Polysciences) in Opti-MEM. One day after transfection, cells were cultured for 24 hours in DMEM containing 10% FBS and 0.01% AMS oil or DMSO. Luminescence was detected using a Nano-Glo® Dual-Luciferase® Reporter Assay System (Promega) following the manufacturer's instructions.

***In-vivo* test of the eyelash serum in human volunteers**

This study employed a double-blind, left-right comparison design to evaluate the effects of 0.1% AMS oil serum and placebo serum on eyelashes. Fifteen Japanese women, aged 27–48 years, without any condition of the eyes, eyelashes, or skin in the application area were recruited. Serums were applied to the roots, upper lashes, and lower lashes. Each participant used the serums twice daily: once in the morning after their skincare routine, and once in the evening after their skincare routine. Participants applied the serums individually to the designated areas, using two doses per application. Observations were conducted at two time points: the initial stage and after four weeks of treatment. The eyelash growth length was quantified after four weeks of treatment using an imaging system (VISIA-Generation 7, Canfield Scientific), and compared with the length on the first day of the trial.

Statistical analysis

Statistical analysis was performed with GraphPad Prism, version 9.5.1.

3. Results

AMS oil promotes hair follicle elongation in organ culture

To analyze the hair growth effects of AMS oil treatment at the organ level, cultures of human scalp HFs were investigated. We found that the hair was significantly elongated in the AMS oil-treated group (Figure 1a, b). The bimatoprost 100 nM-treated group was used as a positive control.

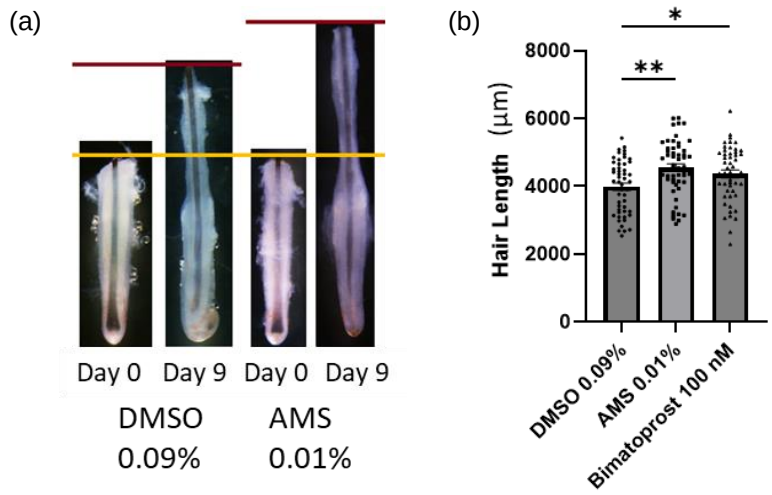


Figure 1. Hair growth induced by *Aleurites moluccana* seed (AMS) oil in hair follicle culture. (a) Representative photos of hair follicles after AMS oil treatment. (b) Graph of the total length of the hair (mean ± SEM; subject number = 10; hair follicle numbers = 52, 53, and 50, respectively; *P < 0.05; **P < 0.01; Dunnett's test).

AMS oil promoted the expression of the *AKR1C* family and proliferation markers

To examine the effect of AMS on hair growth, we focused our analysis on PGF2α synthesis, which has been reported to promote hair growth [8]. We performed western blotting to confirm their expression in HF at the organ level. After AMS oil treatment, *AKR1C1-3* signaling increased (Figure 2a). PTGFR expression was not affected by the treatment (Figure 2a, b). Next, we used HaCaT cells to reveal the mechanism since the expression of AKR1C1 and AKR1C3, as well as the synthesis of PGF2α, has been reported in the outer root sheath and skin keratinocytes [9]. Gene expressions of the *AKR1C* family increased in a dose-dependent manner at each time point (Figure 2c). We further investigated the effects of AMS oil on cell proliferation. Protein expression levels of the proliferation markers ki67 and PHH3, as well as cyclin B1— which is upregulated during the G2M phase—and cyclin D1 and E1, which are required for the G1/S transition, showed an increasing trend after AMS oil treatment (Figure 2d).

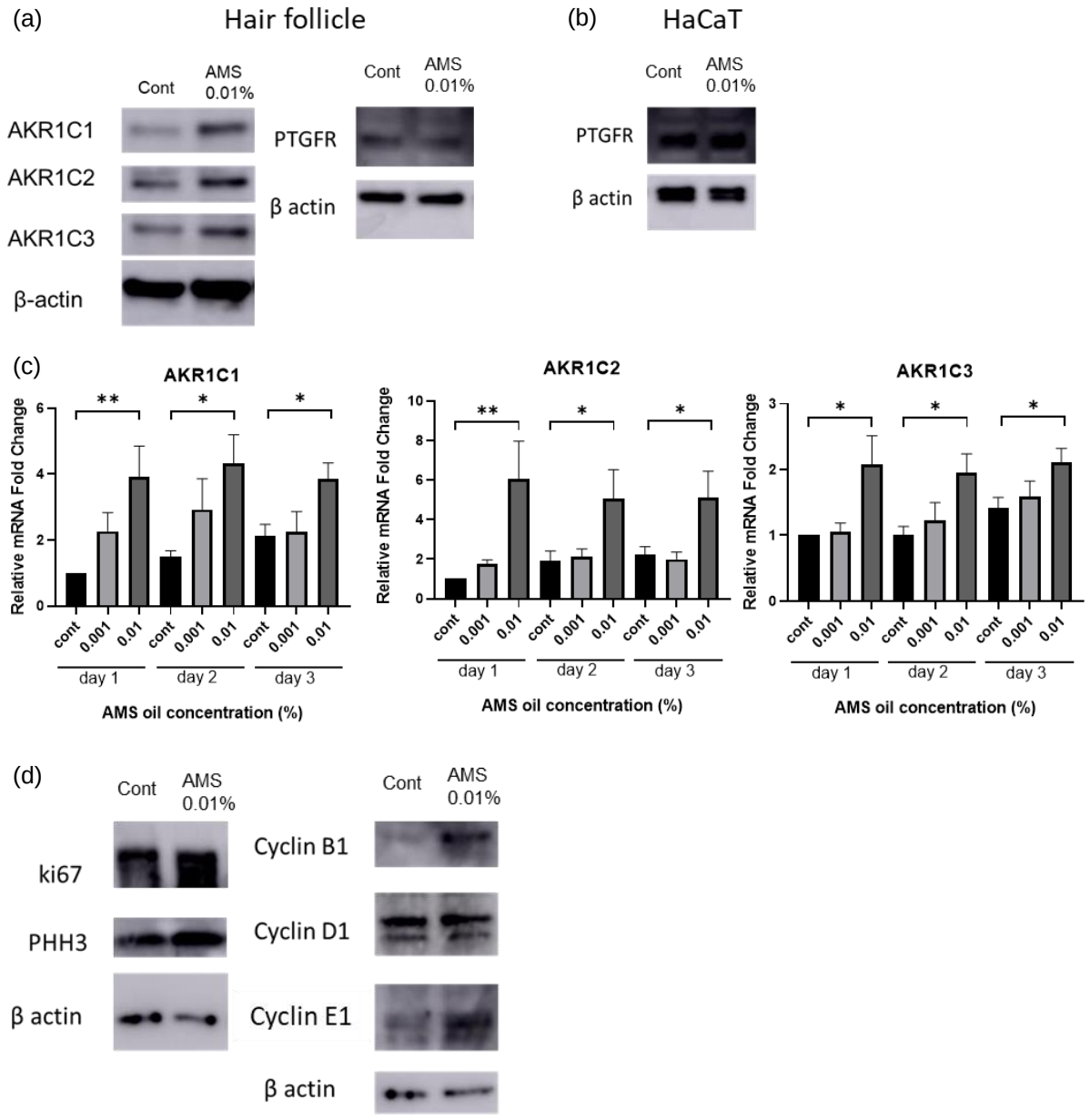


Figure 2. Effect of *Aleurites moluccana* seed (AMS) oil on *AKR1C1-3* expression and proliferation. Protein expression of (a) *AKR1C* family and PTGFR expression in hair follicle, (b) PTGFR expression in HaCaT cells. (c)The relative mRNA expression of *AKR1C1*, *AKR1C2*, and *AKR1C3* in HaCaT cells after induction with 0.001% and 0.01% AMS oil (n = 10, *P < 0.05, **P < 0.01, Dunnett's test for each day of control sample vs treatment sample). (d) Proliferation and cell cycle marker of representative data for the western blot result for HaCaT cells.

AMS oil increases the PGF2α secretion, which was regulated by the AKR1C family

We investigated whether AMS oil contributes to PGF2α production. ELISA analysis revieled an increase in PGF2α concentration following AMS oil treatment compared to the control (Figure 3a). To clarify which isoforms of the *AKR1C* family play an essential role in PGF2α secretion efficacy and specificity, they were successfully knocked down, and PGF2α secretion was investigated. siRNA of the all the *AKR1C* family members successfully decreased the gene expression and PGF2α secretion after AMS oil treatment (Figure 3b, c).

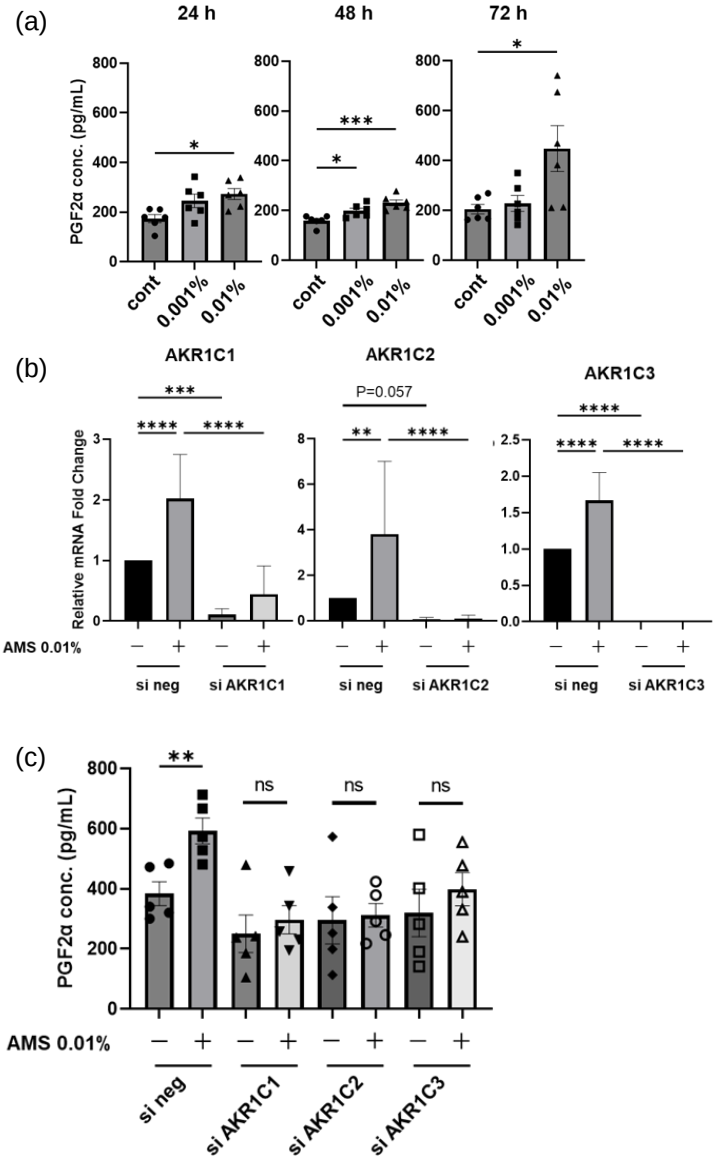
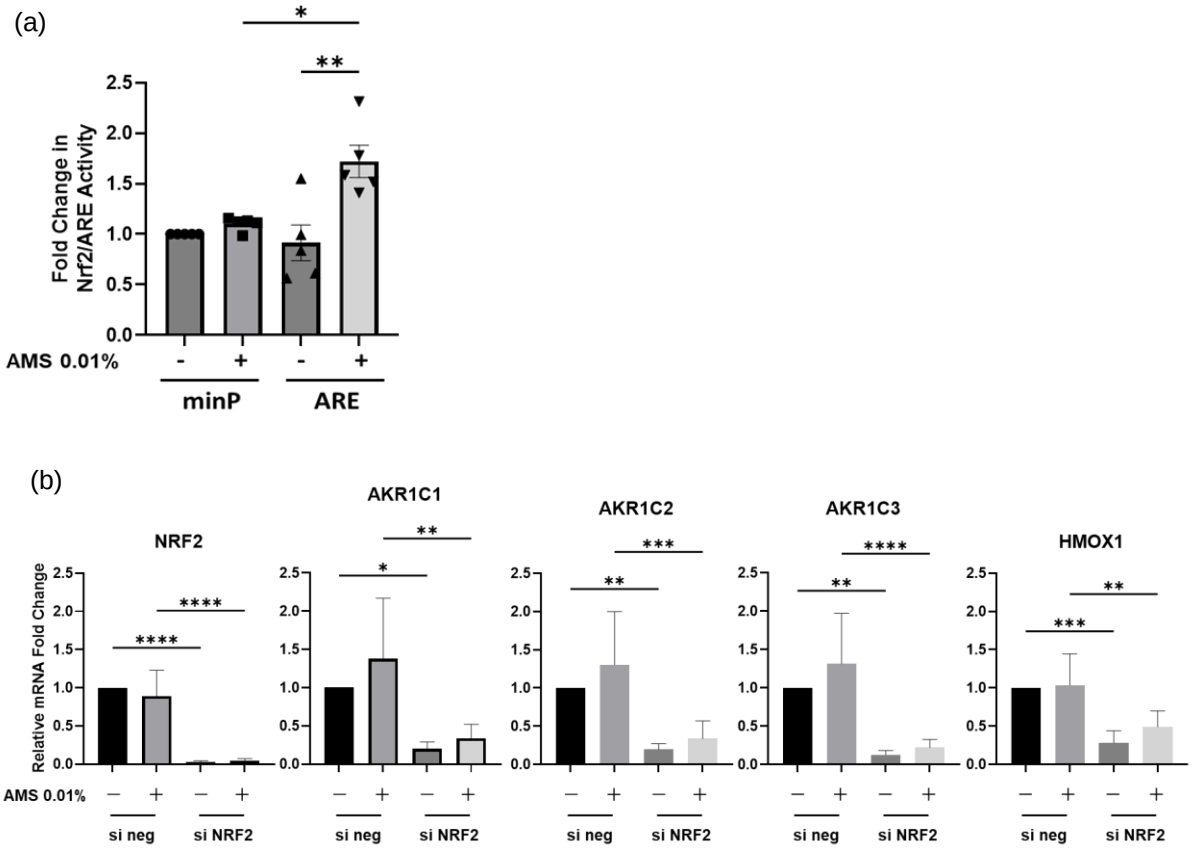


Figure 3. Effect of *Aleurites moluccana* seed (AMS) oil on PGF2α secretion. (a) PGF2α secretion in HaCaT cells after treatment with 0.01% AMS oil for 24, 48, and 72 hours (mean ± SEM, n = 6, Dunnett's test for each treatment time). (b) Gene expression of the target genes treated with each respective siRNA after 0.01% AMS oil treatment (mean ± SD, n = 10, Tukey-Kramer test). (c) PGF2α secretion after siRNA (mean ± SEM, n = 5, unpaired t-test between non-treatment and AMS oil treatment in each siRNA treatment group). ns: non-significance, *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

AMS oil activates the antioxidant response element

The antioxidant response element (ARE) is a transcriptional regulator of *AKR1C1-3*. Therefore, we investigated whether AMS oil regulates the transcriptional activation of ARE in HaCaT cells using a luciferase assay. Treatment with AMS oil increased the luciferase activity of Nrf2/ARE (Figure 4a). Nrf2 binds to ARE and activates downstream gene expression. We performed siRNA targeting of *NRF2* to confirm that *NRF2* regulates the expression of *AKR1C1-3* and *HMOX1*, which are regulated by ARE activation and have antioxidant and anti-inflammatory functions. Knockdown of *NRF2* resulted in down-regulation of *AKR1C1-3* and *HMOX1* expression (Figure 4b). AMS oil treatment had a minimal effect on *NRF2* gene expression (Figure 4b). Furthermore, the impact of *NRF2* knockdown on PGF2α production was investigated. It was observed that *NRF2* knockdown significantly decreased PGF2α production (Figure 4c). This suggests that activation of ARE, which promotes comprehensive expression of the *AKR1C* family of enzymes responsible for PGF2α production, is essential for PGF2α production.



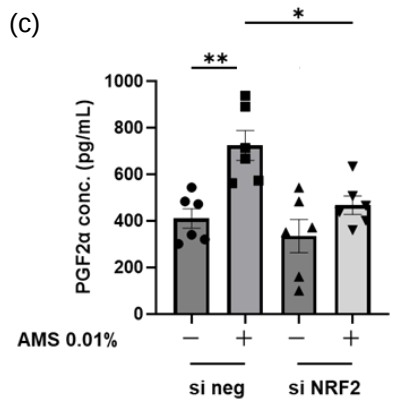


Figure 4. Effect of *Aleurites moluccana* seed (AMS) oil on activation of Nrf2, and *NRF2* knockdown effect on downstream genes and PGF2α secretion. (a) Luciferase activity in HaCaT cells were transfected with the firefly vector containing Nrf2/ARE or an empty vector, minP, as the control. Values are relative to firefly luciferase activity (mean ± SD, n = 5, Tukey-Kramer test). (b, c) HaCaT cells were treated with 0.01% AMS oil following the knockdown of *NRF2*. (b) Expression of Nrf2 and Nrf2/ARE downstream target genes. The bar graph shows the mean values (mean ± SD, n = 6, Tukey-Kramer test). (c) PGF2α concentration in the supernatant (mean ± SD, n = 5, Tukey-Kramer test). *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

AMS oil induces eyelash growth in vivo

To investigate the efficacy of AMS oil *in vivo*, a double-blind, left-right comparison design was employed to evaluate the effects of 0.1% AMS oil serum and control serum on eyelashes. AMS oil serum successfully increased eyelash length. (Figure 5a, b)

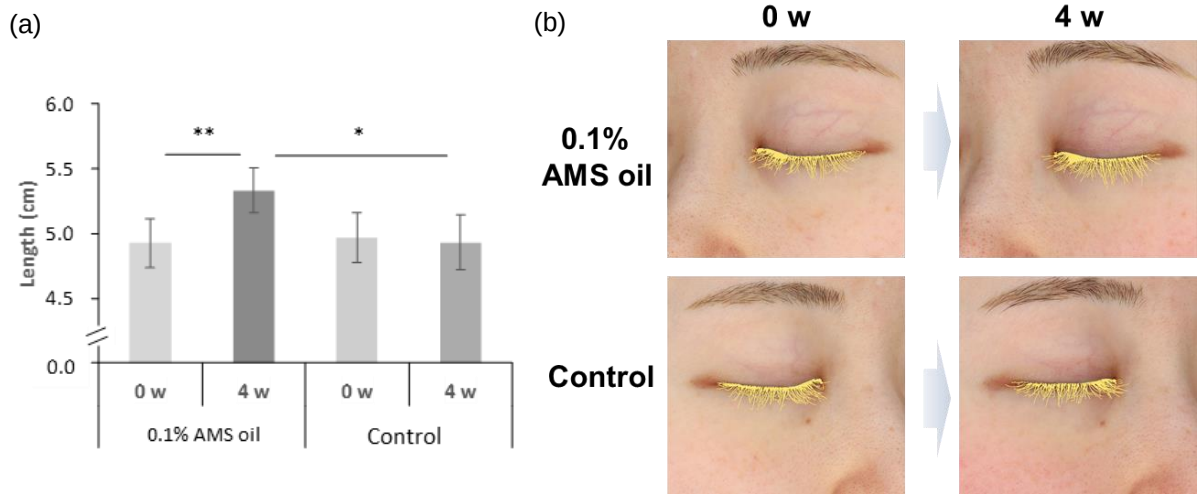


Figure 5. *Aleurites moluccana* seed (AMS) oil induces eyelash lengthening. (a) Difference in eyelash length between placebo and AMS oil application after 4 weeks. (mean ± SEM, n = 15, paired t-test, *P < 0.05, **P < 0.01). (b) Representative images of eyelash growth in treatment with AMS oil.

4. **Discussion**

AMS oil is mainly composed of linoleic acid (ω6 fatty acid), oleic acid (ω9 fatty acid), and linolenic acid (ω3 fatty acid), and contains antioxidants including tocopherols and polyphenols [4,10,11]. Tocopherol, linoleic acid, and linolenic acid have been reported to activate the *NRF2*/ARE pathway [12–14]. These findings suggest that several components of AMS oil activate the *NRF2*/ARE pathway. Because the ARE/*NRF2* pathway is an antioxidant pathway, it has been reported to suppress cellular senescence and increase cell survival by promoting the expression of downstream molecules with anti-inflammatory and detoxifying effects [15,16]. In hair, activation of the NRF2 pathway is reported to rescue the inhibition of hair elongation under oxidative stress [16]. In addition, since AMS oil has been reported to be non-cytotoxic to human conjunctival cells, it can be used not only for hair growth on the head, but also as a safe eyelash hair growth agent [17].

PGF2α derivatives have been reported to be effective in hair growth and regrowth in clinical trials for trichiasis anopia, androgenetic alopecia (AGA), and eyelash alopecia (AA) [8,18,19]. Although PGF2α is known to bind to PTGFR, a G protein-coupled receptor, and prolong the growth phase of hair, the mechanism underlying this effect was unclear. In this study, we focused on cell proliferation and the cell cycle and found that PGF2α increases the G1/S transition and G2M phase of the cell cycle and proliferation markers *Ki-67* and *PHH3*. PTGFR is a type 1 GPCR, and the PGF2α/PTGFR axis is known to promote human retinal microvascular endothelial cell proliferation and tube formation via the Gq/CAMK2G/p38/ELK-1/FOS pathway [20]. PGF2α is also known to promote the cell cycle and thicken the endometrium [21]. These pathways may also be involved in hair growth.

The *AKR1C* family is also involved in the regulation of sex hormones. AGA is the leading cause of scalp hair loss, affecting 60–70% of the population worldwide [22]. One cause of this is excessive 5α-dihydrotestosterone (5α-DHT) production in HFs. 5α-DHT is reduced to 5α-androstan-3α and 17β-diol (3α-diol) by *AKR1C2* and *AKR1C1*, respectively [23]. Thus, *AKR1C2* and *AKR1C1* may suppress male pattern baldness by causing male hormones to become inactive. The PGF2α-independent response is expected to ameliorate AGA caused by AMS oil.

5. Conclusion

In conclusion, AMS oil increases the expression of *AKR1C* family members by activating antioxidant responses via the Nrf2/ARE pathway. It was found to promote the secretion of PGF2α, which exerts hair growth effects *in vitro* and *in vivo*. These results demonstrate the efficacy of naturally derived AMS oil in hair growth. These results will contribute to the cosmetic application of AMS oil for hair growth on the head and eyelashes.

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