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"Mechanism and solutions for hair thinning: licorice leaf extract inhibits the negative influence of sebum peroxidation on the scalp"

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1. Introduction

Hair thinning results from multiple factors, including aging, environmental exposure, and psychological stress. This condition can affect appearance and reduce quality of life, particularly in Asia [1]. Research on preventing hair thinning has been conducted from various perspectives, such as promoting blood flow to sustain the anagen phase of hair follicles and delaying the transition to the catagen phase, as well as balancing the scalp microbiome [2,3].

Hair follicles undergo a unique cycle comprising anagen, catagen, and telogen phases, regulated by cytokines and growth factors [4]. In balding areas of patients with androgenetic alopecia (AGA), elevated production of prostaglandin (PG) D_2 and increased expression of fibroblast growth factor 5 (FGF5), which promote the transition from anagen to catagen, have been observed compared to non-balding areas [5,6]. However, the target cells of PGD_2 and the mechanisms by which they negatively regulate the hair cycle remain unclear.

Compared to other skin, the scalp is characterized by thick hair and highly developed sebaceous glands. Squalene peroxide, a lipid peroxide derived from squalene, induces skin inflammation [7]. Interestingly, hair collected from reddened scalp areas exhibits reduced tensile strength, and exposure to inflammatory cytokines decreases the expression of key a molecule for cuticle strength in outer root sheath cells [8]. These findings suggest that inflammation caused by sebum peroxides may adversely affect the physical properties of hair. However, few studies have established a direct link between sebum peroxide and hair thinning.

This study aimed to elucidate the relationship between PGD_2 and FGF5, which are highly expressed in balding areas. We hypothesized that squalene peroxide, derived from sebum induces PGD_2 and enhances FGF5 production, thereby negatively regulating the hair cycle through a series of molecular regulations. We demonstrated that inhibiting PGD_2 production from epidermal keratinocytes due to squalene peroxide and suppressing FGF5 production in

dermal papilla cells induced by PGD_2 effectively restore the disrupted hair cycle. As a result, Glycyrrhiza Glabra (Licorice) Leaf Extract (LLE) is proposed as a hair-growth ingredient based on an original approach that combines “scalp environmental damage” and “underlying causes of hair thinning” in a novel manner.

2. Materials and Methods

2.1. LLE and its components

Freeze-dried LLE was prepared from the leaves of licorice (*Glycyrrhiza glabra*). The components of LLE, quercetin, rutin (Fujifilm Wako Pure Chemicals), pinocembrin (ChemScene), and pinobanksin (Toronto Research Chemicals), were purchased and their content in LLE was analyzed using high-performance liquid chromatography.

2.2. Cell culture

Normal human epidermal keratinocytes (NHEK) derived from neonatal donors (NHEK-neo) and adult female donors aged 34 years (NHEK-ad) were obtained from Kurabo. Human follicle dermal papilla cells (HFDPC) were purchased from Takara Bio. Cells were cultured in the medium recommended by the manufacturer.

2.3. Preparation of squalene (SQ) peroxide (SQ-OOH)

SQ dissolved in ethanol was irradiated with UVA to produce SQ-OOH, as described previously [8]. Following irradiation, the concentration of malondialdehyde (MDA) in the solution was measured to assess the level of peroxidation using an LPO Assay Kit (Aoxre LLC).

2.4. Treatment with SQ-OOH, LLE and its components on NHEK

NHEK were treated with SQ-OOH, LLE, and its components at specified concentrations for durations indicated in the text. Cell culture media were collected for ELISA. The concentration of soluble PGD_2 in the supernatant was measured using the PGD_2 ELISA kit (Cayman Chemicals). Cell viability was assessed using a BCA protein assay kit (Thermo Fisher Scientific) or a cell counting assay by staining nuclei with Hoechst 33342 (Dojindo).

2.5. Treatment with PGD_2, ramatroban, LLE and its components on HFDPC

PGD_2 (Cayman Chemical), ramatroban (Tokyo Chemical Industry), LLE, and its components were dissolved and used to treat the HFDPC at specified concentrations for durations indicated in the text. Cell culture media and cells were collected for ELISA and real-time RT-PCR analysis. The concentration of soluble FGF5 in the supernatant of cell culture media was measured using an FGF5 ELISA kit (Ray Biotech). Real-time RT-PCR was performed using TB Green Fast qPCR Mix on a Thermal Cycler Dice Real Time System III (Takara Bio) with primer sets obtained from Takara Bio. The number of viable cells was determined using a cell counting assay with Hoechst 33342.

2.6. Human hair follicle organ cultures

Hair follicles isolated from the scalp of a 59-year-old Caucasian female were used for *ex vivo* study. Follicles were photographed on days 0, 5, 9, and 12 of culture, and the length from the hair bulb to the tip was measured using Olympus CellD software.

2.7. Statistical Analysis

Data are expressed as the mean ± standard error of the mean (SEM). Statistical analysis was performed using Student's two-tailed unpaired t-test for comparisons between two groups, and Dunnett's or Tukey's test for comparisons involving more than two groups. A *p*-value less than 0.05 was considered statistically significant.

3. Results

3.1. Effects of SQ-OOH

3.1.1. Preparation of SQ-OOH (quantification of MDA)

The concentration of MDA in the SQ solution increased in a dose-dependent manner following UVA irradiation. Irradiation at 60 J/cm^2 resulted in an MDA concentration of 32 µmol/L (Figure 1). In subsequent experiments, the concentration of SQ-OOH was expressed as MDA equivalents.

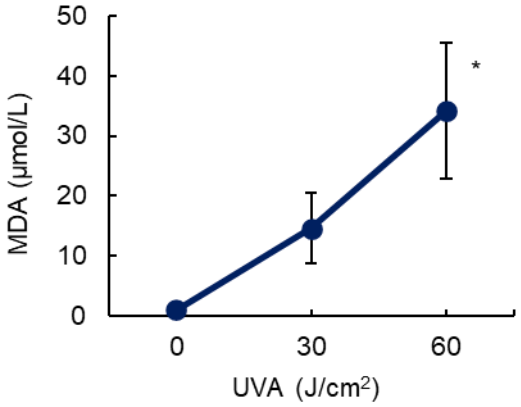

Figure 1. Changes in MDA concentration in squalene following UVA irradiation. MDA was quantified using an LPO Assay kit. Each bar represents the mean ± SEM., n=3, *; $p<0.05$ vs. 0 J/cm^2 using Dunnett's test.

3.1.2. SQ-OOH stimulated PGD_2 production in NHEK-neo and NHEK-ad

SQ-OOH induced PGD_2 production in a dose-dependent manner, with increased cytotoxicity observed in NHEK-neo (Figure 2a, 2b).

Therefore, differences in PGD_2 production responsiveness between NHEK-ad and NHEK-neo were examined at a concentration with minimal impact on cell viability. PGD_2 production was found to be approximately threefold higher in NHEK-ad compared to NHEK-neo (Figure 2c, 2d).

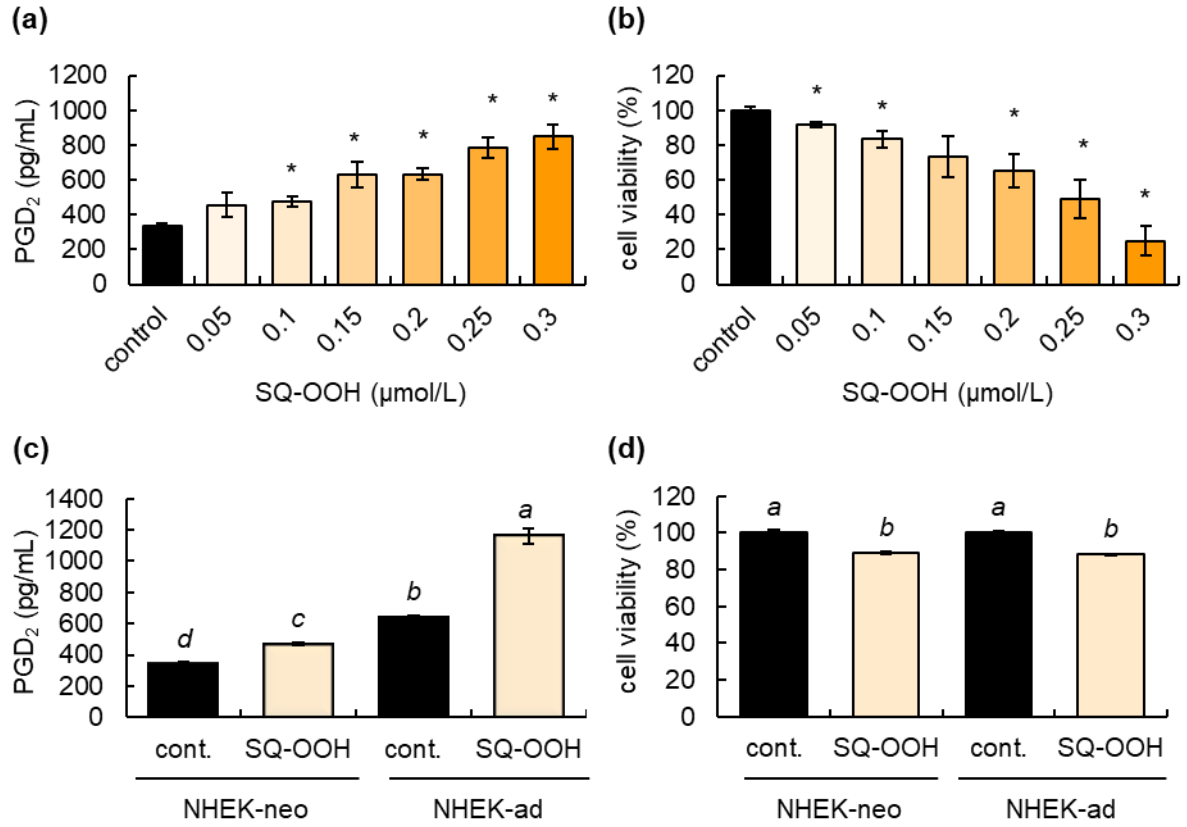

Figure 2. Changes in the PGD_2 production and cell viability with treatment of SQ-OOH in NHEK. PGD_2 was quantified using ELISA. Cell viability was measured using protein assay or cell counting assay. (a, b) NHEK-neo was stimulated by SQ-OOH at 0.05 to 0.3 μmol/L for 24 hours. Each bar represents the mean ± SEM., n=3, *: p<0.05, vs. control using t-test. (c, d) NHEK-neo and NHEK-ad were stimulated by SQ-OOH (0.1 μmol/L MDA equivalents) for 24 hours. Each bar represents the mean ± SEM., n=4, between different signs using Tukey test.

3.1.3. LLE and its components inhibited PGD_2 production induced by SQ-OOH in NHEK-ad

Over 100 plant-derived natural extracts were screened to identify compounds capable of inhibiting SQ-OOH-stimulated PGD_2 production in NHEK-ad. Among these, LLE demonstrated a dose-dependent attenuation of PGD_2 production at concentrations ranging from 0.4 to 10 μg/mL following 24 hours of treatment. The IC_{50} value of LLE was determined to be 0.7 μg/mL (Figure 3a). LLE contained 0.04% quercetin and 10.6% rutin (Figure 3b, 3c). The effects of these components on PGD_2 production were also evaluated. Both compounds were found to inhibit SQ-OOH–induced PGD_2 production, with quercetin exhibiting a more potent inhibitory effect than rutin (Figure 3d, 3e).

3.1.4. Comparison of the mRNA expression of PGD_2 receptors, DP1 and DP2 in NHEK-ad and HFDPC

PGD_2 induced by SQ-OOH may interact with its receptors, DP1 and DP2. To identify the target cells of PGD_2, the mRNA expression levels of these receptors were compared between NHEK-ad and HFDPC. The results revealed that the expression of DP2 mRNA was significantly higher in HFDPC (Figure 4).

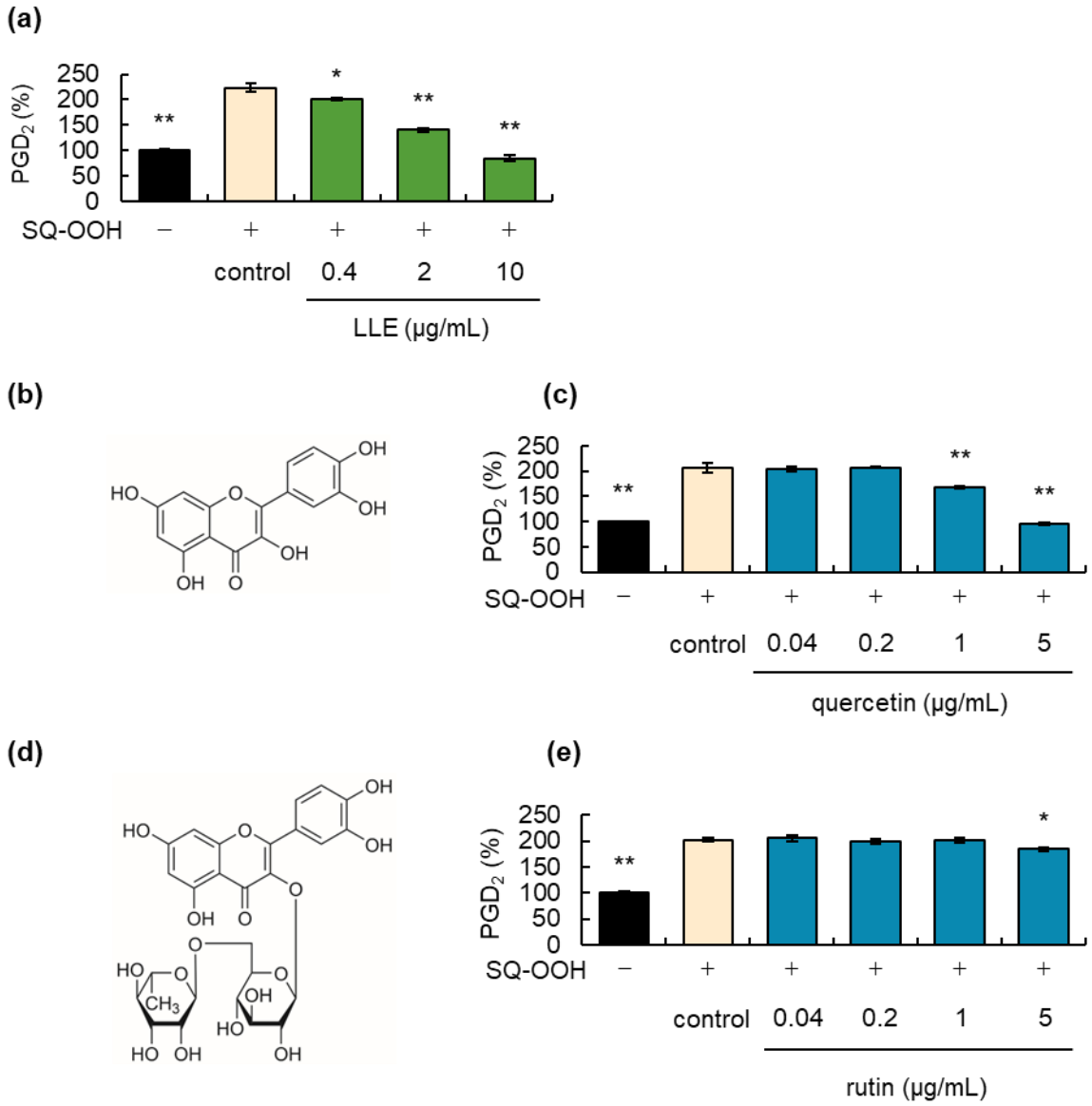

Figure 3. Inhibitory effects of LLE and its components on PGD_2 production in NHEK-ad. (a, c, e) PGD_2 production was analyzed using ELISA, normalized to cell count and expressed as a percentage of SQ-OOH untreated control. Each bar represents the mean ± SEM., n=4, *: p<0.05, **: p<0.01 vs. control using Dunnett's test. Structure of quercetin (b) and rutin (d) .

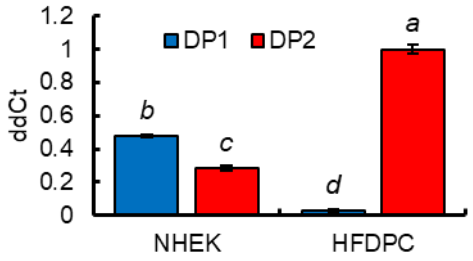

Figure 4. Comparison of DP1 and DP2 mRNA expression in NHEK-ad and HFDPC. DP1 and DP2 mRNA expression were analyzed using qRT-PCR. NHEK-ad and HFDPC were cultured with fresh medium for 24 hours.Each bar represents the mean ± SEM., n=3, between different signs using Tukey test.

3.2. Effects of PGD_2

3.2.1. PGD_2 increased FGF5 mRNA expression and protein production in HFDPC

The effects of PGD_2 on HFDPC were assessed by measuring FGF5 production, which negatively regulates the hair cycle. The results revealed that PGD_2 increased FGF5 mRNA and protein expression without affecting cell viability (Figure 5a, 5b). Ramatroban, a DP2 receptor antagonist, inhibited this effect (Figure 5c).

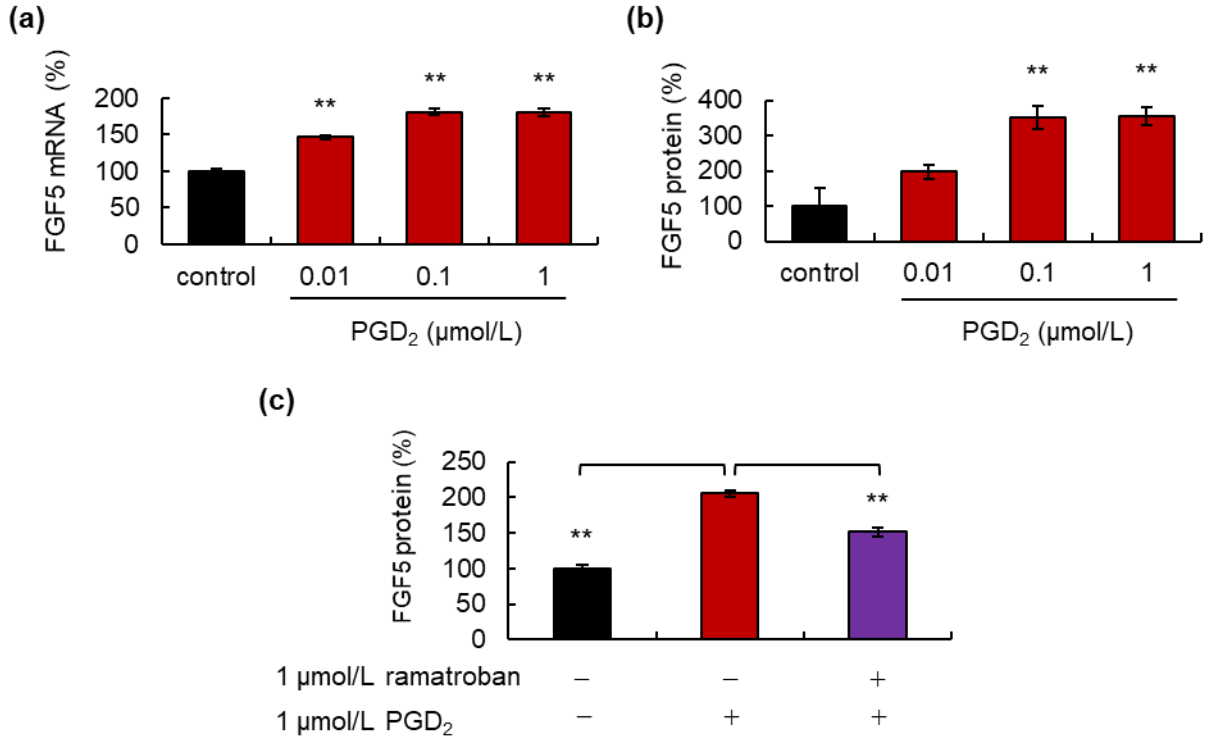

Figure 5. Effect of PGD_2 on FGF5 mRNA expression and protein production in HFDPC.
(a) PGD_2 was treated on HFDPC for 24 hours. FGF5 mRNA expression was analyzed using real-time RT-PCR. (b) PGD_2 was treated on HFDPC for 72 hours. FGF5 protein production was analyzed using ELISA. (c) FGF5 protein production was analyzed using ELISA. PGD_2 and ramatroban were treated at 1 μmol/L on HFDPC for 72 hours. Each bar represents the mean ± SEM., n=3, **: p<0.01 vs. control using Dunnett's test.

3.2.2. LLE inhibited FGF5 protein production induced by PGD_2 in HFDPC

LLE demonstrated a dose-dependent suppression of FGF5 production induced by 1 μmol/L of PGD_2 after 72 h of treatment in HFDPC (Figure 6a), without affecting cell viability. These findings suggest that LLE may help maintain the anagen phase of the hair cycle by inhibiting the production of FGF5 induced by PGD_2. LLE contains quercetin, rutin, 0.8% pinocembrin, and 1.4% pinobanksin as characteristic components, which were evaluated at concentrations corresponding to their content in LLE (Figure 6d, 6e). The results indicated that these components inhibited PGD_2 induced FGF5 protein production (Figure 6f, 6g).

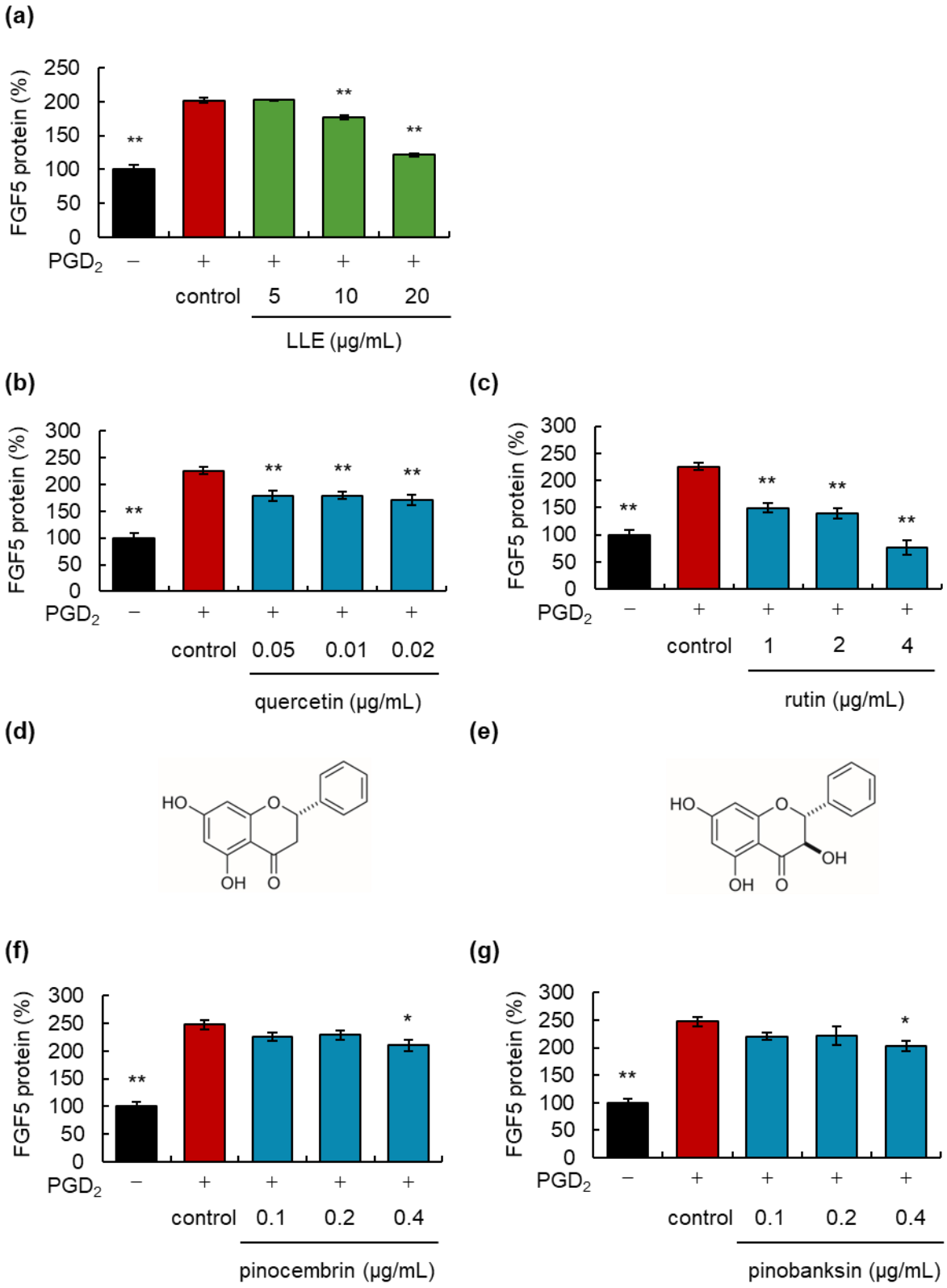

Figure 6. Inhibitory effects of LLE and its components on FGF5 protein production in HFDPC. (a, b, c, f, g) FGF5 protein production was analyzed using ELISA. Structure of pinocembrin (d) and pinobanksin (e) . Each bar represents the mean ± SEM., n=3-4, *: p<0.05, **: p<0.01 vs. control using Dunnett's test.

3.3. LLE promoted hair growth in organ-cultured hair follicles

The hair growth-promoting effects of LLE were investigated in organ-cultured hair follicles. At 0.1 µg/mL, LLE significantly promoted air shaft elongation compared to the control (Figure 7).

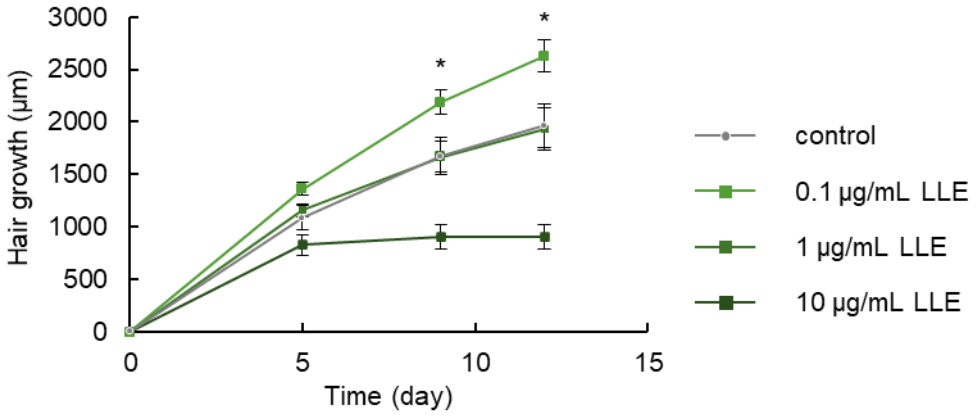

Figure 7. Hair growth-promoting effects of LLE. Organ-cultured hair follicles were treated with LLE, and the length from the hair bulb to the tip of the hair shaft was measured. Each bar represents the mean ± SEM., n=12, *: p<0.05 vs. control using Dunnett's test.

4. Discussion

This study investigated the roles of PGD_2 and SQ-OOH in hair thinning. SQ-OOH prepared via UVA irradiation, was shown to stimulate PGD_2 production in NHEK. SQ-OOH likely induces PGD_2 production by upregulating the expression and activation of cyclooxygenase-2, an enzyme critical to inflammatory responses, through the induction of Interleukin (IL)-1α and IL-8 [6,7]. Notably, PGD_2 production induced by SQ-OOH was higher in adult-derived keratinocytes than in neonatal-derived keratinocytes. Given the reported association between hair thinning and aging, adult-derived cells may be more susceptible to the adverse effects of sebum peroxidation [9]. Previous reports have also indicated that PGD_2 promotes testosterone production via reactive oxygen species, leading to excessive sebum production and subsequent deterioration of the scalp environment through peroxidation [10].

Suppressing PGD_2 production induced by SQ-OOH was explored as a potential strategy for addressing hair thinning by focusing on the scalp environment. Over 100 plant-derived extracts were screened, and LLE was identified as an effective inhibitor of PGD_2 production, with quercetin and rutin as key contributing components. PGD_2, a bioactive substance derived from arachidonic acid, is present in both men and women and is essential for biological functions. The DP1 receptor increases intracellular cAMP levels and exerts anti-inflammatory effects, whereas the DP2 receptor decreases intracellular cAMP levels and promotes inflammation [6]. Although PGD_2 has been implicated in hair thinning via DP2, its target cells had not been clearly identified. To elucidate this, gene expression levels of PGD_2 receptors were compared between NHEK-ad and HFDPC. The results revealed significantly higher expression of the DP2 receptor in HFDPC compared with NHEK-ad, suggesting that PGD_2 may preferentially act through DP2 in HFDPC and contribute to hair thinning. As expected, treatment of HFDPC with PGD_2 significantly increased the production of FGF5, an effect that was attenuated by

ramatroban, a DP2 receptor antagonist. PGD_2-stimulated FGF5 production was also inhibited by LLE and its components, pinocembrin and pinobanksin.

Hair shaft formation is known to involve activation of the Wnt/β-catenin pathway. Recent studies have reported that CXXC-type zinc finger protein 5 (CXXC5), a negative regulator of the Wnt/β-catenin pathway, is upregulated by PGD_2, contributing to hair thinning [11]. In the present study, dihydrotestosterone has been shown to induced CXXC5 expression via PGD_2. These findings suggest that PGD_2 produced by SQ-OOH stimulation may also contribute to CXXC5 induction, and that LLE may interrupt this cascade, thereby promoting hair shaft formation.

In *ex vivo* studies, LLE showed significantly enhanced hair shaft elongation. However, further investigation is required to fully elucidate the roles of PGD_2 and FGF5. Hair thinning is a complex phenomenon influenced by multiple factors, including aging, genetic predisposition, external environment, lifestyle, and psychological stress, affecting both men and women. LLE offers a potential solution for hair thinning driven by inflammatory responses resulting from sebum peroxidation.

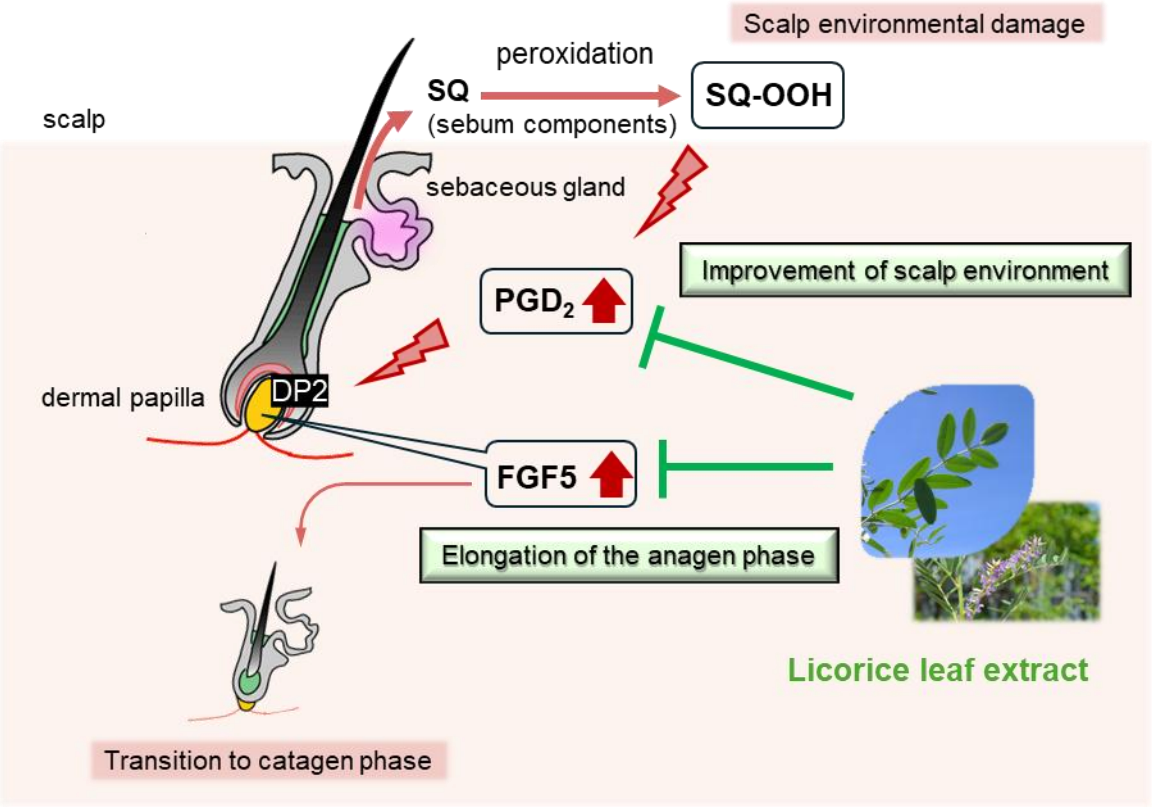

Figure 8. Original mechanism and solutions for hair thinning: licorice leaf extract inhibits the negative influence of sebum peroxidation on the scalp.

5. Conclusion

This study revealed a mechanism for hair thinning related to PGD_2 and FGF5, with sebum peroxidation identified as a significant contributing factor.These findings underscore the importance of maintaining and improving the scalp environment to prevent hair thinning. Furthermore, licorice leaf extract may be effective as a cosmetic ingredient in interrupting the cascade that leads to hair thinning(Figure 8).

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