Unsaturated fatty acid-enriched extract from Hippophae rhamnoides seed reduces skin dryness through up-regulating aquaporins 3 and hyaluronan synthetases 2 expressions

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Abstract

Background: Seed oil of sea buckthorn (SBT) is well known to contain high amount of polyunsaturated fatty acid (PUFA), and PUFA is generally acknowledged to promote skin hydration by reducing trans-epidermal water loss (TEWL).

Aims: The present study is aimed to investigate that skin hydration offered by SBT seed oil is whether through up-regulating AQP3 or HAS2 expression.

Methods: MTT assay was performed to detect cytotoxicity of SBT seed oil, and then, PCR was carried out to explore whether SBT seed oil can increase AQP3 mRNA expression in normal human epidermis keratinocytes (NHEK) cells or not. Immunofluorescence (IF) and Western blot analysis were used to test the protein level expression of AQP3 and HAS2 influenced by SBT seed oil in NHEK cells or in reconstructed epidermis skin model.

Results: According to the result of MTT assay, all test concentration of SBT seed oil showed no cytotoxicity to cells. 10 μg/mL SBT seed oil treatment evidently increased AQP3 mRNA level compared to negative control (NC). IF and Western blot analysis results demonstrated that AQP3 and HAS2 protein levels in NHEK cells treated with 10 μg/mL SBT seed oil were much higher than that of NC. Finally, treatment with 10 μg/mL SBT seed oil substantially up-regulated expression of AQP3 and HAS2 protein in reconstructed epidermis skin model in comparison to NC.

Conclusions: In summary, our study first proved that SBT seed oil can improve skin hydration through increasing AQP3 and HAS2 expressions.

KEYWORDS
AQP3, HAS2, reconstructed epidermis skin model, sea buckthorn seed oil, skin hydration
INTRODUCTION

Skin is the largest and a highly complex organ, acting as the outermost barrier to protect against surrounding environment, including chemical and mechanical threats, pathogens, toxic agents, and ultraviolet radiation; and to maintain homeostasis through regulating hydration and temperature. The skin is a multi-layered organ composed of several compartments: the outermost, stratified, and non-vascularized epidermis, the underlying dermis containing connective tissue, and the deeper hypodermis with adipose tissue. The stratified epidermis mainly consists of keratinocytes, melanocytes, and immunocytes. According to the degree of differentiation of the keratinocytes, the epidermis is divided into the stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB). The dermis is rich in collagen, which harbors fibroblasts and mast cells. The hydration level of the human skin is essential for the function and biophysical properties of the skin. Perturbation or dysregulated of skin hydration in the skin might lead to functional defects and clinical symptoms, such as dryness skin was proved to be susceptible to wrinkled, rough, or scaly properties, with the possible presence of reddening, itching, or cracking. It has been suggested that skin hydration is dependent on several factors, particularly skin barrier and membrane-bound pores (aquaporins) and hyaluronic acid (HA), which facilitate the water transporting and water binding in skin.

Aquaporins (AQPs) are channels that facilitate the water across cell plasma membranes, and 13 family members (AQPO-AQP12) have been identified in human to date. AQPs are expressed in various organs, such as kidney, skin, respiratory, and gastrointestinal tracts. And AQPs are categorized into two groups according to their permeability: AQPO, AQP2, AQP4, and AQP5 are water-selective transporters, AQP3, AQP7, AQP9, and AQP10 are primarily responsible for transporting water, glycerol, or other small solutes including urea. Among them, AQP3 is the most abundant AQP presented in the skin, and abundantly expressed and localized to the spinous and basal layers of the epidermis, which functioned as both a water and glycerol transporter (aquaglyceroporin) in epidermis. Glycerol transported by AQP3 plays an important role in SC hydration, improving barrier function and repair by facilitating biosynthesis of various lipids and promoting desquamation. It is a remarkable fact that there is a continuous reduction of AQP3 level from the SB to the SG in epidermis, and the gradient of AQP3 is consistent with water gradient in the epidermis with a sharp decrease of water content in the SC (10%-15%) compared with the underlying layers (75%), suggesting that AQP3 are important in normal skin physiology, especially in skin hydration process. It has been reported that AQP3-deficient mice displayed selectively reduced glycerol content and water-holding capacity in the epidermis, which cannot be improved by high humidity or skin occlusion. AQP3-deficient mice exhibited a delayed recovery of permeability barrier after acute barrier disruption through tape stripping, while accelerated barrier recovery was observed in transgenic mice overexpressing AQP3. Another study observed that AQP3-deficient keratinocytes can cause problem in cell proliferation, migration, and scratch wound healing. In addition, many evidences indicated that expression of AQP3 was affected by UV exposure or aging can further result in skin dryness. Abnormal expression of AQP3 was involved in a range of skin disease, and it has been demonstrated that expression inhibition of AQP3 was observed in vitiligo, eczema, and psoriatic lesions.

Hyaluronan (HA), a high-molecular-weight glycosaminoglycan, which is composed of alternating unit of N-acetylgalucosamine and glucuronic acid, is a major component of extracellular matrix (ECM), which can retain water effectively, absorbing >1000 times its weight. HA is synthesized by three different HA synthetases (HASs), known as HAS1, HAS2, and HAS3. And it has been proved that HAS2 stands out as a more essential synthetase in vivo compared to HAS1 and HAS3. Because of retains moisture and highly viscous properties, HA is responsible for facilitating skin hydration and further influence cell proliferation, inflammation, and wound healing. Related study indicated that aging and extrinsic stimuli such as UV radiation, air pollutants, and smoking progressively reduces the amount of HA in the body, leading to dry skin and wrinkle formation.

Hippophae rhamnoides L., as a thorny deciduous shrub, naturally grows at high altitude (2500-4000 m) in Europe and Asia and it has orange-yellow fruits. It is usually named as sea buckthorn (SBT), belonging to the Elaeagnaceae family. Because of its multiple medicinal and nutritional properties, such as antioxidant, cardioprotective, and anticancer, SBT has been used in Tibetan, Mongolian traditional medicine for a long time. It has been found that all parts of plant are natural resources of bioactive compounds. Extracts of SBT leaves are rich in flavonoids, triterpenes, and tannins, which has therapeutic effect on wound healing, thrombosis, and hypertension. SBT berries are recognized as a good source of vitamins (C, A, E, and K), polyphenols, organic acids, carotenoids, which might contribute to its antioxidants, antimicrobial properties. Previous studies reported that SBT seed oil has a high content of polyunsaturated fatty acid (PUFA), such as linoleic acid and α-linolenic acid, which are responsible for its cardioprotective and antiatherogenic activities. Recent study suggested that SBT oil had a beneficial effect on AD-like skin lesions via repression inflammation, and SBT seed oil prevented UV-induced impair in lipid metabolism disorders in skin. Oil was generally acknowledged that it can promote skin hydration through reducing trans-epidermal water loss (TEWL). Eicosapentaenoic acid (EPA), a common polyunsaturated fatty acid, proved that it can attenuate UV-induced inhibition of AQP3 in human keratinocytes. SBT seed oil have also been demonstrated that highly enriched in PUFA, but there is still no research investigated whether SBT seed oil affect skin hydration not only by reducing TEWL but also via promotion of AQP3 or HAS2 expression. The present study was aimed to explore whether SBT seed oil treatment offers skin hydration through up-regulating AQP3 or HAS2 expression.
2 | MATERIALS AND METHODS

2.1 | SBT seed oil

SBT seed oil was purchased from Puredia Corporation Limited (Hongkong, China). The oil was extracted from dried SBT seeds using supercritical CO$_2$ extraction. Samples were run in the column using split mode (split ratio = 100:1). The nitrogen carrier gas was programmed to maintain a constant flow rate of 1 mL/min. The oven was initially set to 100°C for a duration of 13 min, then raised to 180°C at 10°C/min increments for a duration of 6 minutes, then raised to 200°C at 1°C/min increments for a duration of 20 minutes, and then raised to 230°C at 4°C/min increments for a duration of 10.5 minutes. Major components of SBT seed oil are listed in Table 1.

2.2 | Cell culture

NHEK cells were purchased from Guangdong Biocell Co., Ltd (Guangdong, China) and incubated with Epilife (Gibico, Thermo Fisher Scientific) containing 60 μM Ca$^{2+}$ and HKGS (Gibico, Thermo Fisher Scientific) at 37°C in a humidified atmosphere with 95% air and 5% CO$_2$. The medium was changed every two days; when the cells were grown to 70% to 80% confluence, the cells were digested and used for the next experiment. The cells were grown for no more than 8 cell generations.

2.3 | Reconstructed human skin models

Episkin™, a reconstructed epidermal model (EPISKIN-SNC, Lyon, France), was used for our research. Skin models upon arrival were immediately transferred to 6-well plates containing 1mL maintenance medium provided by EPISKIN-SNC, and they were incubated in standard condition (37°C, 5% CO$_2$) for 18-24 hours in accordance with manufacturer’s instructions. SBT seed oil was dissolved into DMSO at a final concentration of 10 mg/mL; then, SBT seed oil was applied to maintenance medium (final concentration of SBT seed oil 10 μg/mL) and cultured at 37°C, 5% CO$_2$ for 24 hours.

2.4 | Cell viability assay

The NHEK cells were seeded into a 96-well plate at a density $5 \times 10^4$ cells per well. Then, cells were starved by incubating with HKGS-free media for 12h. SBT seed oil (0.25, 0.5, 1, 10, 100 μg/mL) was used to treat cells for 24 hours at 37°C in a humidified atmosphere with 95% air and 5% CO$_2$. After adding CCK-8 (Beyotime Biotechnology) 10 μL/100 μL, cell viability was evaluated by reading absorbance at 450 nm wavelength (ID5 plate reader, molecular device). The inhibition ratio was calculated, and a growth curve was made. The calculation formula is as follows: Viability (%) = (Optical Density (OD) control group - OD treatment group)/ OD control group 100%

2.5 | Gene expression analysis

Then, total RNA was extracted from the cells by using the Trizol reagent (Invitrogen Life Technologies) according to the manufacturer’s protocol. The concentration and quality of RNA were determined using Qubit 3.0 (Thermo fisher). The extracted RNA was used as a template for subsequent cDNA synthesis with oligo dT primers using the Prime script RT reagent Kit (Thermo Fisher). Initial mRNA levels were quantified using a Premix Taq™ cDNA kit (Takara Bio Inc) in accordance with the manufacturer’s instruction. DNA was first denatured at 98°C for 10s and then annealed at 68°C for 30 seconds. This was repeated for 30 cycles before a final extension at 72°C for 10 minutes. Then, the PCR products were run on a 2% agarose gel; gene expression was assessed with the use of iBright FL1000 image system (Thermo fisher Scientific, USA). The gels were quantified by densitometry using iBright analysis software (Thermo fisher Scientific) and normalized to GAPDH. All values are reported as means ± standard deviation (SD). The following primers were used for amplification: AQP3, forward 5′-CAT CTA GCA CTT AAA GGC TTA-3′ and reverse 5′-GGC TGT GCC TAT GAA CTT GT-3′; GAPDH forward 5′-GGT GAA GGT CGG AGT CAA CG-3′ and reverse 5′-CAA AGT TGT CAT GGA TGH ACC-3′.

2.6 | Western blotting

After treatment, cold phosphate-buffered saline (PBS) was used to wash the cells three times, and M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, USA) containing a protease inhibitor cocktail (1:100; Roche Applied Science) was used to lyse the cells in accordance with manufacturer’s protocol. Protein concentrations were determined using a bicinchoninic Protein Assay kit (Pierce; Thermo Fisher Scientific, USA). The proteins (30 μg) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then proteins were transferred onto a polyvinylidene fluoride membrane (PVDF) using Iblot2 system (Thermo fisher Scientific). Then, Tris-buffered saline-Tween (TBST) containing 5% bovine serum albumin (BSA) was used to block the membrane for 30 minutes.

| Major fatty acids and contents in SBT seed oil |
|-----------------------------------------------|
| **Fatty acids (%)**                           |
| Oleic acid (C18:1) | Linoleic acid (C18:2) | Linolenic acid (C18:3) | Vitamin E (mg/g) | α-tocopherol (mg/g) |
| 22.35 | 36.42 | 27.89 | 1.85 | 0.56 |
at room temperature. After blocking, the membrane was incubated at 4°C for 12 hours with rabbit anti-AQP3 at a dilution of 1:1000 (PA5-78811, Invitrogen) or mouse anti-HAS2 at a dilution of 1:1500 (NBP2-37446, Novusbio) and anti-GAPDH at a dilution of 1:1000 (Invitrogen) antibodies. PBS (phosphate-buffered saline) containing 0.1% Tween-20 was used to wash the membranes at least 3 times (5 minutes each) followed by incubating with anti-rabbit horseradish peroxidase-labeled secondary antibody at dilution ratio of 1:10 000 (31 460, Invitrogen) or anti-mouse IgG H&L (Alexa Fluor® 555) (ab150114, Abcam) in PBST for 2h at room temperature. Protein expression was assessed with the use of iBright L1000 image system (Thermo fisher Scientific, USA). The blots were quantified by densitometry using iBright analysis software (Thermo fisher Scientific) and normalized to GAPDH.

2.7 | Immunofluorescence assay

After treatment, cold PBS was used to wash the treated cells and control cells twice and then fixed with 4% paraformaldehyde for 30 minutes at room temperature. After fixation, the cells were washed twice (5 minutes each time) in PBS and then incubated in 0.05% (v/v) Triton X-100 at room temperature for 30 minutes. Then, TBST containing 5% BSA was used to block fixed cells for 60 minutes at room temperature; primary rabbit anti-AQP3 antibody diluted 1:500 (PA5-78811, Invitrogen), rabbit anti-FLG antibody diluted 1:500 (PA5-83128, Invitrogen), rabbit anti-LOR antibody diluted 1:500 (PA5-30583, Invitrogen) or mouse anti-HAS2 antibody at a dilution of 1:500 (NBP2-37446, Novusbio) were used to incubate cells overnight at 4°C followed by an Alexa 488-conjugated secondary goat anti-rabbit IgG at a dilution of 1:1000 (Invitrogen) or Alexaflour 555-conjugated secondary goat anti-mouse IgG at a dilution of 1:1000 (ab150114, Abcam) for 2 hours in the darkness at room temperature. The nuclei were stained by incubation with 4,6-diamidino-2-phenylindole (DAPI) at a dilution of 1:1000 (Thermo fisher, USA) at room temperature for 10 minutes. PBS was used to wash the fixed cells twice; then, cells were observed under fluorescence microscope (EVOS FL auto; life technology) and analyzed using the EVOS browser imaging software.

2.8 | Statistical analysis

All the values have been indicated as means ± SD (standard deviation). The mean values were calculated based on data from at least three independent replicate experiments. Student’s t test was performed to analyze the data. P value less than 0.05 was considered to be statistically significant. Microsoft Excel (Microsoft) was used to carry out all statistical analysis.

3 | RESULTS

3.1 | Cytotoxicity of SBT seed oil in NHEK cells

CCK-8 assay was performed to analyze the cytotoxicity of SBT seed oil, and then, we choose an appropriate concentration for further experiments. As shown in Figure 1, all test concentration of SBT seed
oil showed no cytotoxicity to cells, and we choose 100 μg/mL and 10 μg/mL SBT seed oil as the concentration for further experiments.

3.2 | The effect of SBT seed oil on the mRNA level of AQP3

PCR (polymerase chain reaction) was carried out to explore the effect of SBT seed oil on gene expressions of AQP3 in NHEK cells. As shown in Figure 2A and B, 10 μg/mL SBT seed oil evidently increased AQP3 mRNA level compared to NC, while AQP3 mRNA level in cells treated with 100 μg/mL SBT seed oil displayed no obvious difference with NC (data not shown). Therefore, 10 μg/mL SBT seed oil was used as the appropriate concentration for further experiments.

3.3 | The effect of SBT seed oil on the protein level expression of AQP3 and HAS2 in NHEK cells

Western blot analysis and IF were carried out to investigate effect of SBT seed oil on protein expressions of AQP3 and HAS2 in NHEK cells. AQP3 and HAS2 protein levels in NHEK cells treated with 10 μg/mL SBT seed oil were measured. According to the result of IF, treatment with 10 μg/mL SBT seed oil substantially up-regulated AQP3 (Figure 3A and B) and HAS2 protein (Figure 3C and D) expressions in comparison with NC. Subsequently, Western blot analysis was performed to further confirm whether SBT seed oil treatment enhanced AQP3 and HAS2 protein expressions in NHEK cells. According to the result of Western blot analysis, treating with 10 μg/mL SBT seed oil evidently increased AQP3 (Figure 4A and B) and HAS2 protein (Figure 4C and D) expressions.

FIGURE 3 SBT seed oil treatment evidently up-regulated AQP3 and HAS2 protein expressions based on result of IF. A, Immunohistofluorescence analysis was executed for AQP3 in NHEK cells. B, Relative optical densities of AQP3. C, IF analysis was executed to HAS2 in NHEK cells. D, Relative optical densities of HAS2. Magnification 20×, data are expressed as mean ± standard deviation (SD). Compared with the negative control, ** represents P < .01. HAS2, Hyaluronic acid synthase 2. Green represents AQP3 protein, red represents HAS2 protein, and blue represents DAPI stained cell nucleus.
in NHEK cells in comparison with NC, suggesting that treatment with 10 μg/mL SBT seed oil notably increased AQP3 and HAS2 protein levels in NHEK cells.

### 3.4 Determination effect of SBT seed oil on the protein expression levels of AQP3 and HAS2 in reconstructed skin model

After demonstrating effect of SBT seed oil on AQP3 and HAS2 expressions in NHEK cells, following prototype experiments were carried out on reconstructed skin model to further confirm promotion effect of SBT seed oil on key protein associated with skin hydration including AQP3 and HAS2. As shown in Figure 5, AQP3 and HAS2 protein expressions in reconstructed skin model treated with SBT seed oil, were substantially up-regulated in comparison with NC. All the results collectively demonstrated that 10 μg/mL SBT seed oil treatment significantly enhanced AQP3 and HAS2 expressions, which were closely related to skin hydration.

### 4 DISCUSSION

In our study, we demonstrated that SBT seed oil can promote skin hydration via up-regulating expression of AQP3 and HAS2, which might provide novel sight into the moisturizing effect of SBT seed oil.

Dry skin is very a common skin problem, which frequently result in a rough, scaly quality to the skin. It will further cause chronic itch and even affect quality of life. Dryness was obvious associated with eczematous or psoriatic skin, and maintaining adequate skin hydration in the skin requires effort from two aspects: (a) skin barrier function improvement, such as reinforcing cornified envelope and order intercellular lamellar lipids to reduce TWEL, or increasing the content of natural moisturizing factors (NMF) in corneocytes; (b) intrinsic hydration capacity enhancement, such as increasing content of endogenous glycerol, which served as a natural moisturizer, and HA in the skin. AQP3 is abundantly expressed in epidermis, served as a channel to transport glycerol and water; HAS2 is also expressed by keratinocyte and located in epidermis, which is responsible for the synthesis of large molecules of HA. Meanwhile, endogenous glycerol and HA play vital role in skin hydration. In this research, we proved that 10 μg/mL SBT seed oil treatment significantly increased expressions of AQP3 and HAS2 in comparison with negative control.

AQP3, as an aquaglyceroporin, can transport water, glycerol, and urea, which has function in keratinocyte proliferation and differentiation. Mature epidermis develops based on the balance of cell proliferation and differentiation; therefore, AQP3 was closely associated with wound healing, disrupted skin barrier recovery, and stratum corneum hydration. The phenotypical research of AQP3-deficient mice demonstrates that AQP3 plays a pivotal role in water and glycerol transport. Overexpression of AQP3 causes more water to be transported from dermis to epidermis to maintains skin hydration. Previous studies indicated that all-trans retinoic acid treatment up-regulates AQP3 expression, which is accompanied by a rise of water movement. AQP3 null mice displayed delayed skin barrier recovery and wound healing.

AQP3 abundantly expressed and localized to the basal layer containing proliferating keratinocytes and spinous layer containing early differentiated keratinocytes, suggesting that AQP3 might involve in cell proliferation and early differentiation. It has been suggested that AQP3 evidently promoted cell proliferation through glycerol transport, because glycerol served as a crucial regulator.
Previous studies have shown that re-expression of AQP3 in AQP3-deficient cells enhanced expression of early differentiating marker K10 (keratin 10) and K1 (keratin 1), as well as terminally differentiated marker LOR (loricrin), and urea transported by AQP3 also has been proved that induced different early differentiating markers expressions. Nevertheless, the role of AQP3 in cell proliferation and differentiation was associated with various skin diseases. Studies suggested that AQP3 expression level was decreased in lesional and peri-lesional psoriasis skin compared with healthy control, which was accompanied by a decline of skin hydration and a rise of TEWL (trans-epidermal water loss). Other studies further suggested that psoriasis is a Th1 cytokine-dominant skin disease with overexpression of Th1 cytokine-TNF-α and TNF receptor-1, which inhibited AQP3 expression. Evident decrease in AQP3 expression was observed in keratinocytes derived from vitiligo lesions, and down-regulation of AQP3 reduced the downstream signaling including PI3K and E-cadherin, which were associated with keratinocyte survival, and further lead to the loss of cell-cell adhesion and decline of keratinocyte-derived growth factors, and finally cause death of adjacent melanocytes. Above results collectively suggested that AQP3 might use as a therapeutic target of dry skin, psoriasis, and vitiligo.

HA, a large glycosaminoglycan, mainly exist in ECM and can absorb water more than 1,000 times its weight, which is critical for skin hydration. According to reports, HAS2 can synthesize HA. It has been previously reported that UV exposure induced loss of HA and inhibition of HAS in skin. It has been suggested that HA was involved in the natural responses of epidermal in wound-healed and barrier recovery in AD, including proliferation and migration of keratinocyte. It has been shown that HA bound to its best-characterized cell surface receptor, CD44, induced LB (lamellar body) formation and secretion, and enhanced expressions of keratinocyte differentiation markers including K10, profilaggrin, which was very important to skin barrier repair and maintains skin hydration.

Seed oil of SBT is known to contain high amounts of PUFA, such as oleic acid (OA), linoleic acid (LA), and α-linolenic acid (ALA). LA is the richest fatty acid in the epidermis and is the precursor to synthesize ceramide, which is a predominant substance responsible for skin permeability barrier. LA was generally acknowledged as an inflammation suppressor, which could be used for the treatment of inflammation-related skin diseases, such as AD. It has been found that LA attenuated pigmentation through anti-inflammation and reducing tyrosinase activity. Moreover, LA inhibits growth of Staphylococcus aureus via influencing synthesis of proteins and nucleic acids. OA has been proved that it can inhibit tyrosinase activity, and it is also used to treat psoriasis. ALA and its derivative have been found that they may contribute to lamellar body formation, cytokine inhibition, lipoxygenase repression, SC differentiation, and maturation, which might relieve inflammatory-related dermatoses including acne, AD, psoriasis, and lupus. SBT seed oil containing abundant OA, LA, and ALA has been validated that had a positive effect on AD through suppression inflammation and repaired lipid metabolism disorders induced by UV exposure.
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