IFSCC 2025 full paper (IFSCC2025-1788)

"Unlocking the mechanism of action of recombinant collagen in preserving dermal structure and delivering anti-aging benefits"

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1. Introduction

Skin aging is a complex, multifactorial process that involves structural, cellular and molecular alterations in both the epidermal and dermal compartments[1]. The aging process is influenced by intrinsic factors such as chronological aging, and extrinsic stressors including ultraviolet (UV) exposure, oxidative stress and environmental pollution[1]. These factors collectively contribute to a decline in fibroblast function, increased collagen degradation and impaired extracellular matrix (ECM) remodeling[2]. As a result, individuals experience visible signs of aging such as reduced skin elasticity, sagging, and the appearance of wrinkles.

A vital component in the aging process is the dermal-epidermal junction (DEJ), a specialized interface that anchors the epidermis to the dermis and mediates essential signaling between keratinocytes and fibroblasts[3]. With age, the DEJ undergoes flattening, accompanied by a decrease in its key structural proteins such as Collagen IV and laminins[4]. This deterioration compromises the integrity of the skin, further exacerbating the signs of aging.

Collagen, the most abundant protein in the ECM, plays a crucial role in providing structural support and regulating cellular functions. While animal-derived collagens are widely used in biomedical applications due to their accessibility, they pose challenges such as variability, immunogenicity and biodegradability[5]. To address these issues, recent research has increasingly focused on recombinant collagen, produced through advanced biotechnological methods. Recombinant collagen is synthesized through controlled expression in microbial or plant-based systems, offering a highly purified, scalable and ethically sustainable alternative that closely mimics the amino acid sequence of native human collagen[6]. These attributes position recombinant collagen as a promising next-generation ingredient for advanced skincare and regenerative dermatology applications[7].

In this paper, we investigate sh-polypeptide-69, a recombinant collagen polypeptide that is produced via fermentation to mimic human Type III collagen. This study aims to elucidate the mechanism of action of this recombinant collagen polypeptide in preserving dermal

architecture and delivering anti-aging benefits. By exploring the intricate interactions between sh-polypeptide-69 and the skin's cellular components, we hope to contribute valuable insights into its potential applications in enhancing skin health and combating the visible effects of aging.

2. Materials and Methods

2.1 Human skin explants

Human skin explants of an average diameter of 10 mm (±1mm) were prepared on an abdominoplasty coming from a 36-year-old Caucasian woman. The explants were kept in culture medium at 37°C in a humid, 5% CO2 atmosphere. Skin explants were treated topically with 0.006% sh-polypeptide-69 or untreated (control) for 48 hours with media and treatment renewals after 24 hours. After 48 hours, the subnatants were collected for ELISA and skin explants were immediately frozen at -80°C for gene expression analysis. All experimental conditions were performed in triplicate.

2.2 Cell culture and treatment

Human primary keratinocytes and fibroblast were kept in culture medium at 37°C in a humid, 5% CO2 atmosphere. Keratinocytes cells were maintained in EpiLife medium containing 60 µM calcium (Gibco) supplemented with 1% Human Keratinocyte Growth Supplement (Gibco), 1% Penicillin-Streptomycin Solution (Gibco). Fibroblast cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) high glucose, GlutaMAX™ Supplement, pyruvate (Gibco, 31966021) supplemented with 10% Fetal Bovine Serum (Gibco) and 1% Penicillin-Streptomycin Solution (Gibco).

The keratinocytes were treated with 0.006% sh-polypeptide-69 or with only EpiLife medium for 24 hours. The supernatants were then collected and subjected to exosome extraction using EXOPrep (Hansabiomed) according to the manufacturer's instructions. The human primary fibroblasts were stimulated with the exosomes obtained from the supernatants of the keratinocytes treated with sh-polypeptide-69 or untreated control for 48 hours. The fibroblasts were then subjected to RNA extraction for quantitative PCR analysis.

2.3 RNA extraction, reverse transcription and Quantitative PCR

The treated skin explants were mechanically homogenized using a Precellys® Evolution homogenizer (Bertin instrument) and total RNA was extracted from each sample using NucleoSpin® RNA kit (Machery-Nagel) according to the supplier's instructions. The mRNA was purified using the DynaBeads® mRNA DIRECT™ kit (Ambion). The complementary DNA was synthesized by reverse transcription of mRNA in the presence of oligoDT and Transcriptor Reverse Transcriptase (Roche). The polymerase chain reaction (PCR) was performed using reagent mix (Ozyme) containing Taq DNA polymerase, SYBER Green 1 and $MgCl_2$ on LightCycler® system (Roche) according to the supplier's instructions. Gene expression levels were normalized to GAPDH, and relative expression was calculated using the 2-ΔΔCt.

Total RNA from fibroblasts were extracted and purified using Purelink™ RNA isolation kit (Invitrogen) following the manufacturer's instructions. Total RNA was reverse transcribed with the Superscript VILO cDNA Synthesis Kit (ThermoFisher) according to the manufacturer's instructions. Quantitative PCR was performed with a Platinum Quantitative PCR SuperMix-UDG Kit (Invitrogen) according to the manufacturer's instructions using the CFX-connect (Biorad). All

the experiments were performed in technical duplicate for each biological triplicate. The results were normalized to endogenous control GAPDH expression, and relative expression was calculated using the 2-ΔΔCt.

2.4 microRNA extraction and quantitative PCR

The microRNAs (miRNAs) from exosomes were extracted and purified using PureLink™ miRNA Isolation Kit (Invitrogen). The total RNA was reverse transcribed with miRCURY LNA RT Kit (Qiagen). miRNA quantitative PCR was performed with a miRCURY LNA SYBR Green PCR Kit (Qiagen) using the CFX-connect (Biorad). The results were normalized against U6 expression.

2.5 Enzyme-linked Immunosorbent Assay (ELISA)

MMP-3 released in the skin explant culture subnatant was measured by ELISA kits (R&D Systems) according to the supplier's instructions.

2.6 Skin microbiome metabolization and high-performance liquid chromatography

sh-polypeptide-69 was incubated with a proprietary microbiome model constituted of 8 species representing skin microbiome for 48 hours. The digested mixture was detected by high-performance liquid chromatography (HPLC).

2.7 Clinical study

A total of 35 females aged between 38 and 58 years old, with mild to moderate facial wrinkles, as determined by scores ranging from 2 to 4 based on the Skin Aging Atlas, were recruited for the study. All participants provided written informed consent prior to enrollment. A wash-out period of 15 days was implemented before the commencement of the study toavoid the effects of any prior treatments. Participants were instructed to apply serum with 0.006% sh-polypeptide-69 or vehicle control twice daily on split face for 60 days after cleansing. Clinical scoring of the different wrinkles on face was performed by an expert dermatologist using the Visual Analogue Scale (VAS). The skin firmness and elasticity assessment were performed using a Cutometer® (Multi Probe Adapter dual MPA 580, C+K Eletronic, Germany) at the beginning of the study and after 30 and 60 days of home use of the investigational products. The parameter Uf (R0) is the total deformation of skin after application of a defined pressure. The increase in skin elasticity was evaluated based on the parameter Ur/Uf (R7) parameter. Ultrasound images of the skin were obtained with a 22 MHz transducer (probe), using the Ultrascan UC22 (Courage & Khazaka, Germany). B-scan Images were obtained over the malar region of the subject. From these images, the dermal density (D) was calculated using specific software. For each subject, the percentage improvement (% Improvement) of the score is calculated as the difference in final and initial scores normalized to the initial scores. The subjects were given questionnaire with the attributes related to investigation products at D60.

2.8 Statistical Analysis

Statistical analysis of the data was performed using Prism Graphpad. Quantitative analysis of *in vitro* and *ex vivo* studies was statistically analyzed using unpaired t-test, and the data were presented as mean values ± SEM. The analysis for clinical study was statistically analyzed by paired t-test, and the data were presented as mean values ± SD. Mean differences were considered significant when p-value < 0.05, p-value < 0.01, and p-value < 0.001.

3. Results

3.1 Evaluation of the effect of sh-polypeptide-69 on extracellular matrix gene and protein expression

Matrix metalloproteinase-3 (MMP-3) is crucial in collagen regulation via its degradation of collagen in the dermis. We examined the effect of sh-polypeptide-69 on MMP-3 expression in skin explant. The treatment with 0.006% sh-polypeptide-69 resulted in a significant inhibition of MMP-3 expression by 74% (Figure 1A). In addition, other ECM-related genes such as LOX, FN1 and COL4A1 were also evaluated. LOX is a gene encoding for lysyl oxidase that plays a role in elastin and collagen cross-linking in the dermis. FN1 encodes fibronectin, a key component of extracellular matrix that interacts with integrins. COL4A1 encodes collagen type IV, which is a part of basement membrane at the dermal-epidermal junction (DEJ). It is observed that 0.006% sh-polypeptide-69 significantly induced LOX gene expression up to 85%, FN1 gene expression up to 115% and COL4A1 gene expression up to 131% (Figure 1B). Taken together, these results suggest that sh-polypeptide-69 plays a role in regulating ECM-related and DEJ genes.

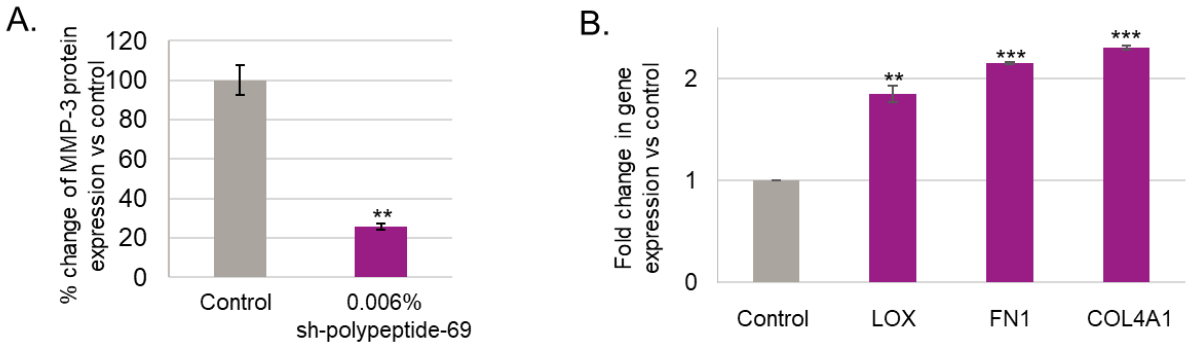

Figure 1: sh-polypeptide-60 regulates extracellular matrix (ECM)-related protein and genes in human skin explant. A) Quantification of MMP-3 protein production in human skin explant by ELISA. Results are expressed in percentage of the control. **p-value < 0.01 vs untreated control. B) Quantification of LOX, FN1 and COL4A1 genes expression in human skin explants via quantitative PCR. Results are expressed in fold change of the control. **p-value < 0.01, *** p-value < 0.001 vs untreated control. LOX: lysyl oxidase; FN1: fibronectin; COL4A1: collagen type IV alpha 1 chain.

3.2 Evaluation of the effect of sh-polypeptide-69 on the cross-talk between keratinocytes and fibroblasts via exosome-delivered miRNA

To investigate the cross-talk between keratinocytes and fibroblasts mediated by sh-polypeptide-69, exosomes derived from the culture media of the sh-polypeptide-69-treated keratinocytes were used to stimulate primary aged fibroblasts. The genes related to matrix protein protection and synthesis such as COL4A, TIMP-1 and P4HA1 were significantly upregulated in the aged fibroblasts (Figure 2A) treated with exosomes from the sh-polypeptide-69-treated keratinocytes compared to control, indicating potential cross-talk between keratinocytes and fibroblasts via exosomal communication. Exosomes are natural carriers of biologically active molecules, including microRNAs (miRNAs)[8]. miRNAs are small non-coding RNA that act as major regulators of genome by downregulating the expression of numerous genes. To investigate the role of keratinocyte-derived exosomal miRNAs in keratinocyte-fibroblast crosstalk, miRNAs from keratinocyte-derived exosomes were extracted and miRNA

expression was analysed. The expression of miR-let7b-5p was downregulated by in exosomes from keratinocytes treated with 0.006% sh-polypeptide-69 (Figure 2B). As COL4A1 has been shown to be a direct target of miR-let7b-5p[9], these results suggest that keratinocyte-derived miR-let7b-5p may suppress COL4A1 synthesis in fibroblast. Taken together, this indicates that sh-polypeptide-69 can trigger a cross-talk between keratinocytes and fibroblasts through exosome-delivered miRNAs.

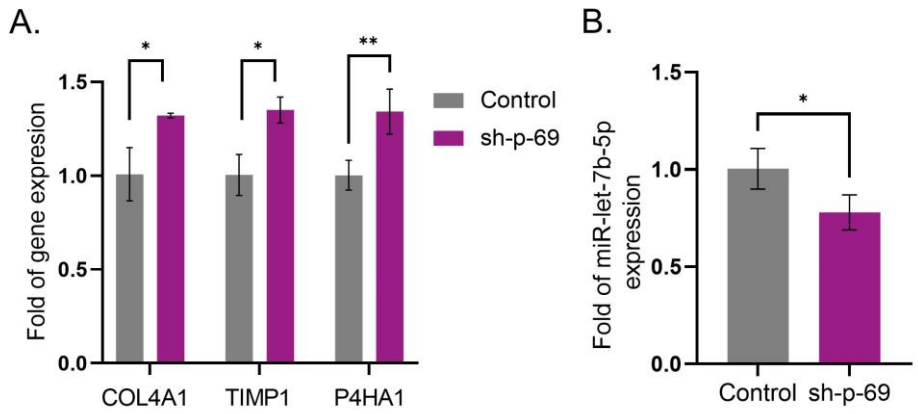

Figure 2: sh-polypeptide-69 regulates dermal epidermal junction (DEJ) protein produced by fibroblasts via keratinocyte-derived exosomal miRNA. A) Quantification of COL4A1, TIMP1 and P4HA1 genes expression in aged fibroblasts treated with exosomes from keratinocytes via quantitative PCR. Results are expressed in fold change of the control. * p-value < 0.05, ** p-value < 0.01. B) Quantification of miR-let7b-5p in the exosomes from keratinocytes treated with sh-polypeptide-69 or untreated control. Results are expressed in fold change of the control. * p-value < 0.05. sh-p-69: sh-polypeptide-69; COL4A1: collagen type IV alpha 1 chain; TIMP1: Tissue inhibitor matrix metallopeptidase 1; P4HA1: prolyl 4-hydroxylase subunit alpha 1.

3.3 Evaluation of sh-polypeptide-69 metabolization by the skin microbiome

To determine the interaction of sh-polypeptide-69 with the skin, we investigate if it can be metabolized by skin microbiome. sh-polypeptide-69 was incubated with a microbiome model constituted with 8 species representative of the skin environment for 48 hours under both aerobic and anaerobic conditions, and analysed by HPLC to determine if there is any metabolization of the polypeptide. Under both conditions, sh-polypeptide-69 was not detected and was considered to be metabolized in the microbiome model (Figure 3). This indicates that the collagen polypeptide is being processed by the skin microbiome.

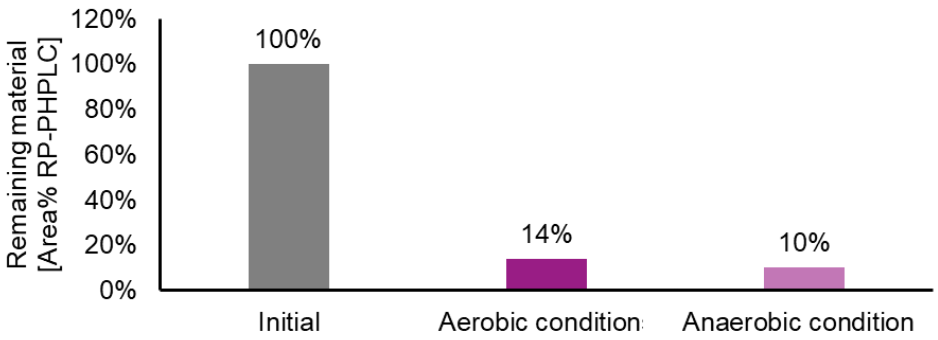

Figure 3: Evaluation of microbiome metabolization by skin microbiota model after 48 hours under aerobic and anaerobic conditions. The culture was analyzed by HPLC to quantify metabolization of the polypeptide.

3.4 Evaluation of the clinical efficacy of sh-polypeptide-69 in delivering anti-aging benefits

To assess the anti-aging efficacy of sh-polypeptide-69, we conducted a monocentric double-blinded randomized hemi-face study with a panel of 35 females aged between 38-58 years old with mild to moderate facial wrinkles using an at-home serum formulation featuring 0.006% sh-polypeptide-69 versus vehicle control over 30 and 60 days. There was a reduction of wrinkles between 7-10% after 30 days, and between 12-17% after 60 days, significantly outperforming the vehicle control for eye corner, frontal, glabella, nasolabial and peribuccal wrinkles based on clinical expert evaluation (Figure 4). In addition, sh-polypeptide-69 significantly improved skin elasticity and firmness at Day 30 and Day 60 versus vehicle control (Figure 5). Ultrasonography revealed notable inceases in dermal density, with 16.6% and 31% increase at Day 30 and Day 60 respectively (Figure 5). The clinical anti-aging efficacy results support the *in vitro* and *ex vivo* effects of sh-polypeptide-69 in improving collagen synthesis and preventing its degradation. In addition, based on the self assessment on perceived efficacy, ≥80% of subjects agree that they observed improvement in different attributes including skin hydration, skin texture, lifting effect, skin flaccidty, skin rejuvenation and overall skin appearance after home use of the serum with sh-polypeptide-69 although no significant difference was observed versus vehicle control (Figure 6).

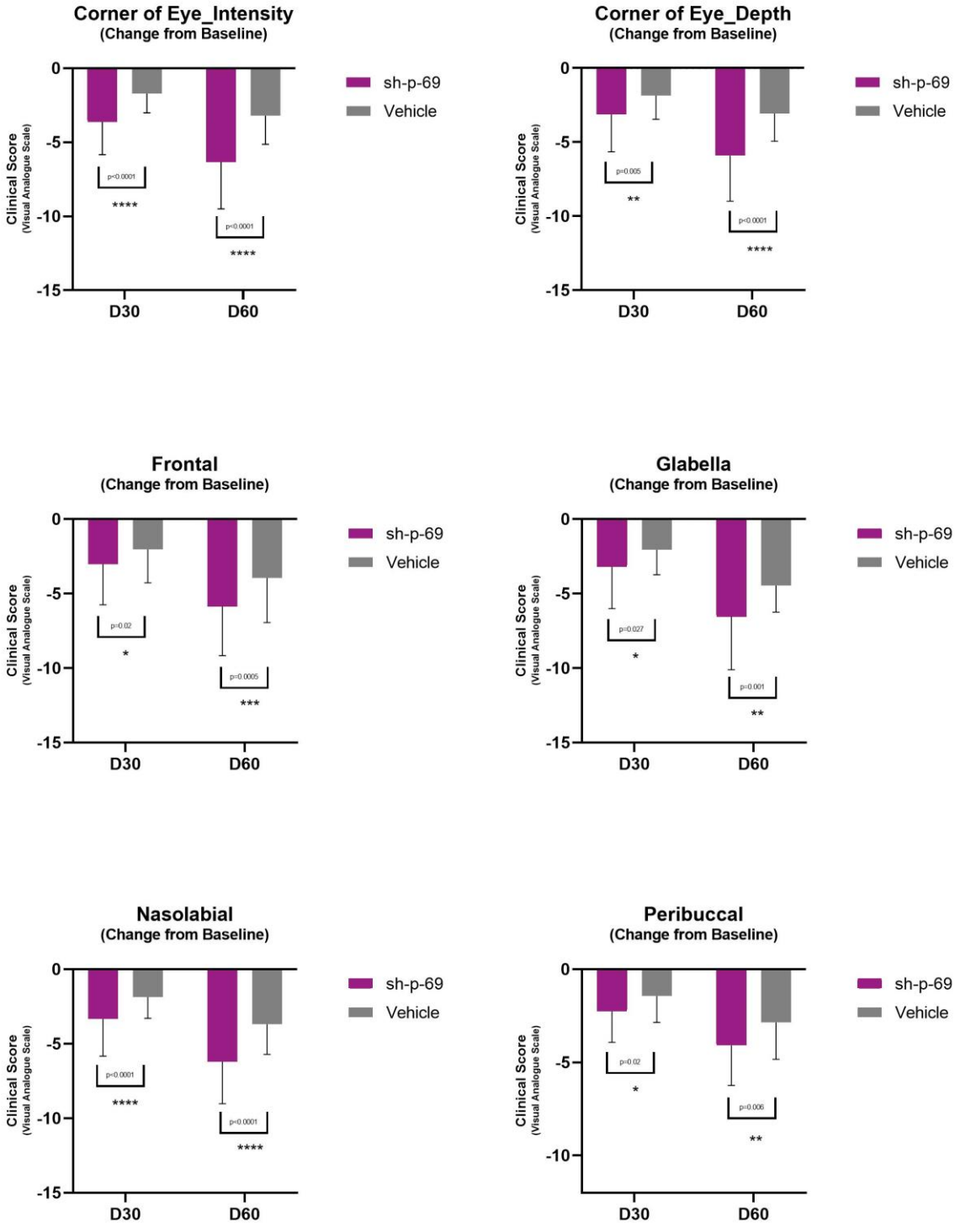

Figure 4: Clinical evaluation of wrinkles at D30 and D60 with 0.006% sh-polypeptide-69 vs vehicle based on visual analogue scale. P-values are indicated vs vehicle. sh-p-69: sh-polypeptide-69; ns: not significant; D30: Difference between D30 and Baseline; D60: Difference between D30 and Baseline.

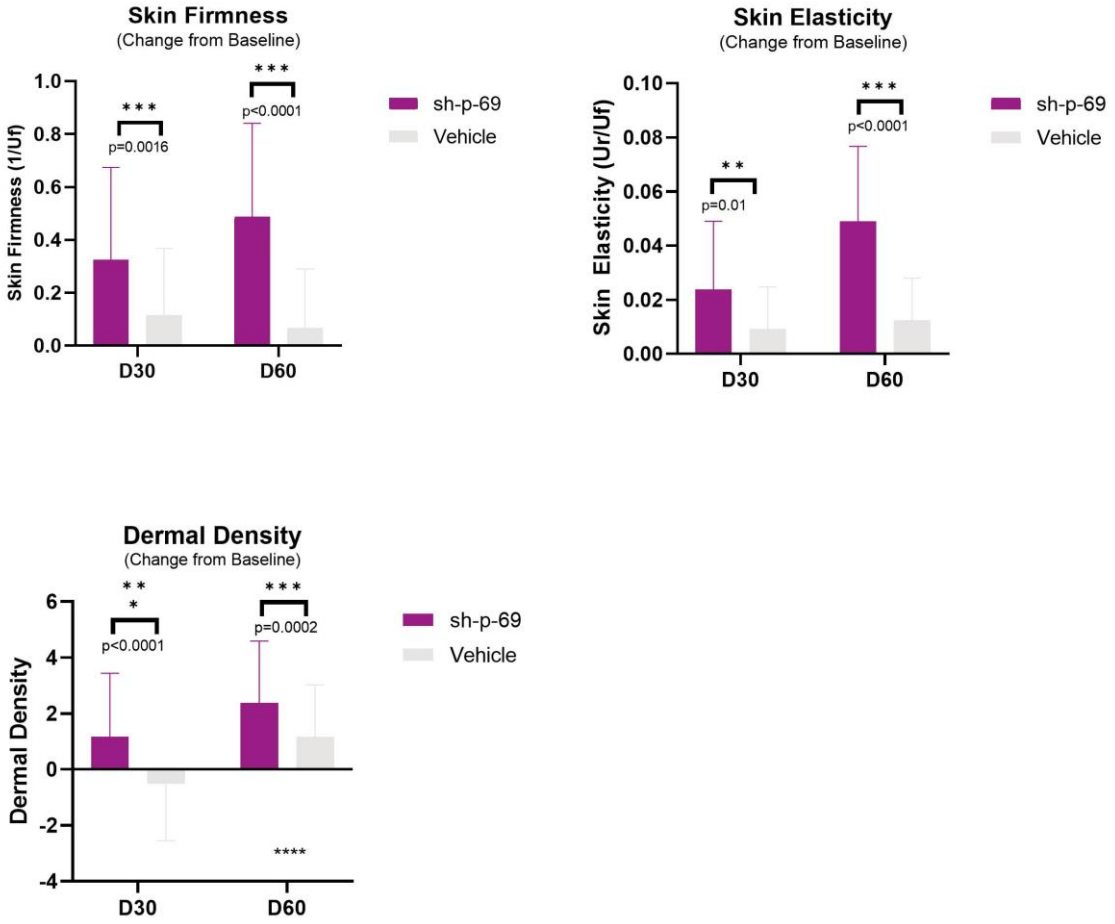

Figure 5: Evaluation of skin elasticity, skin firmness and dermal density at D30 and D60 with sh-polypeptide-69 vs vehicle measured by cutometer and ultrasound imaging. P-values are indicated vs vehicle. sh-p-69: sh-polypeptide-69; ns: not significant; D30: Difference between D30 and Baseline; D60: Difference between D30 and Baseline.

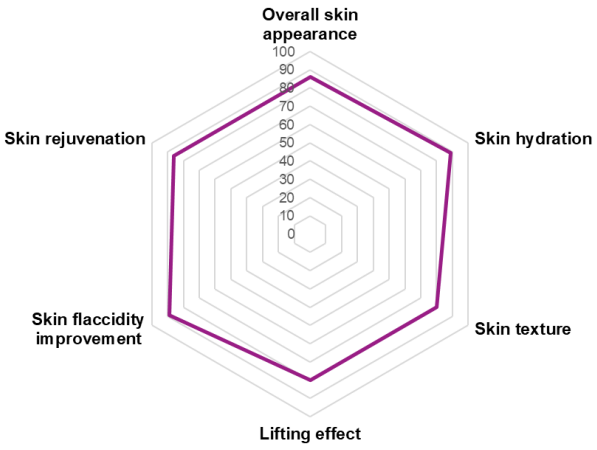

Figure 6. Subjective analysis of perceived efficacy by the research participants after 60 days of using serum with sh-polypeptide-69. The values refer to the percentage of subjects agreeing

with the perceived efficacy with 5-7 rating out of 7-scale rating, with 1 being terrible and 7 being excellent.

4. Discussion

In this study, we demonstrated the multifaceted anti-aging potential of sh-polypeptide-69. Our findings collectively support the role of this recombinant collagen polypeptide in enhancing ECM integrity, facilitating intercellular communication via exosomal miRNA and delivering visible clinical improvement in anti-aging.

MMP-3 is known to mediate collagen degradation and contributes to dermal aging[10]. The observed inhibition of MMP-3 protein expression by sh-polypeptide-69 indicates its potential to prevent dermal matrix breakdown and preserve collagen integrity in the skin. Concomitant upregulation of *LOX*, *FN1* and *COL4A1* further underscores the ECM-stabilizing effects of the peptide. *LOX* facilitates collagen cross-linking[11], FN1 plays a pivotal role in cell-matrix adhesion[12], and *COL4A1* supports the structural framework of the basement membrane[4]. These findings suggest that sh-polypeptide-69 may aid in dermal remodeling and DEJ reinforcement, key processes in counteracting age-associated skin deterioration.

The role of exosomes in skin aging and regeneration has gained increasing interest in recent years. Current literature has primarily focused on the therapeutic effects of stem cell-derived exosomes in photoaging[13], but studies investigating keratinocytes-derived exosomes remained limited. Our data show that exosomes from sh-polypeptide-69-treated keratinocytes can significantly upregulate ECM-related genes in aged fibroblast, suggested a novel form of crosstalk. The observed downregulation of miR-let7b-5p, a known repressor of *COL4A1*[9], supports the hypothesis that sh-polypeptide-69 modulates fibroblast function through epigenetic mechanisms. This provides new insight into polypeptide-based modulation of skin homeostasis via intercellular communication and addresses a gap in current understanding of keratinocyte-derived exosomal miRNA function in skin aging.

Another important finding is the metabolization of sh-polypeptide-69 by a representative skin microbiome. This observation raises new questions about the interaction between topical bioactive polypeptide and skin-resident microbes. While many topical polypeptide face limitations in skin penetration, the role of microbial biotransformation as a route to enhanced bioactivity is underexplored in dermatological research. Our study suggests that the microbiome may serve as a bioactivator of sh-polypeptide-69, although the identity and function of its metabolites remain to be elucidated. Future studies involving metabolomic profiling are warranted to understand the downstream biological effects and to confirm bioavailability *in vivo*.

Clinically, the application of sh-polypeptide-69 demonstrated significant improvement in wrinkle reduction, skin elasticity, firmness and dermal density over 60 days. These outcomes align with *in vitro* and *ex vivo* findings, and support the potential of this recombinant collagen polypeptide as a non-invasive anti-aging agent.

5. Conclusion

Taken together, regarding sh-polypeptide-69 underscore its potential as a powerful agent in dermal ECM enhancement. By effectively protecting against ECM degradation and stimulating the biosynthesis of crucial components like collagen and fibronectin, this polypeptide demonstrates a dual mechanism of action that could significantly benefit skin health. Furthermore, its role in facilitating intercellular communication between keratinocytes and fibroblasts through exosome-mediated microRNA regulation highlights its innovative approach to skin rejuvenation. The observed clinical improvements in wrinkles, skin elasticity, firmness and density further validate the efficacy of sh-polypeptide-69, positioning it as a promising ingredient in advanced skincare formulations.

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