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## ***Synergy of Powerhouses: Unveiling the New Barrier Repair Profile of Retinol and A New Peptide and Competing with the Anti-Aging Efficacy of IPL***

**Wangwang Lu<sup>1</sup>, Mengping Wang<sup>2</sup>, Xiaojun Tang<sup>2</sup>, Kan Bao<sup>2</sup>, Xia Jiang<sup>3</sup>, Xianghua Qu<sup>3</sup>, Mathilde Garnier<sup>4</sup>, Eric Jourdan<sup>4</sup>, Jianglu Zhou<sup>2</sup>, Fanqi Kong<sup>2</sup>, Jing Cheng<sup>2</sup>, Jiabin Sun<sup>2,\*</sup>**

<sup>1</sup> Yatsen Global Innovation R&D Center, Guangzhou, China; <sup>2</sup> Yatsen Global Innovation R&D Center, Shanghai, China; <sup>3</sup> Lubrizol Life Science, Lubrizol Management (Shanghai) Co. Ltd, Shanghai, China; <sup>4</sup> GALÉNIC COSMETICS LABORATORY SAS, R&D Center, Toulouse, France

### **1. Introduction**

Skin aging has long been a major concern among consumers, spurring the emergence of a wide range of skin care approaches to address this issue. Chronological and photoaging processes accumulate gradually over time, with photoaging acting in parallel with intrinsic aging to modify skin structure and function [1-2]. Anti skin aging strategies can be systematically categorized into two primary frameworks: primary preventive approaches, which target the initiation of aging processes before the emergence of clinical aging phenotypes, and secondary intervention strategies [2]. Among the numerous active ingredients for anti-skin aging, retinol has long been regarded as one of the most effective and classic anti-aging components [3]. The main mechanism is that after it is converted into retinoic acid in the body, it binds to retinoic acid-binding proteins and enters the cell nucleus to regulate various genes (such as those related to collagen, elastin, matrix metalloproteinases, and cell differentiation, etc.). Consequently, retinol is well recognized for its ability to improve the appearance of fine lines and defend against the detrimental effects of photoaging. However, the molecular structure of retinol contains multiple conjugated double bonds, rendering it highly unstable. It is particularly sensitive to high temperatures, light exposure, and oxygen, and is prone to denaturation and inactivation [4-5]. To address this issue, numerous raw material developers have attempted to enhance the stability of retinol-based raw materials by modifying the retinol molecule or encapsulating retinol. Examples of such approaches include the use of retinol palmitate, retinol propionate, or liposome-encapsulated retinol [6].

Another major category of skin anti-aging ingredients is peptides. The application of peptides in skin cosmetology and anti-aging has achieved widely recognized breakthroughs [7-8]. In terms of the mechanism of action, they can be roughly classified into carrier peptides, neurotransmitter inhibitor peptides, signal peptides, etc [9]. Cosmetic peptides have clear mechanisms, high bioactivity, and safety. In addition to their anti-aging effects, they are widely used in various fields such as anti-oxidation, wrinkle removal, skin whitening, anti-allergy and soothing, repair, and hair growth [10].

To maximize the anti-aging potential, we formulated a novel compound complex, including a synthesized tetrapeptide-1 and an encapsulated retinol. This study aims to explore whether the tetrapeptide-1 and retinol exhibit a synergistic effect, and to determine if a new mechanism of synergistic action can be discovered, thereby providing a guiding basis for subsequent product development and application. Given the high popularity of intense pulsed light (IPL) skin rejuvenation treatments in the market, we also investigated whether a facial serum formulated with the combination of the tetrapeptide and the retinol can achieve similar effects, offering consumers more options.

## 2. Materials and Methods

### Materials

The encapsulated retinol formulation contains around 9% retinol (CelluCap RL, Tagra) by weight. It is encapsulated using a combination of materials: cellulose acetate butyrate, trioctanoin, pentaerythrityl tetra(bis-tert-butyl hydroxyhydrocinnamate), silylated silica, silica, and magnesium stearate.

A new tetrapeptide-1: Tetrapeptide-1 (Uplevity™ e-Lift peptide, Lubrizol).

The testing serum (batch No. SCZSE002-70) includes the encapsulated retinol and the new peptide.

### *In vitro* test

The 3D full thickness skin model (EFT-400s, EpidermFT™) was topically treated with 0.25% encapsulated retinol, 0.005% Tetrapeptide-1, and their combination (combo) separately for 24 hours. Each treatment condition was set up in triplicate. Subsequently, the tissues were harvested, and the total RNA from each sample was extracted, purified, and then reverse-transcribed into cDNA. RNA sequencing was carried out, and the resulting data were analyzed using GeneMarker software.

To investigate the underlying mechanisms, we performed enrichment analysis on differentially expressed genes (DEGs). Utilizing the 'clusterProfiler' package in R software, we carried out Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) enrichment analyses. The GO analysis was comprehensive, encompassing biological process (BP), cellular component (CC), and molecular function (MF). For each of these categories, we reported the top 10 most significantly enriched terms. To effectively visualize all the enrichment results, we employed the 'ggplot2' package in R.

The 3Dskin model was used to evaluate the protein level of COL 1 (Anti-Collagen I antibody [COL-1] - ab6308, Abcam) and Elastin (Anti-Elastin antibody [BA-4], Abcam) after cultured with or without the combo (retinol and Tetrapeptide-1) for 48 hours. Goat Anti-Rabbit IgG H&L (Alexa Fluor® 488, Abcam) was used as the 2<sup>nd</sup> antibody.

### *In vivo* assay

A double blind, randomized, comparative controlled trial design was adopted for the clinical trial. Seventy female volunteers, aged 42-64, were recruited. They were randomly assigned to two separate groups, ensuring that each group had a minimum of 30 participants. For Group 1, the intense pulsed light (IPL)-based photorejuvenation procedure employed in this investigation represents a form of photoelectric therapy. The treatment apparatus utilized was the Lumenis M-22 (Courage & Khazaka electronic GmbH, Cologne, Germany) [11]. The operational parameters included a filter wavelength of 640 nm, three consecutive pulses, a pulse width of 6.0 ms, an inter-pulse delay of 50 ms, and an energy fluence of 14-16 J/cm<sup>2</sup>. Meanwhile, the subjects in Group 2 were directed to apply a particular serum across their full

facial area, with an application frequency of once every three days over a 28-day course. The facial images of all volunteers were taken by VISIA-CR 5.0® (Canfield Imaging Systems Europe, Utrecht, Netherlands) at day 0 (baseline, BL), day 1 (T1d), day 7(T7d), day 14 (T14d), and day 28 (T28d). The skin glossary and moisture condition were evaluated using a multifunctional skin physiology monitor (Courage & Khazaka electronic GmbH, Cologne, Germany) and probes, skin-Glossymeter GL 200 and Corneometer® CM 825, which determines skin glossary through reflection of light emitted to the skin, and capacitance measurement of a dielectric medium. The wrinkle length and wrinkle area of each volunteer were calculated by VISIA images. The Nasolabial folds area and Jawline Positive area were observed by three-dimensional skin imaging system PRIMOS CR (Canfield Scientific, United States). Meanwhile, safety and efficacy evaluation was done by Dermatologists. This study was conducted in accordance with the principles of the Declaration of Helsinki, and written informed consent with the approval of the Ethic Committee at Shanghai China-non Quality Technical Service Co., Ltd was signed by all volunteers.

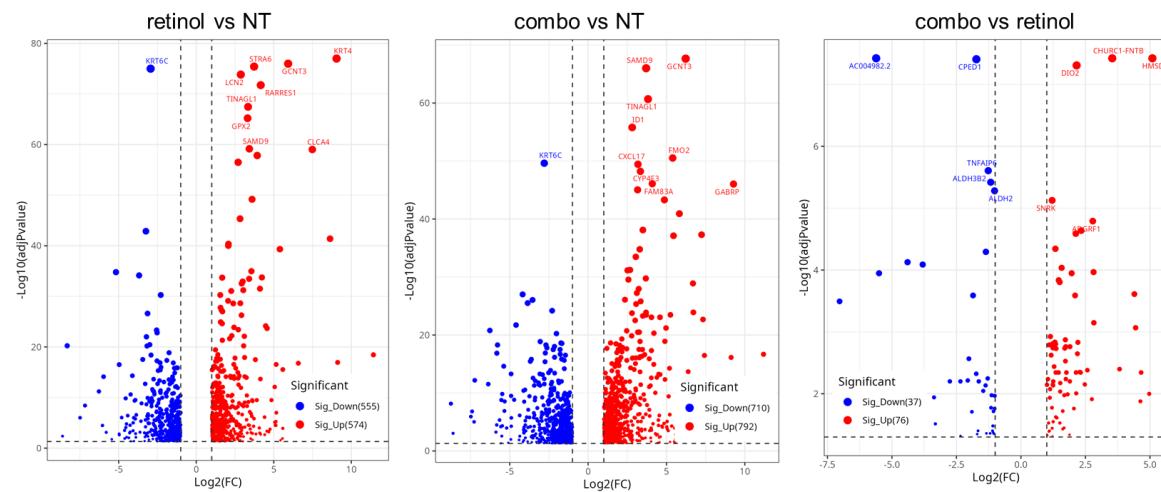
### Statistics analysis

During the GO and KEGG analyses, hypergeometric tests were the statistical approach chosen to calculate p-values for assessing the enrichment significance of various biological pathways and gene functions. To address the issue of inflated false positive rates due to multiple comparisons, the False Discovery Rate (FDR) procedure was then employed. This procedure recalibrated the calculated p-values, resulting in adjusted p-values that offer a more robust For the assessment of efficacy in both *in vitro* and *in vivo* experiments, the mean values with standard error (SE) were used to represent group characteristics. For comparing differences between groups, an independent samples T-test was employed for parametric data. When the data deviated from normal distribution or violated the assumption of equal variances, the non-parametric Mann-Whitney U test was used. A p-value less than 0.05 was regarded as statistically significant, with \* for  $p<0.05$ , \*\* for  $p<0.01$ , and \*\*\* for  $p<0.001$ .

## **3. Results**

### 3.1 In vitro test

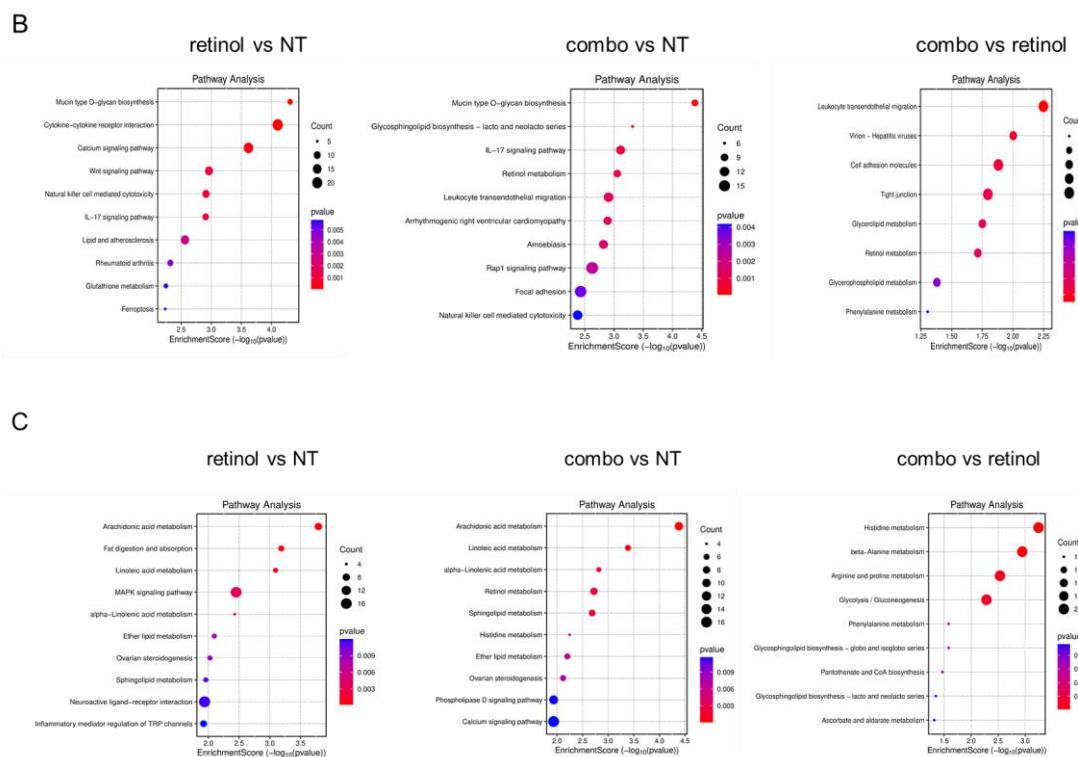
Topical applications of retinol, Tetrapeptide-1, and the combination (combo) were administered to the 3D full thickness skin model for 24 hours each. RNA sequencing was carried out. More than 60,000 gene were analyzed per sample. Genes that were upregulated and downregulated were compared respectively in the pairs of retinol and non treatment (NT), combo and NT, as well as combo and retinol. As shown as in the volcano plot (Figure 1), genes depicted in red were upregulated. In both the retinol vs. NT and combo vs. NT comparisons, over 500 genes were either upregulated or downregulated. Conversely, when comparing the combo and retinol directly, fewer than 80 genes exhibited significant differential expression.



**Figure 1.** A volcano plot was used to display the upregulated genes (in red) and downregulated genes (in blue) with statistical differences among different pairs of groups. The horizontal axis represents the difference in the expression of genes between groups, denoted by Log2F, and the vertical axis represents the statistical significance of the expression of differentially expressed genes.

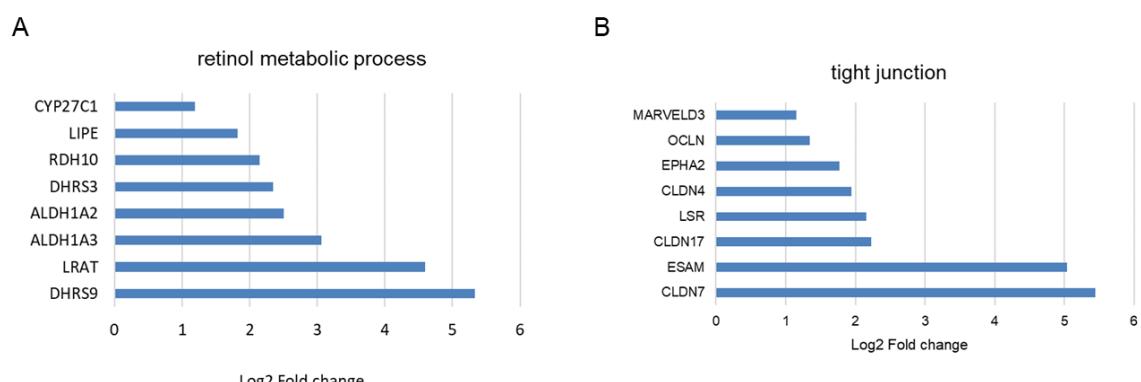
In order to explore the pathways, KEGG enrichment analysis was conducted on the differentially expressed genes (DEGs). In Figure 2A, the gene numbers were plotted on the horizontal axis. Different groups were represented by distinct categories of pathways, which are displayed along the vertical axis. The pathway categories are color-coded for easy identification. Among the pathways, there were six categories of pathway demonstrated. Furthermore, the different pathways from the different pairs of groups were shown on upregulated in Figure 2B, and downregulated in Figure 2C. The pathways on cell adhesion, tight junction and Retinol metabolism were upregulated in the comparison between combo and retinol. Meanwhile, histidine metabolism pathway was downregulated in the comparison between combo and retinol.





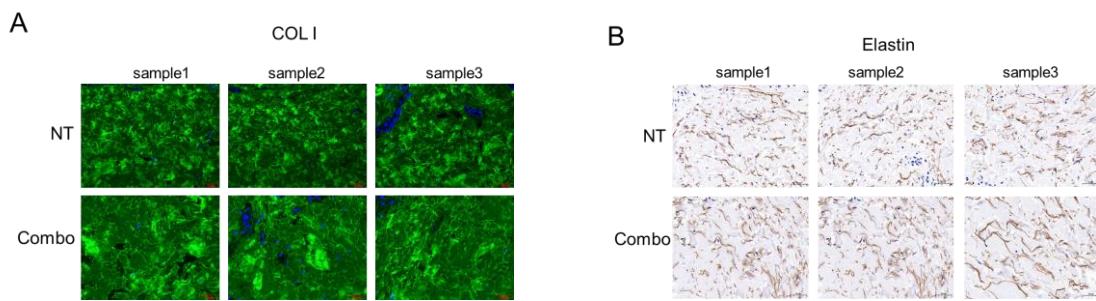
**Figure 2.** KEGG enrichment analysis. A. In the bar charts, distinct colors were used to denote pathways derived from different paired-group analyses. B. The bubble charts were utilized to illustrate the top pathways associated with the **upregulated** gene counts in the different pairs of groups. C. The bubble charts were utilized to illustrate the top pathways associated with the **downregulated** gene counts in the different pairs of groups.

Based on the pathway patterns identified through KEGG analysis, the genes associated with collagen synthesis, cell turnover, and antioxidant defense were upregulated by retinol and its combination treatment. Moreover, genes related to the retinol metabolic process and tight junction process stood out in terms of relative expression levels compared to other gene categories. As demonstrated in Figure 3A, when compared to retinol treatment alone, the combination treatment induced the upregulation of eight genes within the retinol metabolic process pathway. Similarly, eight genes from the tight junction pathway were assessed and found to be upregulated by the combination treatment in Figure 3B.



**Figure 3.** The relative expression of genes from retinol metabolic process pathway (A) and tight junction pathway (B).

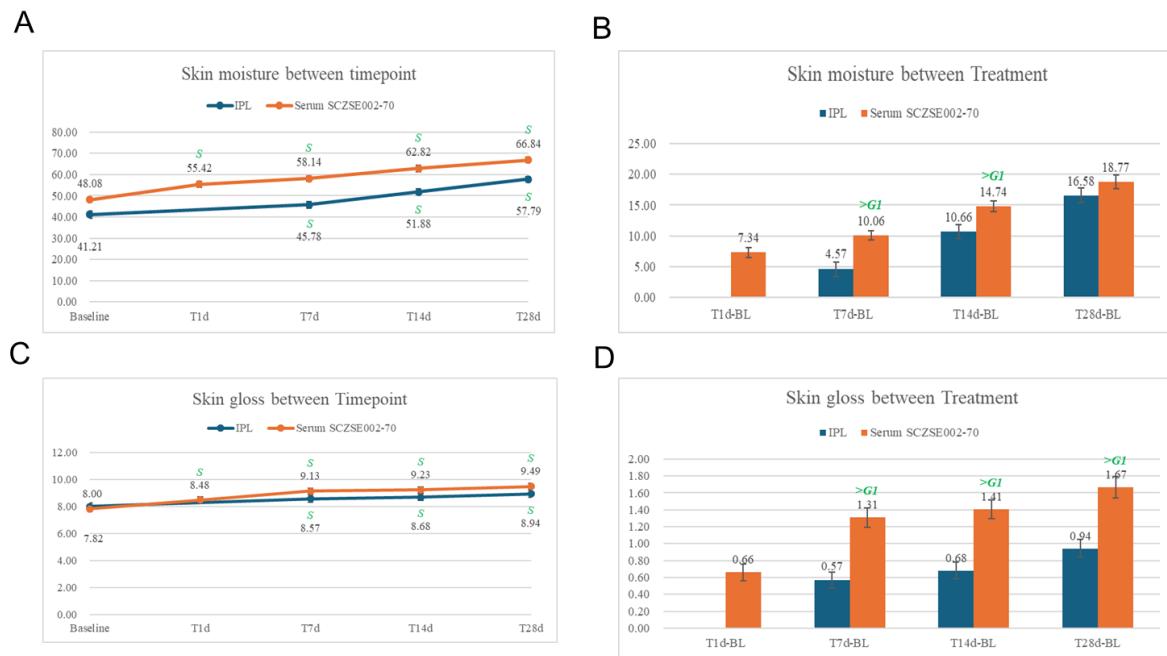
To rigorously validate the efficacy of the combination of retinol and Tetrapeptide-1, an 3D skin model was employed. This model enabled us to assess the protein levels of type I collagen (COLI) and elastin following treatment with the aforementioned combo. As vividly illustrated in Figure 4, the levels of both COLI and elastin were upregulated, providing compelling evidence of the synergistic anti-aging effects of this combo.



**Figure 4.** The protein level of COL I (A) and Elastin (B) in 3D skin model with or without treated with the combo (retinol and Tetrapeptide-1).

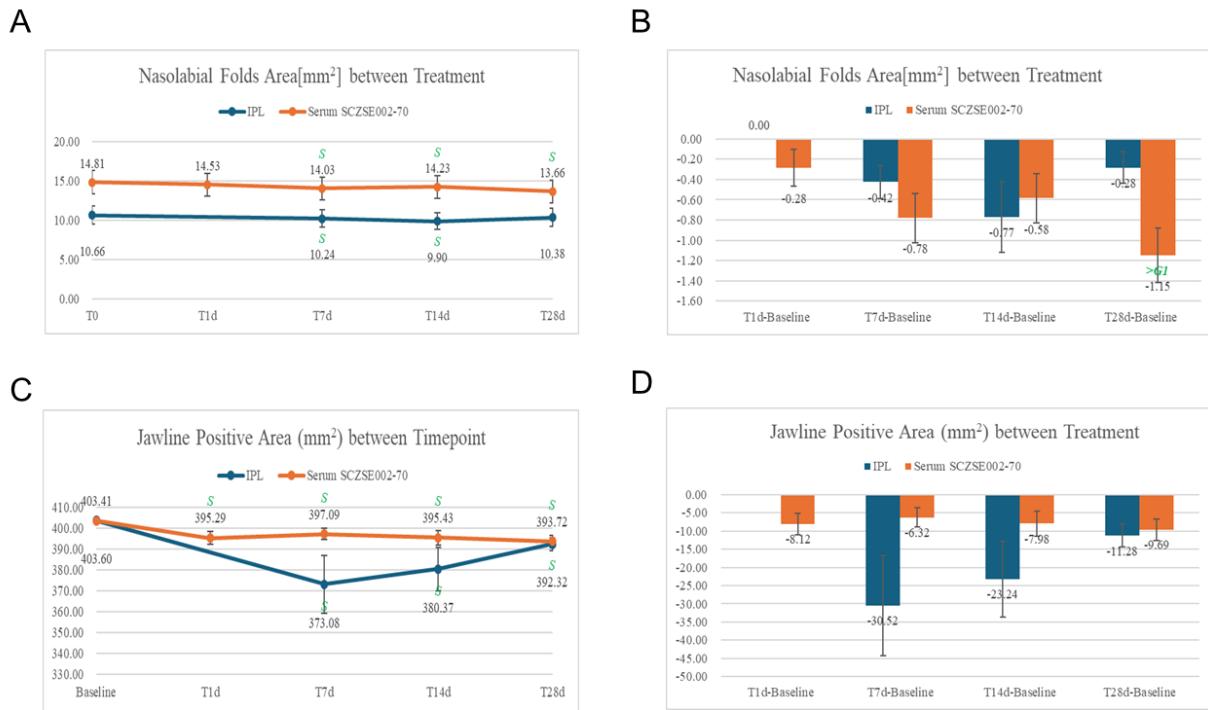
### 3.2 *In vivo* assay

In the previous study we found that Tetrapeptide-1 could be a booster to promote the metabolism of retinol, thus the efficacy of the combination (retinol and Tetrapeptide-1) was validated to enhance the protein level of COL I and Elastin from *in vitro* test. In order to evaluate the powerful efficacy of the serum with the combination, the clinical study was run. There were two groups of volunteers. One group received Intense Pulsed Light (IPL) treatment on the entire face, and the other group used the serum. As shown in Figure 5, both IPL treatment and application of the serum significantly enhanced skin moisture (Figure 5A and 5B) and skin gloss (Figure 5C and 5D) when compared to baseline measurements. Notably, at the 7-day (T7d), 14-day (T14d), and 28-day (T28d) time points, the serum demonstrated superior efficacy in improving skin moisture and gloss levels compared to IPL treatment.



**Figure 5.** Skin moisture (A-B) and skin gloss (C-D) were monitored by Corneometer at baseline (BL), the 1st day (T1d), the 7th day (T7d), the 14th day (T14d) and the 28th day (T28d). Note: “S” indicates statistically significant improvement compared to **baseline values** ( $p < 0.05$ ), “>G1” indicates statistically significant improvement **compared to IPL** ( $p < 0.05$ ).

Further to evaluate the anti-wrinkle efficacy, The Nasolabial folds and Jawline Positive area were measured. In Figure 6A, compared to baseline, the area of Nasolabial folds was decreased by application of IPL and the serum. Only at T28d, the efficacy of decreasing Nasolabial folds of the serum was significantly better than that of IPL application, as shown as shown in Figure 6B. The Jawline positive area was measured by Evaface. The efficacy of decreasing Jawline area was enhanced by application of IPL and the serum, compared to BL. There were no significant differences of efficacy between IPL application and the serum application, as shown ad in Figure 6C and 6D.



**Figure 6.** The Nasolabial folds and Jawline Positive area were measured. The analysis of Nasolabial folds area was measured by three-dimensional skin imaging system PRIMOS (CR) between timepoint (A) and treatment (B). The analysis of Jawline positive area was measured by Evaface between timepoint (C) and treatment (D). Note: “S” indicates statistically significant improvement **compared to baseline values** ( $p < 0.05$ ), “>G1” indicates statistically significant improvement **compared to IPL** ( $p < 0.05$ ).

#### 4. Discussion

Globally, hundreds of millions of individuals aged 35 and older face a confluence of aging accelerators: dynamic lifestyles, prolonged blue light exposure, and accelerated ozone depletion - the latter two amplifying UVA and UVB penetration into the skin [12]. With the growing demand for anti-aging beauty products, skin rejuvenation has become the primary selling point. Retinol and peptides are two crucial components that can effectively promote collagen synthesis, helping to rejuvenate the skin and restore its youthful look.

Vitamin A and its derivatives, with retinol being particularly notable, are some of the most efficient substances for delaying the aging phenomenon. Retinol, which is fat soluble, can penetrate the stratum corneum and, to a certain extent, the dermis [13-14]. Increasing the penetration of retinol is vital since it can increase its spectrum of activities. Within the epidermis, retinoids have the potential to modulate the secretion of transcription and growth factors. These substances play a pivotal role in promoting the proliferation of the epidermis' viable layer, fortifying its protective capabilities, and minimizing excessive transepidermal water loss (TEWL). Furthermore, retinoids safeguard collagen from breakdown by suppressing metalloproteinase activity and encourage the formation of new blood vessels in the papillary dermis [13,15]. Nevertheless, the tendency of vitamin A and its derivatives to cause skin irritation and their poor stability are major drawbacks that limit their utilization in cosmetics and drugs [16]. Further investigations into retinol's performance within different cosmetic formulations are essential. These studies aim to pinpoint the formulation that elicits the least skin irritation and to precisely determine how retinol concentration modulates its effects on the skin [17]. In our research, we discovered that a novel tetrapeptide-1 significantly enhances the efficacy of retinol, resulting

in a synergistic anti-skin-aging effect. Intriguingly, when compared to retinol alone, the combination of tetrapeptide-1 and retinol prominently upregulates the retinol metabolism pathway and the tight junction pathway, making them the most significantly activated pathways. The heightened expression of retinol metabolic genes indicates a more efficient processing and utilization of retinol within the cells, potentially amplifying its bioactivity. Meanwhile, the upregulation of tight junction-related genes may contribute to enhanced skin barrier function, facilitating better retention of moisture and protection against external insults. These findings highlight the multifaceted molecular effects of retinol and its combination treatment, providing insights into the underlying mechanisms driving their observed anti-aging efficacy.

Intense Pulsed Light (IPL) therapy is a highly effective non-invasive cosmetic procedure designed to address a wide range of skin concerns and enhance overall skin tone and texture [18]. Leveraging broad-spectrum light, IPL specifically targets pigment related imperfections such as sun induced spots, age associated lesions, freckles, and erythema from broken capillaries. As the light energy permeates the skin, it stimulates the production of collagen and promotes the turnover of skin cells, thereby fostering a more balanced complexion and restoring skin's elasticity [19]. Our study demonstrates that, when compared to IPL therapy, the topical application of a serum formulated with a combination of tetrapeptide-1 and retinol yields superior results in terms of skin hydration and gloss. These enhanced effects were observed at both the 7-day and 14-day assessment time points. Regarding the nasolabial folds and the positive area along the jawline, both the serum treatment and IPL therapy induced a reduction in fold area, suggesting a parallel impact on these specific aging areas. The results of this study have verified that the serum formulated with a combination of tetrapeptide-1 and retinol possesses excellent clinical efficacy. Significantly, they reveal that topical cosmetics applications can achieve results on par with, or sometimes exceeding, those of conventional cosmetic treatments, opening up new frontiers for cosmetic research and development.

## 5. Conclusion

In conclusion, the novel tetrapeptide-1 effectively enhances retinol's anti-aging power. When combined, they produce a potent anti-skin-aging effect, with results comparable to or better than those of Intense Pulsed Light (IPL) therapy. These findings strongly support an innovative anti-aging approach, providing a solid basis for future research and development. This discovery has the potential to transform the anti-skin-aging market by offering consumers a more convenient and effective alternative treatment. Further studies on its mechanisms, formulation optimization, and long term safety and efficacy are needed to fully realize its potential in cosmeceuticals.

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