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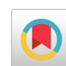

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Effectiveness of Krill Oil in Regulating Skin Moisture

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Abstract This study aims to explore the impact of Krill Oil (KO, Superba^{TM} Boost) on skin moisturization regulation. The research involved five groups: an intact control, a reference group (L-AA 100 mg/kg), and KO groups (400, 200, and 100 mg/kg), each comprising ten mice. Oral administration was conducted for 8 weeks (56 days), during which changes in body weight, hyaluronan, collagen type 1 (COL1), transforming growth factor-β1 (TGF-β1), ceramide, and water contents were analyzed in dorsal back skin tissue. Real-time PCR was employed to assess gene expression related to hyaluronic acid synthesis (*HAS1, HAS2, HAS3*), COL1 synthesis (*COL1A1 and COL1A2*), and *TGF-β1*. Results demonstrated that KO administration significantly increased hyaluronan content, hyaluronic acid synthesis (*HAS1, HAS2, HAS3*), COL1 content, COL1 synthesis (*COL1A1 and COL1A2*), TGF-β1 content, *TGF-β1 mRNA* expression, ceramide content, and water content in a concentration-dependent manner compared to the intact control. Importantly, no discernible disparities were noted between the KO and L-AA groups, even though they received equivalent oral dosages. This study accentuates the potential utility of exogenous KO in the regulation of skin moisture, thus positioning it as a promising avenue for the development of nutricosmetics. Future research endeavors should delve into the role of KO in safeguarding against both intrinsic and extrinsic aging-related skin manifestations, as well as its potential to ameliorate skin wrinkles, in conjunction with its moisturizing attributes.

Keywords Ceramide · Collagen · Hyaluronic acid · Skin water contents · Transforming growth factor-β

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Introduction

The skin is composed of three layers: epidermis, dermis, and subcutaneous tissue. The outermost layer of the epidermis, known as the stratum corneum, is recognized to play a crucial role in regulating skin water barrier homeostasis and water-holding capacity [1]. The stratum corneum is comprised of three major lipids: ceramides, cholesterol, and free fatty acids [2], which notably differ from the lipids found in the cell membranes of living cells [3]. Ceramides, constituting 35-40% of the key structural components of these lipids, particularly contribute to moisture maintenance. As a result, a deficiency or loss of ceramides in the skin can lead to a nonfunctional hydrolipidic barrier [4] and it has been suggested that the ceramides produced can influence the improvement of hydration in dry skin [5, 6]. While ceramides are naturally produced within the body, it is anticipated that they can also be effectively obtained from nutricosmetic products.

Recently, along with ceramides, a component of the epidermal intercellular cement, unsaturated fatty acids have been proposed to contribute to the prevention of trans-epidermal water loss [7]. Kendall and colleagues speculated that docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) could influence the ceramide profile in the epidermis and dermis, thereby potentially contributing to a robust lipid barrier and ceramide-mediated regulation of skin function [8]. Essential fatty acids like DHA and EPA, which are not synthesized within the human body, must be supplied through the diet as their source.

Omega-3 fatty acids (O3FA) consist of alpha-linolenic acid (ALA) and two active metabolites, namely DHA and EPA. While

ALA is found in vegetable oils, walnuts, flaxseeds, and soy products as an essential nutrient, marine-derived products such as fish oil (FO) and krill oil (KO) rich in DHA and EPA have gained attention in the market due to the limited ability of the human body to convert ALA into DHA and EPA after consumption [9]. Particularly, KO has been reported to have higher bioavailability of O3FA compared to FO [10,11], suggesting its potential superiority in enhancing a robust lipid barrier and ceramide-mediated regulation of skin function when compared to FO. Watanabe and colleagues analyzed the correlation between skin hydroxyceramide and plasma total plasmalogen, plasma total alkyl phospholipid, and plasma total phospholipid through the administration of KO and FO using the NC/Nga mice model. Their findings revealed significant correlations of $r=0.615$ ($p=0.002$) for plasma total plasmalogen and $r=0.655$ ($p<0.001$) for plasma total alkyl phospholipid, indicating that KO could potentially fortify the stratum corneum lipid barrier more effectively than FO [12]. Furthermore, our preliminary research has shown that KO administration, when compared to 100 mg/kg of Vitamin C (L-ascorbic acid, L-AA), leads to improvements in skin COL1 contents, hyaluronic acid contents, and skin water contents following UV irradiation (unpublished) [13]. This suggests that KO could not only enhance diminished skin moisturization caused by intrinsic and extrinsic skin aging but also provide protective effects in skin moisturization.

The purpose of this study is to investigate the skin moisturizing effects induced by KO (Superba™ Boost) in normal ICR mice, without artificially inducing intrinsic and extrinsic aging. Additionally, the study evaluates the potential of KO as a nutricosmetic product through comparison with well-established L-AA, known for its functions in enhancing keratinocyte differentiation, reducing differentiation-dependent oxidative stress, and maintaining skin barrier integrity, ultimately aiding in the prevention of skin water loss [14,15].

Materials and Methods

Krill oil (KO) preparation

The Antarctic KO commercial product (Superba™ Boost) was manufactured by Arker BioMarine (Houston, TX, USA) and obtained from SC Science (Goyang, Korea) for this investigation. The extraction process of KO from Euphausia superba involved steam heating followed by ethanol extraction. Filtration separated solid particles, subsequently refined through the addition of ion exchange resin and NaOH. Further processing of the refined KO occurred at 60 ºC and 360 mmHg vapor pressure for 1 hour to eliminate ethanol after centrifugation. A secondary evaporation and filtration were performed, resulting in the final product, KO (Superba™ Boost). The composition of KO included 51.2% (wt/wt) phospholipids, consisting of 44.9% phosphatidylcholine, 3.6% 1-palmitoyl-2-hydroxy-glycero-3-phosphocholine, 2.1% phosphatidylethanolamine, and 0.6% N-Acyl-phosphatidylethanolamine. EPA and DHA contents were quantified in KO. The outcomes of the KO test material were juxtaposed against those of L-AA administered at 100 mg/kg (Fig. 1). This comparison was conducted, considering the well-documented skin moisturizing effects demonstrated by L-AA in an in vivo context, a notion corroborated by the current experiment.

Gas Chromatography (GC) analysis

Methanol-treated KO underwent sodium hydroxide and boron trifluoride esterification, followed by dissolution in isooctane for Gas Chromatography (GC) analysis. An Agilent gas chromatograph with an SP®-2560 capillary GC column (100 m×0.25 mm, 0.20 μm) and a Flame Ionization Detector (GC/FID) was employed. Helium served as the carrier gas with a flow rate of 0.75 mL/min and a split ratio of 200:1. Injector and detector (FID) temperatures were set at 225 and 285 ºC, respectively. The column temperature commenced at 100 ºC for 4 min, then increased to 240 ºC at 3 ºC/

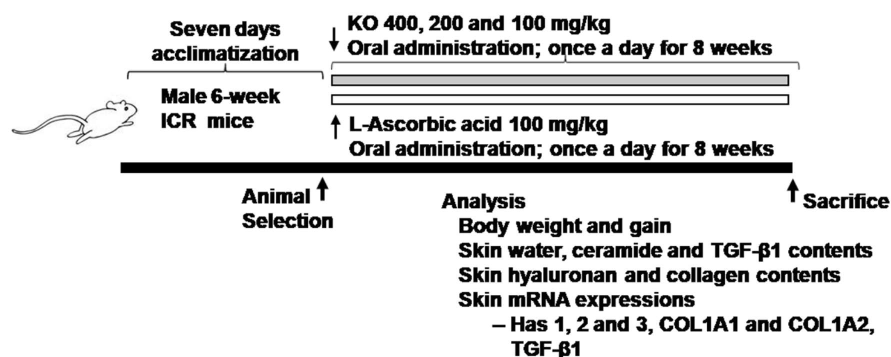

Fig. 1 Experimental design

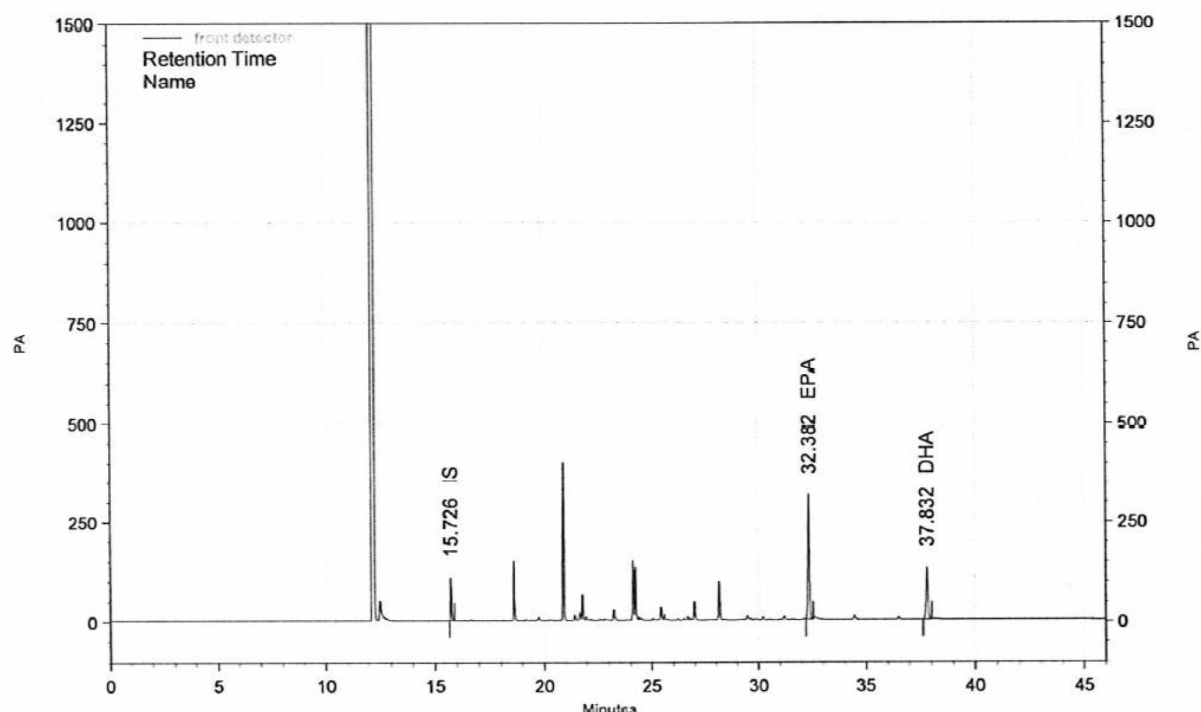

Fig. 2 Gas chromatography analysis of KO (Superba^{TM} Boost) for EPA and DHA contents

min. Quantification involved calculating peak areas of fatty acids from test and standard solutions, alongside internal standard substance peak areas. KO's EPA and DHA contents were determined as 296 mg/g (Fig. 2). It was dissolved in distilled water and stored at −20 °C. Substances such as L-AA, RA, PP, and arbutin were procured from Sigma-Aldrich (St. Louis, MO, USA), while TGF-β1 was sourced from R&D Systems (Minneapolis, MN, USA).

Experimental animals

A total of 80 healthy male SPF/VAF Outbred CrljOri:CD1[ICR] mice (OrientBio, Seongnam, Korea) were employed for the study. Following a 7-day acclimatization period, the mice were categorized into 5 groups, each comprising 10 mice, based on their body weight (average 31.75±1.42 g, range 29.50 to 34.20 g). The animal experiment adhered to the ethical guidelines of Daegu Haany University (Approval No. DHU2021-107, December 09, 2021). The mice were grouped as follows: Normal Medium Control Group (Control group with medium, oral administration of sterilized distilled water 10 mL/kg), L-AA Group (Group receiving oral administration of L-AA 100 mg/kg), KO 400 Group (High-dose experimental group receiving oral administration of KO 400 mg/kg), KO 200 Group (Intermediate-dose experimental group receiving oral administration of KO 200 mg/kg), and KO 100 Group (Low-dose experimental group receiving oral administration of KO 400 mg/kg).

Experimental Substance and Administration

KO was dissolved in sterilized distilled water to generate concentrations of 40, 20, and 10 mg/mL. Employing a metal cannula affixed to a 1 mL syringe, doses of 10 mL/kg (400, 200, and 100 mg/kg) were administered orally once daily over an 8-week span, encompassing 56 days. Similarly, L-AA was dissolved in sterilized distilled water to establish a concentration of 10 mg/mL, and doses of 10 mL/kg (100 mg/kg) were orally administered once daily for the same 56-day duration. The selection of dosage levels for KO (400, 200, and 100 mg/kg) was guided by preceding efficacy evaluation experiments employing a hairless mouse model for UVB-induced skin photoaging, akin to previous studies conducted by the authors (unpublished) [13]. The determination of the L-AA dosage (100 mg/kg) was predicated on prior in vivo experimental findings reported by other researchers [16-18]. To ensure consistent stress conditions within the normal medium control group, sterilized distilled water (medium) was orally administered at an equivalent volume of 10 mL/kg for the same duration, rather than the experimental substances. The experimental agents were prepared at least once weekly and stored in a refrigerator at 4 °C until usage.

Observation parameters

Alterations in body weight and weight gain, levels of skin moisture within a 6 mm diameter region post-shaving on the dorsal side, fluctuations in TGF-β1, ceramide, COL1, and hyaluronan contents

Table 1 Primer sequences for real-time RT-PCR

Target	5'-3'	Sequence	NCBI accession No.
Has 1	Sense Antisense	GCATGGGCTATGCTACCAAGTAT AGGAGGGCGTCTCCGAGTA	NM_008215
Has 2	Sense Antisense	GACCCTATGGTTGGAGGTGTTG ACGCTGCTGAGGAAGGAGATC	NM_008216
Has 3	Sense Antisense	AGACCGAGCTAGCCTTCCTAGT TAATGGCCAGATACAGCATGAG	NM_008217
COL1A1	Sense Antisense	GCGGTAACGATGGTGCTGTT CTTCACCCTTAGCACCAAC	NM_007742
COL1A2	Sense Antisense	ATTGTCGCCAGTGAG CTGGTCCTGCTGGT	NM_007743
TGF-β1	Sense Antisense	GCAACATGTGGAACTCTACCAGAA GACGTCAAAAGACAGCCACTCA	NC_000073
β-actin	Sense Antisense	AGCTGCGTTTTACACCCTTT AAGCCATGCCAATGTTGTCT	NM_007393

within skin tissue, as well as modifications in gene expression patterns revealed via real-time RT-PCR for *TGF-β1*, genes related to hyaluronan synthesis (*Has 1, Has 2, and Has 3*), and genes associated with collagen synthesis (*COL1A1 and COL1A2*) are documented in Table 1.

Measurement equipment and kits

For the determination of moisture content, an automated moisture analyzers balance (MB23, Ohaus, Pine Brook, NJ, USA) was employed. To quantify TGF-β1 levels, the Mouse TGF-β1 ELISA Kit (MBS175818, MyBioSource, San Diego, CA, USA) was utilized. The evaluation of ceramide levels was accomplished by combining the Quick Start™ Bradford Protein Assay Kit (Cat No. 5000201 Bio-Rad, Hercules, CA, USA) with the anti-ceramide antibody (Cat. No. C8104, Sigma-Aldrich). The assessment of COL1 levels involved the use of the Pro-COL1 C Peptide Assay Kit (Cat No. MK101, Takara Bio, Tokyo, Japan). To measure hyaluronan levels, the Mouse Hyaluronic Acid ELISA Kit (Cat No. MBS161603, MyBioSource) was employed. Real-time RT-PCR analysis was conducted using the DNA-free DNA Removal kit (Cat No. AM1906, Thermo Fisher Scientific Inc., Rockford, IL, USA), the High-Capacity cDNA Reverse Transcription Kit (Cat. No. 4368813, Thermo Fisher Scientific Inc.), and the CFX96TM Real-Time System (Bio-Rad). Additionally, primers were utilized for the amplification of TGF-β1, Has 1, Has 2, and Has 3, COL1A1, COL1A2, and β-actin genes (Table 1).

Statistical analysis

All measurements were presented as the mean ± standard deviation (SD) for a sample size of 10 mice per group. Multiple comparison tests were performed on the samples treated with different concentrations. The homogeneity of variances was assessed using the Levene test. If the Levene test showed no significant deviations from homogeneity of variances, the collected data were subjected to one-way analysis of variance, followed by Tukey's honest significant difference test. This was done to identify pairs of group comparisons that exhibited significant differences. In cases where significant deviations from variance homogeneity were observed in the Levene test, the Dunnett's T3 test was applied to identify significantly different pairs of group comparisons. Statistical significance was considered when $p < 0.05$. The statistical analyses were conducted using SPSS for Windows (Release 18.0, SPSS Inc., Chicago, IL, USA).

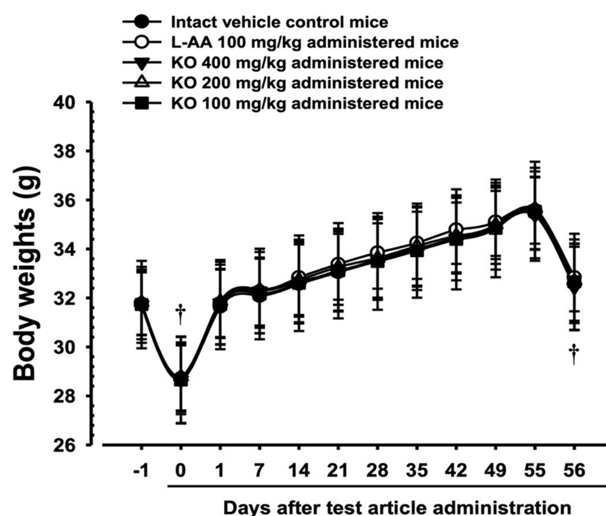

Fig. 3 Bodyweight changes on the days after intact vehicle and oral administration. The mice oral administration or not for 56 days. L-AA (100 mg/kg) or KO (400, 200, and 100 mg/kg) was orally administrated once a day for 56 days. All mice were overnight fasted prior to both first administration and sacrifice (†). The body weights were measured every week. Data are presented as mean ± SD (n = 10, significance compared with intact control)

Results and Discussion

Effects of KO on body weight change

Before the initial administration of the test substance and at the time of termination, all animals were subjected to an overnight fasting period lasting approximately 18 hours, during which they had access to water. This fasting strategy was implemented to reduce potential variations arising from individual feeding patterns followed by our previous study [19]. The body weight gains during the 56-day experimental period were calculated using the following equation.

Body weight gains (g) = (body weight at sacrifice) – (body weights at the day of initial test material administration) [19]

The average weight of the intact control group was 31.74±1.42 g one day prior to oral administration, and it decreased to 28.72±1.47 g on the fasting day at the onset of administration (Day 0). The average weight of the intact control group gradually increased, reaching an average of 32.58±1.51 g on the final day of administration (Day 56) (Fig. 3). There were no significant differences detected in body weight when comparing the administration of L-AA with the intact control group. The administration of L-AA or KO did not result in significant differences in body weight changes compared to the intact control group.

Effects of KO on skin hyaluronic acid and COL1 change

Hyaluronic acid and COL1 are recognized as key molecules in maintaining skin moisture and play a significant physiological role in regulating skin's moisture homeostasis as extracellular matrix components. Both KO and L-AA demonstrated a significant increase in hyaluronan contents and skin COL1 compared to the intact control group (Fig. 4A, B). Furthermore, the mRNA

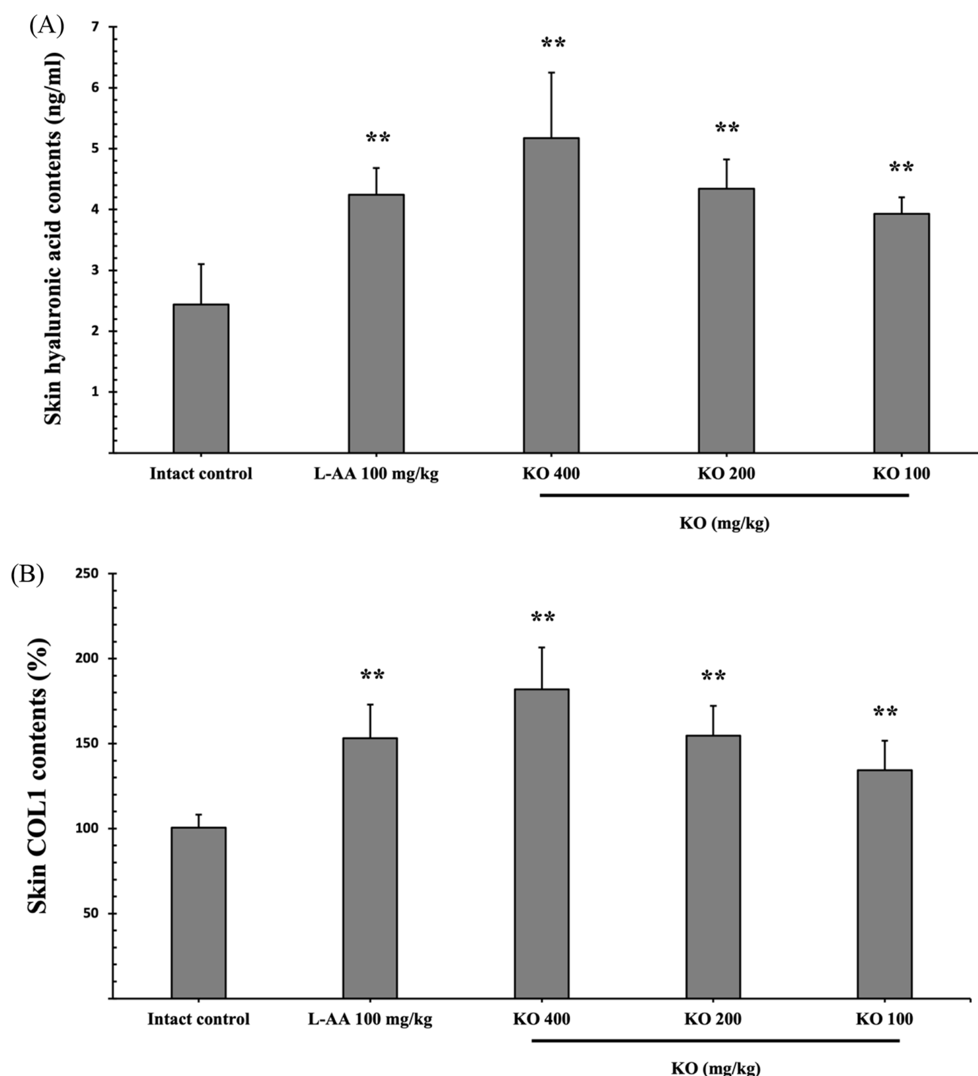

Fig. 4 Effects of KO on hyaluronan (A) and COL1 (B) contents in dorsal back skin tissues. Results are presented as mean ± SD (n = 10, significant difference vs. intact control; ** p <0.01). L-AA, L-Ascorbic acid (reference). KO, Krill oil (Superba^{TM} Boost). COL1, Collagen type I

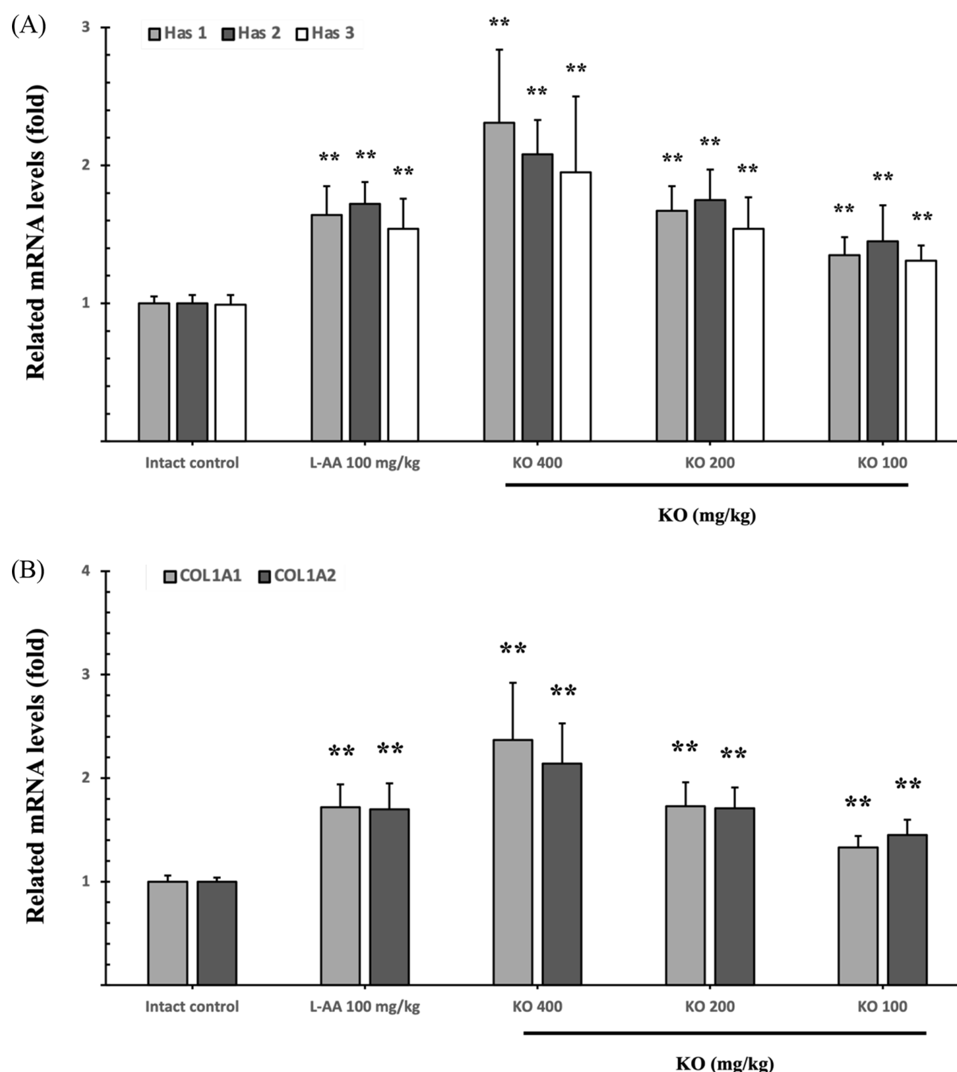

Fig. 5 Effects of KO on hyaluronic acid synthesis (HAS1, HAS2, and HAS3) (A) and COL1 synthesis (COL1A1 and COL1A2) (B) gene expression in dorsal back skin tissues. mRNA expression levels were quantified through real-time PCR analysis. The data are normalized to intact/β-actin and presented as mean ± SD (n = 10, significant difference vs. intact control; $^{**}p$ <0.01). L-AA, L-Ascorbic acid (reference). KO, Krill oil (Superba^{TM} Boost). COL1, Collagen type I

expression of genes associated with hyaluronic acid synthesis (*HAS*) and COL1 (*COL1A*) significantly increased with both KO and L-AA, compared to the intact control group (Fig. 5A, B).

The most prominent characteristic observed in skin aging and environmental stress is the decrease in hyaluronic acid levels, leading to impaired skin moisture retention due to the degradation of the ability to maintain water molecules within the skin [20]. Therefore, replenishing hyaluronic acid intake is suggested to aid in the recovery of moisture loss in the stratum corneum of the face [21] Consequently, the increased synthesis of hyaluronic acid (*HAS1, HAS2, and HAS3*) by KO is deemed to make a substantial contribution to assisting in moisture recovery. This assessment is grounded in the fact that intrinsic and extrinsic skin aging results in the downregulation of *HAS2 gene* expression in skin fibroblasts, leading to decreased hyaluronic acid synthesis [22]. As a result, the contribution of mediators such as hyaluronic acid and KO in nutricosmetics designed for hyaluronic acid synthesis is of significant consideration in the market. Although we did not observe improvements in reduced hyaluronic acid due to skin aging and environmental stress through oral administration of KO, our verification of increased hyaluronic acid synthesis and hyaluronan contents in normal skin tissue following KO administration unmistakably confirms the potential for enhancing skin moisture maintenance attributed to hyaluronic acid synthesis, which regulates the behavior of epithelial cells and fibroblasts [23].

Furthermore, the degradation of hyaluronic acid in the human body is reported to be a result of oxidative damage caused by reactive oxygen species [24,25]. Therefore, it is speculated that

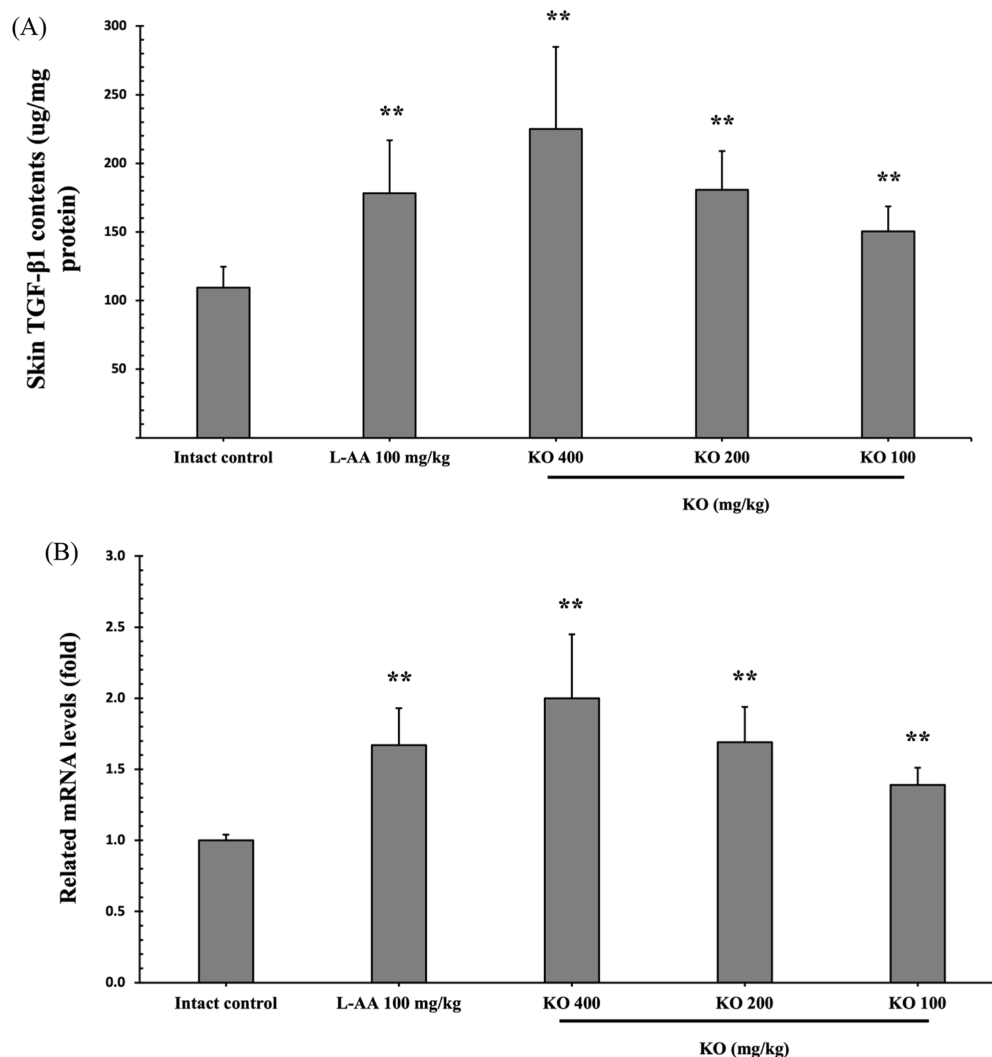

Fig. 6 Effects of KO on TGF-β1 contents (A) and TGF-β1 mRNA expression (B) in dorsal back skin tissues. mRNA expressions levels were quantified using the real-time PCR analysis. The data are presented as mean ± SD and normalized to intact/β-actin. The sample size was n = 10, and significant differences compared to the intact control are indicated as $^{**}p < 0.01$. L- AA refers to L-Ascorbic acid (reference), and KO stands for Krill oil (Superba^{TM} Boost). TGF denotes Transforming Growth Factor

KO administration might effectively regulate the inhibition of oxidative stress, potentially leading to the improvement of hyaluronic acid synthesis and function. This prediction is grounded in the fact that metabolites of O3FA (such as epidermal 15-lipoxygenase transforming EPA into 15-hydroxyeicosapentaenoic acid; 15-HEPE and DHA into 17-hydroxydocosahexaenoic acid; 17-HDoHE) can accumulate in the normal epidermis following KO consumption [26].

The hydration brought about by hyaluronic acid is known to transduce the structure of collagen fibrils. Particularly, type I collagen, being a major component responsible for forming a physical barrier between the skin's environment and body fluids, equips the skin with the capacity to resist water loss [27]. It is commonly recommended by experts to supply marine-derived fish collagen for the provision of type I collagen due to its relevance. This is because the primary collagen type in fish collagen is type I collagen. The primary fatty acids in type I collagen are reported to be palmitic acid (C16:0), oleic acid (C18:1), and DHA (C22:6) [28]. Hence, the intake of fatty acids is believed to contribute to the increase in skin COL1 contents and COL1 synthesis. Consistently, our current study's outcomes reveal that the administration of KO in normal mice results in the increase of skin COL1 contents and COL1 synthesis, indicating that KO's higher bioavailability of O3FA [10,11] likely contributes to the alteration of the structure of collagen fibrils due to hydration. Cho and colleagues [29] reported that administration of collagen peptide reduced hyaluronic acid degradation, *HAS2 mRNA* expression, improved skin hydration, and inhibited water loss in the skin of UVB-irradiated SKH-1 mice. This suggests that hydration, driven by the crosstalk between collagen and hyaluronic acid, likely

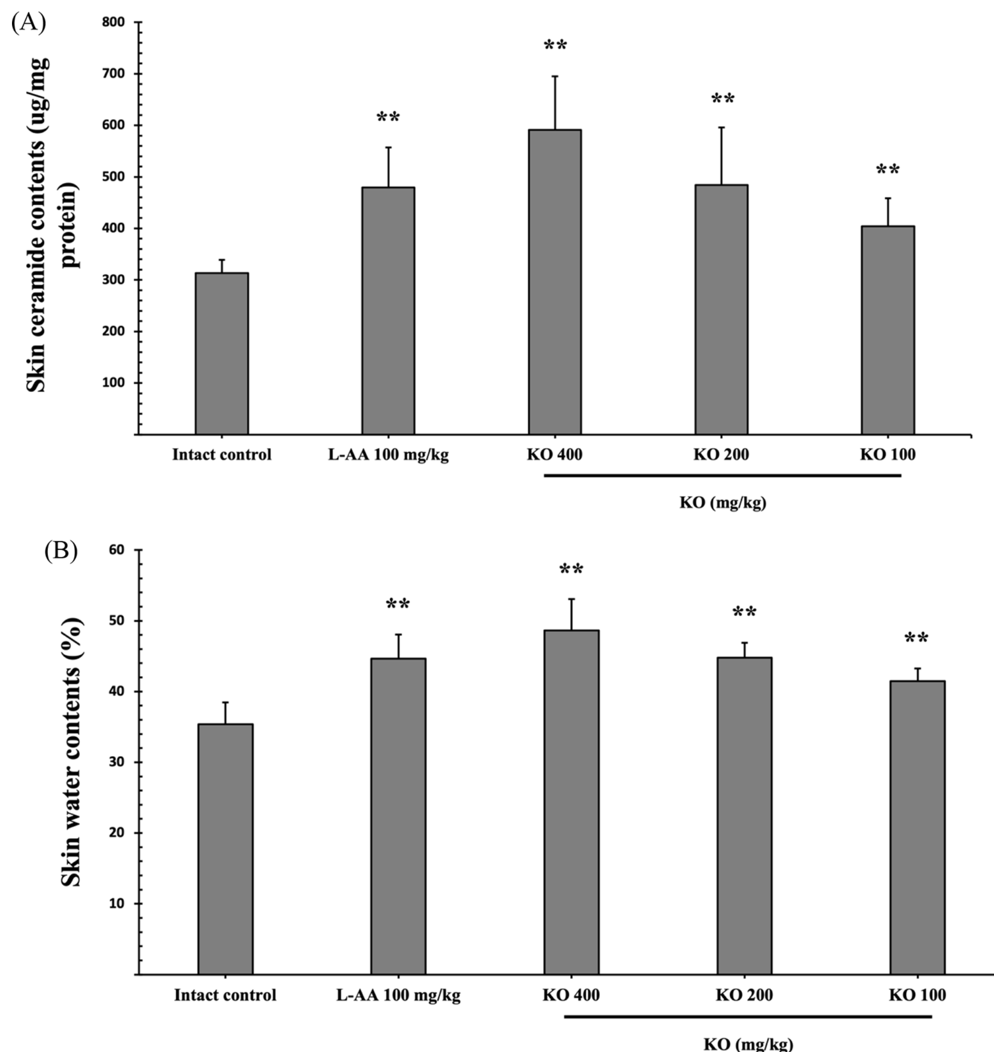

Fig. 7 Effects of KO on ceramide contents (A) and water contents (B) in dorsal back skin tissues

inhibits water loss through alterations in the structure of collagen fibrils. The crosstalk between collagen and hyaluronic acid might be attributed to increased hyaluronic acid synthesis and skin hyaluronan contents resulting from the fatty acids that constitute collagen. Consequently, this interplay is believed to bring about changes in the structure of collagen fibrils, contributing to the inhibition of water loss.

Effects of KO on skin TGF-β1 contents and *TGF-β1* mRNA expression in skin tissue

TGF-β promotes collagen formation through various cellular functions [30], and TGF-β1 is known to stimulate the synthesis of hyaluronan via *HAS 1* and *HAS 2 mRNA* expression [31,32]. KO and L-AA exhibited a significant increase in skin TGF-β1 contents and *TGF-β1 mRNA* expression compared to the intact control group (Fig. 6A, B).

Previous studies have documented that healthy epidermis generally exhibits a certain level of *TGF-β1* expression and a predominant presence of *TGF-β3* at the basal cellular layer. Notably, during wound healing, *TGF-β1* expression plays a pivotal role in fibroblasts through TGF-β signaling [33,34]. Interestingly, in the present study, exogenous KO and L-AA led to an increase in *TGF-β1 gene* expression in skin tissue, supported by the elevation in skin TGF-β1 contents. This suggests that KO contributed to epithelial homeostasis maintenance. Dysregulation of the TGF-β signaling pathway could lead to unrestrained proliferation of epithelial cells and promote malignant transformation [35]. However, in line with our findings, up-regulation of TGF-β can potentially mitigate malignant transformation [36]. Particularly, the up-regulated *TGF-β1* induced by exogenous KO in our study is inferred to have influenced not only the increase in *HAS mRNA* expression and skin hyaluronan contents in normal skin but also the promotion of collagen formation. Although the precise relationship between KO's essential fatty acids and TGF-β

remains unclear, the potential modulation of *TGF-β1* by O3FA has been reported selectively in various cell types [37], suggesting the need for further research on exogenous O3FA and epithelial homeostasis.

Effects of KO on skin ceramide contents and skin water contents

Ceramides constitute a significant component of the stratum corneum [2], and contribute to moisture maintenance [5,6]. Given that ceramides are produced endogenously, the potential impact of exogenous nutricosmetics on changes in skin ceramide and skin water contents has not been conclusively established. However, through this study, it has been observed that the administration of KO significantly increases skin ceramide and skin water contents compared to the intact control group (Fig. 7A, B). Furthermore, there were no notable differences between the KO-administered group and the L-AA-administered group when considering an equal oral dosage.

The increase in skin ceramide contents due to exogenous KO is believed to have contributed to the enhancement of ceramide-mediated regulation of skin function, resulting in the increased skin water contents. This prediction is grounded in the potential for changes in ceramide profile between the epidermis and dermis caused by O3FA, leading to trans-epidermal water replenishment [8] and hydration of dry skin through the generated ceramides [5,6]. Moreover, through this study, we have confirmed the increased contributions of KO to hyaluronic acid synthesis, COL1, and TGF-β1, all of which can influence changes in skin water contents. This suggests that exogenous KO can effectively regulate skin moisture.

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Declaration of Conflict of interest The authors declare no conflict of interest.

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