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"Novel Plant-derived Polycarbon-chain Ceramide: An Ingredient that Promotes Endogenous Moisturizing and Repairing Effects"

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1. Introduction

Dry skin is a major concern in skin care. The normal skin stratum corneum has a water content of 15-20%, and the skin becomes rough once it drops below 10%[1]. The stratum corneum, which is the core of the skin barrier, comprise multiple layers of keratinocytes and intercellular lipids. Once the skin barrier is damaged, the integrity of the stratum corneum is destroyed, intercellular lipids and natural moisturizing factor (NMF), which reduces water loss, are intensified, and the skin becomes dry. Prolonged dryness makes the stratum corneum brittle and functional, further weakening the skin barrier and forming a vicious cycle [2]. Therefore, maintaining a healthy skin barrier and stratum corneum is key to preventing and improving skin dryness.

Studies have shown that ceramide deficiency in the stratum corneum may cause atopic dermatitis, skin barrier disruption, and dry skin. Ceramides are important components of the "brick wall" structure of the skin barrier and play an important role in regulating the skin's moisture barrier homeostasis and water-holding capacity [3]. When intradermal homeostasis is disturbed, glucosylceramides and sphingomyelins conversion to ceramides is inefficient, thus weakening the barrier function and upregulating skin dryness [4]. Further studies have shown that in addition to the total ceramide content, the different types of ceramides and the ratio between them have an influential effect on normal skin barrier function. HUANG et al. discovered that the skin barrier repair effect was most pronounced when ceramides 1 and 3 were used in a complex [5, 6], and thus, supplementing formulations with various ceramides repairs the skin barrier more effectively. This may be because ceramides, by virtue of their large number of hydrophilic groups, promote epidermal hydration, reduce water evaporation, enhance cellular cohesion, and prevent dry and flaky skin [7]. Ceramide 3 is the most abundant in the skin, accounting for about 22.1%. It consists of one molecule each of fatty and amino acids linked by amide bonds. The most common methods of preparing ceramide 3 are chemical synthesis or enzyme-catalyzed reactions. Chemical synthesis typically involves the reaction of stearic/oleic acid and phytosphingosine to obtain the structure of the target ceramide 3. Using the fatty acid portion of vegetable oils, fats, and phytosphingosine for preparing ceramides has been less well studied. Natural plant-derived ceramides have more sustainable and environmentally friendly source of raw materials that are similar to the composition of dermal ceramides. Hence, they can better repair the skin barrier to prevent water.

Peony seed oil is made from peony seed kernel through certain extraction and refining processes. It contains more than 80% polyunsaturated fatty acids, such as α-linolenic acid and linoleic acid, which have numerous skincare benefits, such as moisturizing, antioxidant, inflammation reduction, and skin repair [8, 9]. Studies on plant-derived ceramide 3 or its

complex compositions obtained from peony seed oil are few. In this study, we aimed to address the problem of dry skin by determining the difference between ceramide 2,5,6, and 10 in dry and normal skin using lipidomic analysis. Using microbial fermentation to obtain phytosphingosine, combined with fatty acids from peony seed oil, a natural synthesis process is employed to produce Multi-carbon chain ceramide 3. Furthermore, in vitro three-dimensional (3D) skin modeling experiments were performed to investigate the mechanism of action of peony seed oil-derived ceramide 3 in repairing dry skin and its synergistic effect with various ceramides in repairing the skin barrier.

2. Materials and Methods

2.1 Reagents, materials, and instruments

Filaggrin (FLG) and cysteinyl aspartate specific proteinase 14 (Caspase 14) primary antibodies and fluorescent secondary antibody were purchased from Abcam, Human interleukin-6 (IL-6) enzyme-linked immunosorbent assay (ELISA) kit was purchased from Lenovo, Human tumor necrotic factor-alpha (TNF-α) ElISA kit was purchased from PhD, nuclear factor-kappa beta (NF-κB) primary antibody was purchased from CST, pyrrolidone carboxylic acid standard was purchased from Sigma, cis-uranic acid standard was purchased from TargetMol, and trans-uranic acid standard was purchased from McLean.

2.2 Lipidomics analysis

(1) Volunteer recruitment and grouping: 70 women aged 18-40 were recruited in Beijing, China. Based on the overall dry skin score (ODS), the women were grouped into dry skin (ODS=2-4) and healthy control (ODS=0)groups of 35 each.(Ethical Review Approval Number:EWISH-IEC-015-2022)

(2) Lipid sample collection and extraction: Skin lipid samples were collected using a peeling tape on the outer side of the volunteers' calves 10 times repeatedly and stored at -80℃. The lipids were extracted using the methyl tertiary butyl ether method.

(3) Ultra Performance Liquid Chromatography and mass spectrometry analysis: a reversed-phase T3 column with a column temperature of 35°C and a flow rate of 0.4 ml/min was used. Water and 0.1% formic acid were used as the mobile phase A, acetonitrile and 0.1% formic acid were used as the mobile phase B. The gradient of change were 0-0.5 min, 5% solvent B; 0.5-7 min, 5%-100% solvent B; 7-8 min, 100% solvent B; 8-8.1 min, 100%-5% solvent B; 8.1-10 min, 5% solvent B).

2.3 Preparation of ceramide 3 and quadruple ceramide complexes from peony seed oil source

Peony seed oil and phytosphingosine were mixed thoroughly in a beaker. Subsequently, the mixture was subjected to continuous stirring at 100°C and 200 rpm for 3 h. The ceramide composition was made in emulsified form. For further isolation and refinement, hexane was added to induce precipitation. The mixture was filtered, and the precipitate was collected to obtain the peony seed oil-derived ceramide 3 of higher purity.Using peony seed oil-derived ceramide 3 as the core ingredient, a ceramide complex containing ceramide 1, ceramide 2, ceramide 3, and ceramide 6 was prepared using emulsification homogenisation technology.

2.4 Moisturizing and Repairing Efficacy Test

2.4.1 Filaggrin and cysteinyl aspartate specific proteinase 14 expression

Based on the Skinovo®-Epi epidermal model, UVA irradiation was performed in the UV irradiation, positive control, and sample groups. After completion, 25 μL of peony seed oil-derived ceramide 3 and quadruple ceramide complex was added to samples from the sample group. The samples were incubated for 24 h at saturated humidity, 5% CO2, and 37°C.Subjected to FLG and Caspase 14 immunofluorescence staining. The samples were imaged using a fluorescence microscope, and the average fluorescence intensity of FLG and Caspase 14 was calculated using Image J software.

2.4.2 Moisturizing factor content test

Based on the epidermal model, the sample group was surface spiked with 25 μL of peony seed oil-derived ceramide 3 and quadruple ceramide complex. The machine was used to detect Pyrrolidone Carboxylic Acid (PCA) and Urocanic acid (UCA). Where: UCA content = cis-UCA content + trans-UCA content.

2.5 Soothing efficacy of ceramide 3 from peony seed oil source tested

2.5.1 Nuclear factor kappa-B assay

Based on the epidermal model, UVB irradiation was performed in the UV-irradiated, sample, and positive control groups. The sample group was spiked with 25 μL of ceramide 3 from peony seed oil， subjected to immunofluorescence staining for NF-κB. The samples were photographed by a fluorescent microscope and the average fluorescence intensity of NF-κB was calculated using the Image J software.

2.5.2 Inflammatory Factor Testing

Based on the Skinovo®-Epi epidermal model, the positive control, negative control, and sample groups were irradiated with UVB before spiking. After irradiation, the samples were surface-administered with 25μL of ceramide 3 from peony seed oil. After 24 h, the culture medium of each group was collected, centrifuged, and the culture supernatants were analyzed for IL-6 and TNF-α according to the manufacturer's instructions of the ELISA kit. The release inhibition rate was calculated.

2.6 In vitro skin whitening based on 3D skin modeling with peony seed oil-derived ceramide 3

Based on the Skinovo®-Mela melanin model, the positive control, negative control, and sample groups were irradiated with UVB before spiking. The surfaces of the samples were treated with a gas-liquid form of 25 μL of peony seed oil-derived ceramide 3 on days 1 and 8.

1) Apparent colorimetry: Individual models were placed in the circle of the colorimetric card, camera parameters were adjusted, and the samples were photographed.

2) Determination of the apparent brightness L* value: The model was placed on photo paper with the cuticle placed face up and tested thrice in parallel using a colorimeter.

3. Results

3.1 Lipid composition analysis

3.1.1 Trend analysis of lipid subgroups

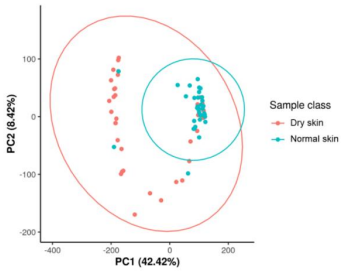


Figure 1. PCA scores of skin surface lipids between dry and normal skin

Figure 1 shows the PCA scores from the lipid analysis. Dry skin samples were relatively dispersed, while normal skin samples were relatively concentrated. This suggests that normal skin has a consistent lipid metabolism, while that of dry skin varies widely.

3.1.2 Trend analysis of lipid subgroups

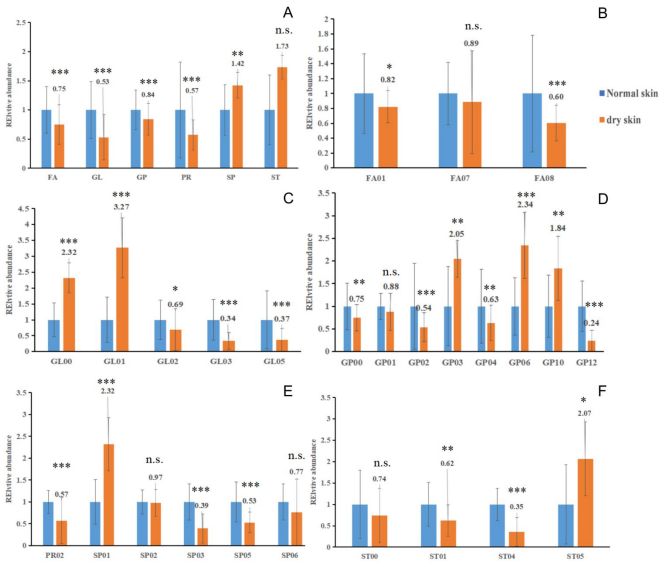


Figure 2. Comparison of relative abundance in dry and normal skin. ((A)Total,(B)fatty acyls, (C)glycerolipids, (D)pentenolipids, (E)sphingolipids, (F)sterolipids [ST]) (***P<0.001; **P<0.01; *P<0.05)

Overall, 624 lipid components were detected in 70 samples. The lipid content of dry skin differed significantly from that of normal skin. The relative abundance of fatty acyls [FA], glycerol lipids [GL], glycerophospholipids [GP], and prenyl lipids [PR] were much lower than that of normal skin, whereas sphingomyelin lipids [SP] were significantly higher, and sterol lipids [ST] did not show any significant difference in abundance.

3.1.3 Analysis of the relative content of different lipids

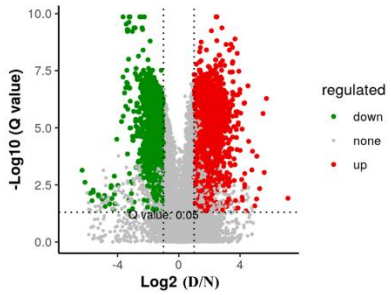


Figure 3 Differential lipid volcano map (D: dry skin; N: normal skin)

Figure 3 shows an up-regulation of several lipid contents in dry skin compared with normal skin, while other contents were significantly decreased and differentiated. Of the lipid components on the skin surface, ceramides are important because they play key roles in skin hydration. Our analysis revealed six different ceramides: Cer-NS, Cer-NdS, Cer-NP, Cer-AS, Cer-AP and Cer-AdS (Table I). Of these, Cer-NS, Cer-NdS, Cer-AP, and Cer-AS were significantly absent in dry skin, whereas Cer-AdS was significantly elevated. Cer-AS, Cer-NS, Cer-AP, Cer-NP and Cer-EOP are listed in the catalog of used cosmetic ingredients (2021).

Studies have shown that Cer-EOS decreases in healthy people with age[10], while CEREO content decreases in patients with Atopic dermatitis, especially CEREOS [11]. Considering the above studies and findings, we compounded 4 heavy ceramides (CERNS, CERAP, CERNP, and CEREOS) to solve the dryness problem fundamentally.

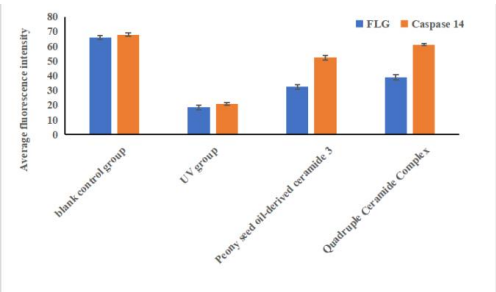
Table I Rarest individual lipid types in dry skin

DESCRIPTIVE	KIND	FOLDING CHANGE	P
MGDGO-9:0_18:5	GLYCOSYLDIACYLGLYCEROLS [GL0501]	0.060	0.000
CER21:1;2O/4:0	N-ACYLSPHINGOSINES (CERAMIDES) [SP0201]	0.153	0.000
CER10:0;2O/17:1	N-ACYLSPHINGANINES (DIHYDROCERAMIDES) [SP0202]	0.156	0.000
SM14:2;2O	CERAMIDE PHOSPHORYLCHOLINE (SPHINGOMYELIN) [SP0301]	0.157	0.000
TG8:0_8:0_8:0	TRIACYLGLYCEROL [GL0301]	0.192	0.000
NAORN18:5/14:1	NITRAMIDE [FA0802]	0.203	0.000
PE-CER17:3;2O/18:4	CERAMIDE PHOSPHOETHANOLAMINE [SP0302]	0.206	0.000
MGDGO-16:4_10:0	GLYCOSYLDIACYLGLYCEROLS [GL0501]	0.233	0.000
MLCL16:0_20:3_20:4	MONOACYLGLYCEROPHOSPHOGLYCEROPHOSPHOMONORADYLGLYCEROLS [GP1207]	0.239	0.000
TG8:0_10:0_10:0	TRIACYLGLYCEROL [GL0301]	0.251	0.000

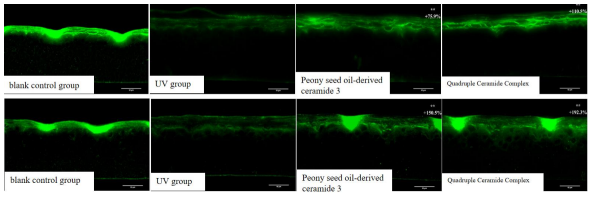
3.2 Results of moisturizing and repairing efficacy test

3.2.1 FLG and Caspase 14 assay results

FLG is synthesized by keratin-forming cells, and its precursor is pre-filament polyprotein. When keratinocytes move from the granular layer to the stratum corneum, the pre-filament polyprotein is rapidly dephosphorylated to FLG, which can be degraded to NMF. Caspase 14, a key enzyme that regulates the dephosphorylation of the pre-filament polyprotein to FLG, is an important factor in the formation of the skin barrier and plays an important role in moisturizing and maintaining the skin barrier.



(**a**)



(**b**)

Figure 4. (a)Immunofluorescence results of FLG and Caspase 14 protein expression;(b)Fluorescence intensity.The green color represent FLG and Caspase14. The brighter the green marking, the higher the protein content of the skin barrier.

As shown in the experimental results, compared with the UV-irradiated group, peony seed oil-derived ceramide 3 and the quadruple ceramide complex could significantly promote FLG and Caspase 14 expression, indicating that both had significant moisturizing and repairing effects on the skin barrier damage induced by UV stimulation. The quadruple ceramide complex had a better moisturizing and repairing effect than the peony seed oil-derived ceramide 3, which proves that the quadruple ceramides have synergistic effects on skin barrier repair.

3.2.2 Results of PCA and UCA content assays

PCA and UCA are the main components of NMFs in the skin. PCA has a high hydration capacity and can absorb water from the surrounding environment and lock moisture inside the skin. In contrast, UCA helps the skin retain moisture. It absorbs water directly and enhances the overall moisturizing effect by interacting with other NMFs and skin components.

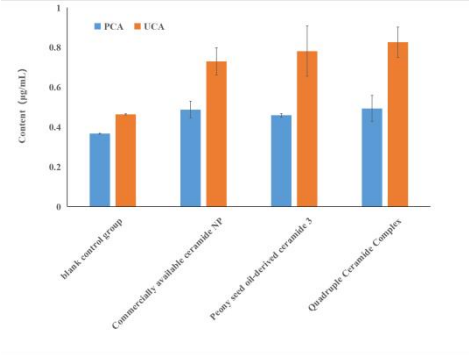


Figure 5 Results of PCA and UCA content detection.

As shown in the test results, compared with the blank control group, peony seed oil-derived ceramide 3 and quadruple ceramide complex significantly increased the PCA and UCA contents in the epidermal model. The enhancement rate of peony seed oil-derived ceramide 3 was 24.9% and 68.7% for PCA and UCA, respectively, while that of the quadruple ceramide complex was 34.2% and 78.1%, respectively, which further proved that the quadruple ceramide complex had synergistic effects with ceramides 1, 2, and 6 in repairing the skin barrier.

3.3 Results of in vitro soothing efficacy assay of ceramide 3 from peony seed oil source

3.3.1 NF-κB assay results

NF-κB is an important intracellular nuclear transcription factor involved in the body's inflammatory and immune responses. When excessive UV irradiation and other irritants are applied to the skin, they cause NF-κB activation in keratinocytes and other cells, which in turn triggers an inflammatory response.

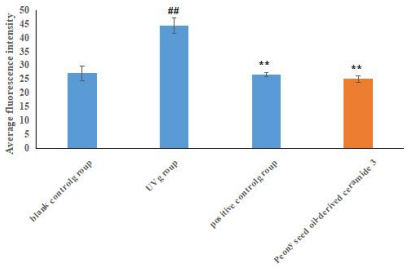
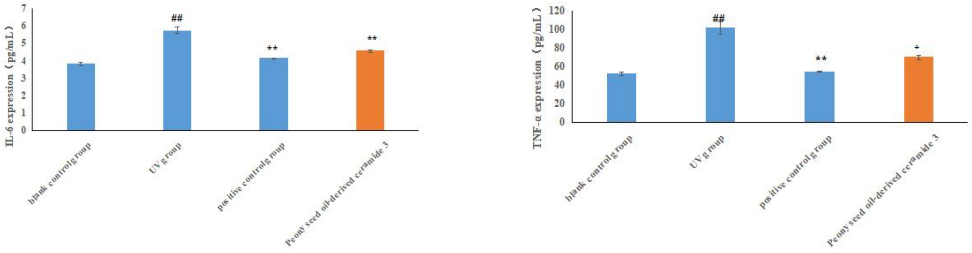


Figure 6 Immunofluorescence results of NF-κB expression

The peony seed oil-derived ceramide 3 inhibited NF- κ B expression in the UV-irradiated epidermal model extremely significantly by 43.5% compared with the UV-irradiated group (Figure 6).

3.3.2. IL-6 and TNF-α expression

When skin models are exposed to external stimuli (such as UV irradiation and air pollution), an inflammatory response is induced in the skin. TNF-α andIL-6 are important cytokines in the early stages of the inflammatory response.



(**a**) (**b**)

Figure 7. Results of IL-6 (a)and TNF-α(b) assays.

Compared with the negative control group, the sample of peony seed oil-derived ceramide 3 inhibited IL-6 expression significantly (P<0.01), with an inhibition rate of 20.8%±0.9%, and TNF-α expression (P<0.05) with an inhibition rate of 31.1%±2.4%. Ceramide 3 from peony seed oil, when applied to UV-irradiation-induced epidermal model for 24 h, reduced NF-κB expression and inhibited IL-6 and TNF-α expression, thus exerting soothing effect.

3.4 Results of whitening efficacy assay of ceramide 3 from peony seed oil source

(**a**) (**b**)

Figure 8 Results of Apparent Colorimetry Test.(a)blank group;(b)blank group;(c)positive control group;(d)peony seed oil-deried ceramide 3.

The overall color of the melanin model was obtained by taking pictures with the color gradient of the colorimetric ring card. The whiter the overall color, the better the whitening effect of the sample. The results showed that the apparent coloration of the negative control model was darker than that of the blank control model, indicating that the model can darken in response to external UVB irradiation. The apparent coloration of the peony seed oil-derived ceramide 3 model became whiter compared than that of the negative control model.

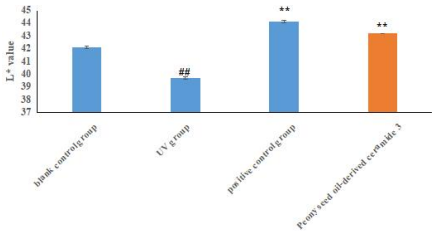


Figure 9. Apparent brightness L* value detection results.## P<0.01 compared with blank control group; **: P<0.01 compared with negative control

Brightness is characterized by the L* value measured by a chromaticity system (Lab Chromaticity System) colorimeter. The change indicates the change in black-white chromaticity of the skin. The larger the value, the more the color is inclined to white, which indicates that the whitening effect of the samples is better. As shown by the test results, the sample peony seed oil-derived ceramide 3 can significantly increase the modeled apparent brightness L* value compared to the negative control.

4. Discussion

Skin dryness has long plagued people and its causes are complex. CERN-(tetracosanoyl)-phytosphingosine (CERNP), the major ceramide isoform in the healthy stratum corneum and one of the most vulnerable to loss, is significantly reduced in patients with atopic dermatitis. Increasing the CerNP level to normal is the main method for restoring the skin barrier and the first choice to address dry skin [12]. The present study comprehensively demonstrated the efficacy of peony seed oil-derived ceramide 3 in

enhancing endogenous moisturization and repairing the skin barrier. Our results elucidate the mechanism of action of peony seed oil-derived ceramide 3 in upregulating key skin structural proteins, modulating inflammatory pathways, and synergizing with other ceramides to treat dry skin.

This study showed that peony seed oil-derived ceramide 3 repaired skin barrier damage caused by UV irradiation, resulting in a 75.9% and 150.5% increase in FLG and caspase 14 expression, respectively. FLG is an important structural protein and a major source of moisturizing factors. Therefore, we further verified the promotional effect of peony seed oil-derived ceramide 3 on NMF and found that the PCA and UCA content increased by 24.9% and 68.7%, respectively, suggesting that peony seed oil-derived ceramide 3 enhanced the hydrolysis of FLG proteins and the synthesis of NMF to improve the hydration and elasticity of the stratum corneum, achieving endogenous moisturization and repair. The observed effects may be attributed to the structural similarity between peony seed oil-derived ceramide 3 and endogenous ceramides, which can be integrated into the lipid matrix to stabilize the cohesion of keratinocytes and promote their terminal differentiation. In addition, we found that peony seed oil-derived ceramide 3 inhibited NF-κB activation, leading to reduce the expression of inflammatory factors IL-6 and TNF-α and significantly increased the skin's epidermal brightness and advanced skin barrier repair.

Further, we used non-targeted lipidomics technology to analyze the differences in lipid composition of the surface of dry calf skin in volunteers, and discovered that ceramides 2, 5, 6, and 10 were significantly absent in dry skin. Acne, dermatitis, and other disorders are related to the absence of ceramides 1, 2, 3, and 6. Combining the above studies and our findings, we compounded 4 ceramides (CERNS, CERAP, CERNP, CEREOS) to address the root cause of dryness. The natural composition of the stratum corneum was mimicked by supplementation with 4-ceramides, which addressed quantitative and qualitative lipid deficiencies. Skin modeling results showed a 110.5% and 192.3% increase in FLG and caspase 14, effectively demonstrating that tetra-ceramide supplementation is more effective in restoring the "brick and mortar" structure of the skin barrier than mono-ceramide therapy. These results emphasize the synergistic interaction between peony seed oil-derived ceramide 3 and ceramides 1, 2, and 6, and highlight the importance of ceramide diversity in skin barrier restoration.

5. Conclusion

This study revealed that plant-derived ceramide 3 significantly enhanced PCA and UCA synthesis and endogenous barrier repair by promoting FLG and caspase14 expression. In addition, we discovered that peony seed oil-derived ceramide 3 can inhibit NF-κB activation and IL-6 and TNF-αexpression, and significantly increase the apparent brightness of the skin. We also validated that it works synergistically with ceramides 1, 2, and 6 to optimize the lipid composition of the stratum corneum and stabilize the "brick wall structure.” Peony seed oil-derived ceramide 3 mimics the structure of natural ceramides and repairs UV-induced

barrier damage, providing theoretical support for developing plant-derived skincare regimens based on the modulation of lipid metabolism.

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