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"Synergistic Anti-Aging Effects of Yeast/Rice Fermentation Filtrate and Sialic Acid Combination on Collagen Synthesis and Skin Inflammation"

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1. Introduction

Skin aging is a multifactorial process characterized by fine lines, wrinkles, reduced elasticity, and loss of firmness. Extrinsic factors such as ultraviolet (UV) radiation, oxidative stress, and inflammation accelerate this process. Collagen, the primary structural protein in the extracellular matrix (ECM), is essential for maintaining skin firmness and elasticity. Type I collagen constitutes approximately 80–90% of total collagen, followed by Type III (8~12%) and Type V (~5%) [1]. During aging, excessive reactive oxygen species (ROS) activate matrix metalloproteinases (MMPs), leading to collagen degradation, ECM disorganization, and impaired fibroblast function. This creates a self-perpetuating cycle of reduced ECM synthesis and increased matrix breakdown [2,3].

While the TGF-β/Smad pathway is a well-established regulator of collagen gene transcription [4,5], post-transcriptional regulation remains less understood. The IL-17 signaling pathway, which plays a central role in inflammation, accelerates skin aging by inducing pro-inflammatory mediators through IL-17A receptor activation [6,7]. Recent evidence highlights the role of RNA-binding proteins (RBPs), such as HuR, in modulating mRNA stability and translation of IL-17-related genes [8,9]. By binding to the 3′ untranslated regions (3′UTRs) of target mRNAs, HuR enhances transcript stability, including that of collagen genes [10–12], suggesting it as a potential target for anti-aging interventions.

Rice fermentation filtrate (RFF), a bioactive ingredient rich in amino acids, peptides, polysaccharides, polyphenols, organic acids, and vitamins, has demonstrated multiple skin benefits, including hydration, barrier repair, antioxidation, anti-inflammation, and anti-aging effects [13,14]. Our recent clinical study showed that 8 weeks of RFF-containing cream significantly improved periorbital wrinkles, firmness, and smoothness [15]. N-Acetylneuraminic acid (sialic acid, SA), a naturally occurring sugar in vertebrates, is known for its roles in cell signaling, adhesion, and immune modulation [16–18], and is increasingly used in cosmetics for its hydrating, antioxidant, and anti-aging properties [19].

Although both RFF and SA have shown efficacy in promoting ECM-related functions, their combined effects and underlying mechanisms remain unclear. In this study, we investigated the synergistic anti-aging effects of RFF and SA. We focused on their dual regulatory mechanisms, where RFF enhances collagen mRNA stability through HuR modulation in the IL-17

signaling pathway, and SA promotes collagen gene transcription via the TGF-β pathway. These findings provide new insights into collagen regulation and offer promising strategies for anti-aging skincare.

2. Materials and Methods

2.1. Cell culture conditions

HFF-1 human fibroblasts (ATCC CRL-3216) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; catalog no. S711-001, LONSERA). All cells were cultured at 37°C with 5% CO2. When not specified, human fibroblasts (HFF-1) were treated with 0.5% RFF (RFF group), 0.5% SA (SA group), and a combination of 0.25% RFF plus 0.25% SA (RFF and SA group).

2.2. RNA immunoprecipitation assay

RNA immunoprecipitation (RIP) was performed as described previously. Cells (1×10^6) were crosslinked with 1% paraformaldehyde, quenched with 0.125 M glycine, and sonicated using a Bioruptor Plus (25 cycles). Immunoprecipitation was carried out using 5 μg antibodies and 15 μl pre-blocked protein A/G magnetic beads. After washing, complexes were reverse-crosslinked in elution buffer (with proteinase K), and DNA was purified via phenol-chloroform extraction and ethanol precipitation.

2.3. RNA-Sequence

Total RNA was extracted using RNeasy (Qiagen), quality-checked via Bioanalyzer 2100, and libraries prepared with the KAPA RNA Hyper Prep Kit. Sequencing (150 bp paired-end) was performed on the Illumina HiSeq X platform. Reads were aligned to GRCh38 (Hisat2 v2.1.0), normalized to RPM, visualized as bigwig files, and analyzed using DESeq2 (v3.6.0) and rMATS for differential expression and splicing (criteria: $|\Delta PSI| > 0.05$, FDR < 0.05).

2.4. Real-time PCR for collagen mRNA detection

Total RNA was extracted from the transfected cells using TRIzol reagent (Invitrogen) following the protocol provided by the manufacturer. The reverse transcription PCR was carried out using the cDNA Reverse Transcription Kit (Takara). The primers used for detecting collagen mRNAs were designed based on previously published methods [20, 21]. Collagen mRNA levels were detected using a SYBR Green Master Mix Kit (Vazyme), and fold changes were calculated using the $2^{-\Delta\Delta Ct}$ method. GAPDH (human) were used as endogenous controls.

2.5. Luciferase reporter assay

For functional mechanistic analyses of SA and RFF, we predicted several Smad targets using the bioinformatics software Targetscan and Miranda and validated the candidates with the luciferase reporter assay. We generated 2 sets of vector (pMIR-GLO Plasmid #212613) containing the luciferase cDNA fused to the TGFB1, COL1A1, COL4A1 and CXCL8 3′-UTR (pMIR-GLO-COL1A1-3'UTR, pMIR-GLO-COL4A1-3′-UTR, pMIR-GLO-TGFB1-3′-UTR and pMIR-GLO-CXCL8-3′-UTR). HFF-1 cells were co-transfected with 0.4μg of the reporter construct, 0.015μg of the PGL3-basic (Plasmid #48743) or pMIR-GLO control vector. After 48h, the cells were harvested and assayed with the Dual Luciferase Assay Kit (Promega, Madison, WI), according to the manufacturer's instructions. All transfection assays were performed in triplicate.

2.6. ELISA assay

The protein levels of CXCL1, CXCL5, CXCL7, CXCL8, CXCL17, and MMP1 in the culture supernatants of treated HFF-1 cells were measured using enzyme-linked immunosorbent

assay (ELISA) kits specific for each cytokine and MMP1 (CUSABIO, CSB-E17286m/ CSB-E08178h-IS/ CSB-E04562h/ CSB-RA582227A0HU/ CSB-EL006246HU/ CSB-E04672h). The assays were performed according to the manufacturer's instructions.Briefly, culture supernatants were collected after 24 hours of treatment and centrifuged at 1,500 rpm for 10 minutes to remove cellular debris. The supernatants were then aliquoted and stored at -80°C until analysis. ELISA plates were coated with capture antibodies specific to each target protein and incubated overnight at 4°C. After washing with PBS containing 0.05% Tween-20 (PBST), the plates were blocked with 1% BSA in PBST for 2 hours at room temperature. Supernatant samples were added to the wells and incubated for 2 hours at room temperature. Detection antibodies were then added and incubated for an additional 1 hour, followed by the addition of streptavidin-conjugated horseradish peroxidase (HRP) for 30 minutes. The substrate solution (TMB) was added, and the reaction was terminated by adding 2M sulfuric acid. The absorbance was measured at 450 nm using a microplate reader (BioTek). The concentrations of each protein were determined by comparing the absorbance values to a standard curve generated from recombinant proteins of known concentrations.

2.7. Nucleocytoplasmic Separation

Nucleocytoplasmic fractionation was conducted using the NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's protocol. Briefly, treated HFF-1 cells were washed twice with cold PBS and lysed in cytoplasmic extraction buffer containing protease inhibitors. After centrifugation at 700×g for 5 min at 4°C, the supernatant (cytoplasmic fraction) was collected and stored at −80°C. The nuclear pellet was resuspended in nuclear extraction buffer, vortexed intermittently for 15 min, and centrifuged at 16,000×g for 5 min at 4°C. The resulting supernatant (nuclear fraction) was also stored at −80°C. Protein concentrations were determined using the BCA Protein Assay Kit (Thermo Fisher Scientific). Equal amounts of protein (30 μg) from each fraction were separated by 10% SDS-PAGE and transferred to PVDF membranes (Millipore). Membranes were blocked with 5% non-fat milk in TBST (Tris-buffered saline with 0.1% Tween-20) for 1 h at room temperature and incubated overnight at 4°C with primary antibodies against HuR (1:1000), GAPDH (1:5000, cytoplasmic marker), and Lamin B1 (1:2000, nuclear marker). After washing, membranes were incubated with HRP-conjugated secondary antibodies (1:5000) for 1 h, and signals were detected using enhanced chemiluminescence (Bio-Rad) and analyzed with ImageJ..

2.8. Western blot

Whole-cell lysates were subjected to Western blot analysis. Rabbit polyclonal anti-COL1A1 antibody (6994-1; 1:1000) and anti-COL4A1 antibody (ab35962; 1:1000) that from Epitomics (Abcam, Cambridge, MA), or a GAPDH antibody (60004-1-Ig, Proteintech, Rosemont, IL; 1:100,000) were used as the primary antibodies and incubated in 1× TBST containing 5% BSA for 2h at room temperature (RT) with continuous shaking. After washes with 1× TBST for 15 mins at RT, the membranes were incubated with an HRP-conjugated goat anti-rabbit secondary Ab (Thermo Fisher Scientific, San Jose, CA) for 1 h at RT. After a final washing step with 1× TBST for 15 mins at RT, the immunoreactive bands were visualized by enhanced chemiluminescence (ECL) (Amersham Biosciences, Piscataway, NJ) at indicated exposure time.

2.9. Statistical analysis

All data was presented as the means ± standard deviation (SD) of three experiments. Analysis was performed using Student's t-test or ANOVA when it's appropriate. P values of <0.05 were considered significant.

3. Results

3.1. Combination of RFF and SA Significantly Enhances Collagen Expression

To determine the optimal synergistic concentration of RFF and SA, we first tested different concentrations of the SA and RFF combination, ranging from 0.1% to 0.7%, to identify the optimal formulation. The expression of the COL1A1 gene in HFF-1 cells was analyzed using RT-qPCR on total mRNA extracted from the cells. The results show that 0.5% SA significantly promoted the expression of COL1A1 genes compared to the control group, while RFF showed only a slight enhancement of COL1A1 expression at concentrations below 0.5% (Figure 1A). Notably, 0.25% SA exhibited a superior effect in promoting COL1A1 gene expression. This effect was further enhanced when 0.25% SA was combined with 0.25% RFF, resulting in a 20.8% increase (Figure 1B). This result suggests that in this combination, RFF may synergistically enhance COL1A1 gene expression through a molecular mechanism distinct from that of SA, thereby amplifying the effect of SA on COL1A1 gene expression.

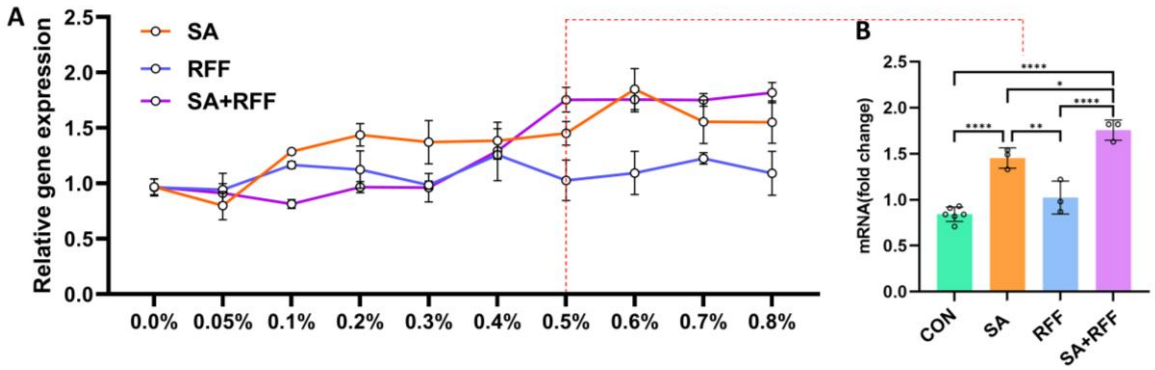

Figure 1. Combination of RFF and SA significantly enhance collagen expression. (A-B) mRNAs level of COL1A1 were measured using real-time PCR in (SA, RFF, SA+RFF)-treated HFF-1 cells. Data represent means ± SEM from three independent experiments. *p < 0.05.

3.2. RFF and SA Synergistically Regulate Anti-Aging Genes via IL-17 Signaling

To elucidate the molecular mechanisms underlying the synergistic effects of RFF and SA, we examined their impact on collagen-related gene expression using previously optimized concentrations. Principal component analysis (PCA) of RNA-seq data demonstrated high concordance among biological replicates (Figure 2A). A radar plot highlighted 10 genes significantly altered in the RFF+SA group compared to the control (Figure 2B). As shown in Figure 2C, the combination treatment upregulated multiple collagen-related transcripts (log2FC > 0.5, q < 0.05). Notably, increasing SA concentrations led to distinct changes in gene expression profiles. KEGG analysis of 636 differentially expressed genes (log2FC > 0.5, q < 0.05) revealed enrichment in the ECM–receptor interaction, cytokine–cytokine receptor interaction, and IL-17 signaling pathways (Figure 2D). Among these, the ECM–receptor interaction pathway—typically regulated by TGF-β/Smad signaling—was significantly enriched. While RFF alone showed limited impact on TGF-β/Smad signaling, enrichment analysis indicated that RFF primarily modulated genes involved in the IL-17 pathway in the combination group.

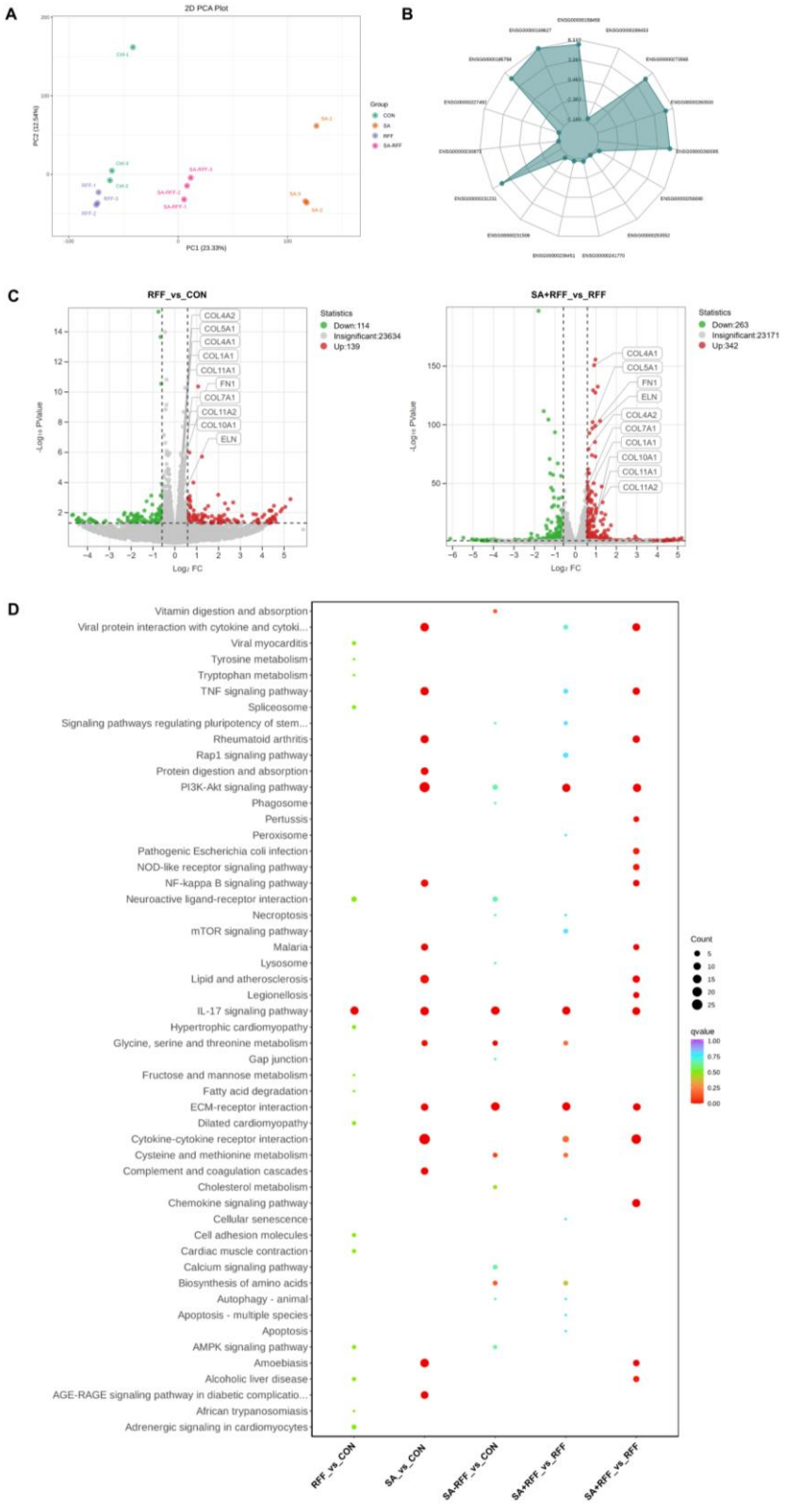

Figure 2. Combination of RFF and SA regulate anti-aging genes through IL-17 signaling pathway. (A) PCA of gene expression in treated vs. control cells. (B) Radargram of top 10 upregulated genes. (C) Volcano plots of differential genes vs. SA. (D) KEGG pathway enrichment based on RNA-Seq.

3.3. Differential Effects of SA and RFF on Collagen Gene Promoter Activity

HFF-1 cells were treated with SA, RFF, or their combination, and the expression of key ECM-related genes was analyzed by RT-PCR. SA significantly upregulated COL1A1, COL3A1, COL4A1, COL7A1, COL17A1, and COL24A1, while RFF alone showed no significant effect. However, the combination treatment markedly enhanced the expression of these genes compared to control ($P < 0.05$). Western blot analysis confirmed that protein levels were consistent with mRNA expression (Figure 3A, B). To assess transcriptional regulation, promoter fragments of COL1A1, COL3A1, COL4A1, and COL7A1 were cloned into the PGL3 vector (Figure 3C), and luciferase reporter assays were conducted. Promoter activity in the combination group was comparable to that of SA alone, indicating no synergistic transcriptional enhancement (Figure 3D). These results prompted us to investigate an alternative mechanism—post-transcriptional regulation via mRNA stabilization mediated by the RNA-binding protein HuR within the IL-17 signaling pathway.

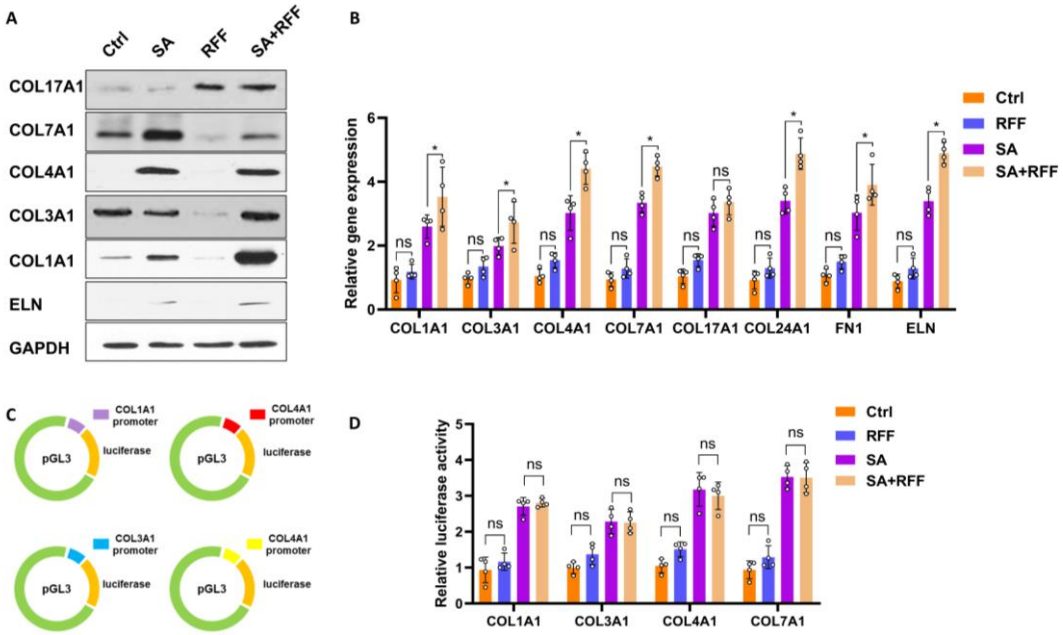

Figure 3. SA activates collagen expression and promoter activity. (A) Protein and (B) mRNA levels of collagen-related genes in treated HFF-1 cells. (C) Schematic of collagen promoter reporter constructs. (D) Promoter activity by luciferase assay.

3.4. RFF and SA Synergistically Induce HuR Nuclear Translocation in Fibroblasts

HFF-1 cells were treated with SA, RFF, or their combination, and the expression of IL-17 pathway downstream genes (CXCL1, CXCL5, CXCL7, CXCL8, CXCL17, and MMP1) was analyzed by RT-PCR. RFF significantly suppressed the expression of these genes, while SA showed mild inhibition. The combination treatment did not exhibit greater suppression than RFF alone (Figure 4A). ELISA results confirmed the inhibitory effect of RFF at the protein level (Figure 4B). Given HuR's role in stabilizing IL-17-related mRNAs via binding to their 3′UTRs, we assessed its cellular localization following treatment. Western blot analysis of cytoplasmic and nuclear fractions revealed that the RFF+SA combination promoted HuR translocation from the cytoplasm to the nucleus, with RFF being the dominant contributor (Figure 4C).

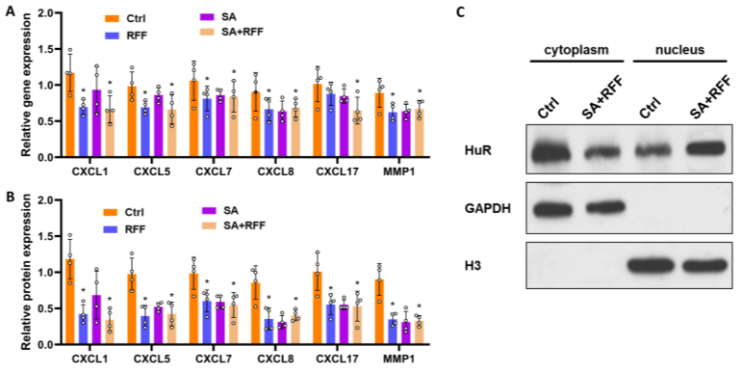

Figure 4. Combination of RFF and SA promotes HuR nuclear translocation. (A) mRNA levels of CXCLs and MMP1 in treated HFF-1 cells by qPCR. (B) Protein levels assessed by ELISA. (C) HuR distribution analyzed by nucleocytoplasmic fractionation and western blot.

3.5. Combination of RFF and SA Increases COL1A1 mRNA Stability

We treated HFF-1 cells with the global methylation inhibitor 3-deazaadenos ine (3-DAA) and separately treated the cells with SA, RFF, and RFF plus SA. Then, HFF-1 cells were transfected with pMIR-GLO-COL1A1-3'UTR, pMIR-GLO-COL4A1-3′-UTR, pMIR-GLO-TGFB1-3′-UTR and pMIR-GLO-CXCL8-3′-UTR for 24 hours in the presence or absence of 3-DAA. Luciferase mRNA stability was measured in HFF-1 cells transfected with these constructs in the presence or absence of 3-DAA. RFF group and SA plus RFF group displayed a significant increase in luciferase mRNA stability compared with the control (Figure 5A). We then stuided

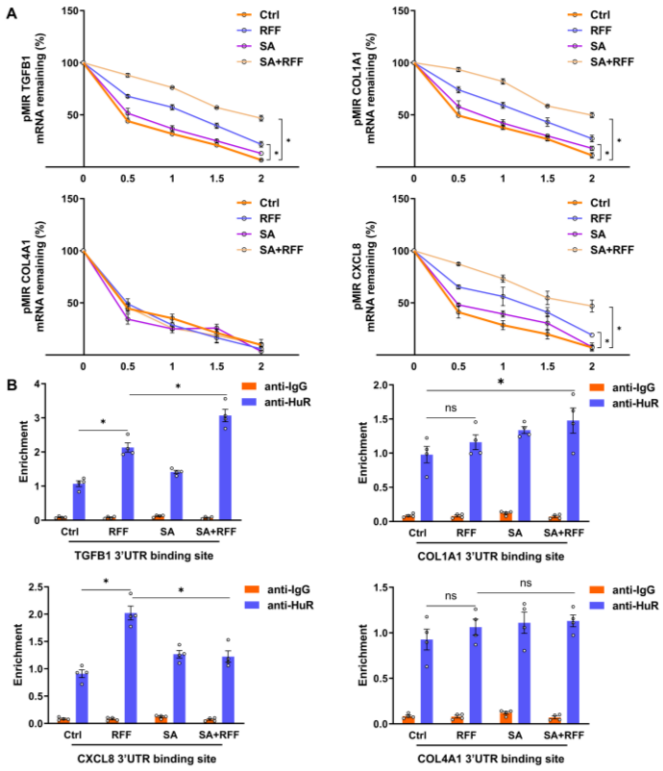

Figure 5. The combination of RFF and SA increase COL1A1 mRNA stability. (A) The stability of COL1A1, COL4A1, TGFB1, CXCL8 mRNA was calculated in HFF-1 cells treated with SA, RFF, SA+RFF. (B) RIP was used to detect the physical association between HuR and (COL1A1, COL4A1, TGFB1 or CXCL8) mRNAs in HFF-1 cells. *p < 0.05.

the binding of HuR protein and COL1A1-3'UTR in cells using RIP, and the results showed that SA and RFF could significantly promote the binding of HuR and COL1A1-3'UTR (Figure 5B). The results showed that, in contrast to SA, RFF inhibited collagen mRNA degradation by increasing mRNA stability.

3.6 SA and RFF Enhance Skin Cell Resistance to Inflammation and Oxidative Stress

The proliferation of cells in the SA, RFF, and SA plus RFF groups was examined, and the results showed that SA plus RFF had stronger efficacy in promoting cell proliferation (Figure 6). The ROS generated by the cells were detected using flow cytometry, and the combination of SA plus RFF was effective in reducing ROS generation in the cells. Besides, PCR and ELISA results showed that SA and RFF could also effectively reduce the mRNA and protein expression of these inflammatory factors, such as IL1β, IL6, IL8, PEG2 and NO.

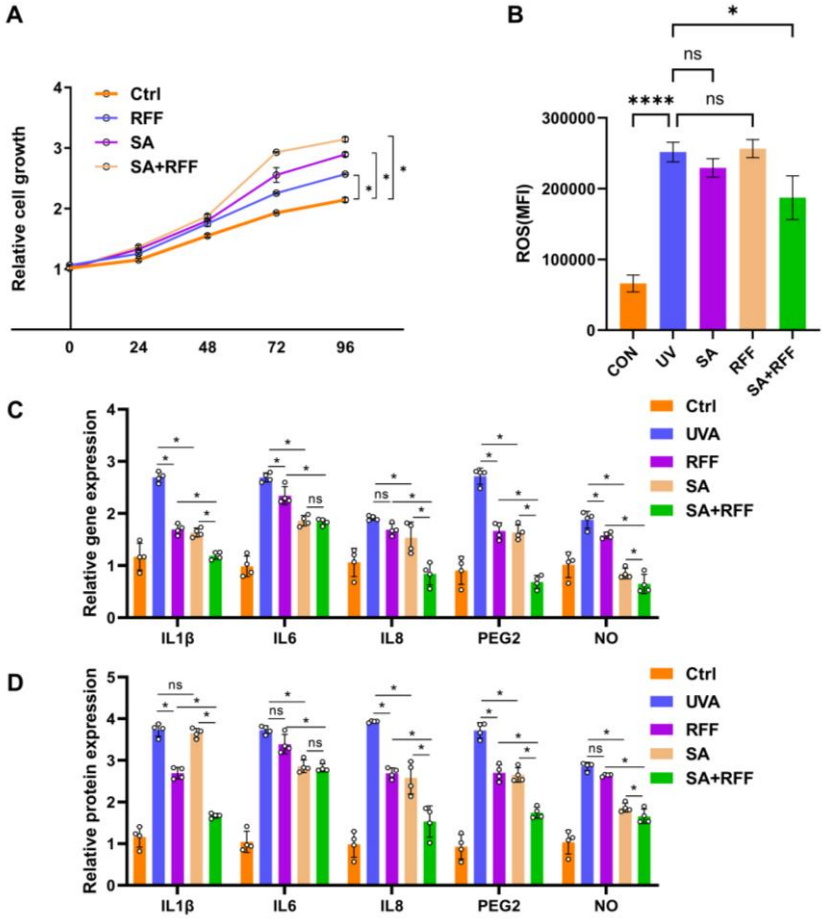

Figure 6. SA and RFF enhance skin cell resistance to inflammation and oxidation factors.

4. Discussion

Skin aging is a complex, multifactorial process characterized by the appearance of fine lines, wrinkles, laxity, and uneven pigmentation [1]. Key extrinsic factors such as ultraviolet radiation, oxidative stress, and inflammation accelerate this process. Collagen, the primary structural

protein in the ECM, is crucial for maintaining skin firmness and elasticity [3]. With aging, the accumulation of ROS activates MMPs, leading to collagen degradation and ECM disorganization. This disruption reduces fibroblast spreading and mechanical tension, promoting an aged phenotype marked by decreased ECM protein synthesis and increased expression of MMPs. This ECM imbalance promotes continuous collagen fibril fragmentation, thereby establishing a self-reinforcing cycle.

In this study, we investigated the synergistic anti-aging effects of RFF and SA, focusing on their roles in collagen production, inflammation modulation, and underlying molecular mechanisms. Our results demonstrated that the combination of RFF and SA significantly enhanced collagen synthesis compared to either agent alone. RNA-seq and qRT-PCR analyses revealed that RFF stabilized collagen mRNA by regulating the RNA-binding protein HuR through the IL-17 signaling pathway, while SA increased transcription of collagen genes via the TGF-β pathway. These findings elucidate complementary mechanisms by which RFF and SA promote collagen production and suppress degradation, with HuR playing a central role. Moreover, the combination treatment reduced the expression of key pro-inflammatory mediators (e.g., IL-1β, IL-6, IL-8, PGE2, and NO) and enhanced the resistance of HaCaT cells to oxidative and inflammatory stress.

Despite these promising findings, this study has certain limitations. All experiments were conducted in vitro, and further validation in in vivo models is required. While we identified involvement of the IL-17 and TGF-β pathways, the detailed regulatory networks remain to be fully elucidated. Future studies should focus on in vivo validation and long-term effects on skin health, as well as a deeper investigation into the roles of HuR and other RNA-binding proteins in regulating collagen homeostasis.

5. Conclusion

In conclusion, our study demonstrates that the combination of RFF and SA synergistically enhances collagen production via dual mechanisms. RFF promotes collagen mRNA stability by modulating the RNA-binding protein HuR through the IL-17 signaling pathway, while SA upregulates collagen gene transcription via the TGF-β pathway. Together, they not only stimulate collagen synthesis but also enhance skin resistance to inflammation and oxidative stress. These findings underscore the potential of RFF and SA as promising anti-aging agents and provide a novel strategy for promoting collagen production and maintaining skin health.

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