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# *"Next-Generation Green Cosmetics: Development and Application of Rice Callus-Derived Proteins and Vesicles"*

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## 1. Introduction

The cosmetic industry is increasingly transitioning toward sustainable, animal-free, and clinically validated ingredients, driven by consumer demand and regulatory advancements [1]. Plant cell suspension cultures, combined with synthetic biology technologies, offer a safe, scalable, and sustainable alternative for bioactive compound production. Compared with mammalian and microbial systems, plant-based platforms reduce the risk of pathogen and endotoxin contamination, ensure batch-to-batch consistency, and enable the production of structurally bioactive molecules without ethical concerns, supporting future innovations and next-generation advancements in the cosmetic and biomedical industries [1-4].

Rice callus is a rich source of antioxidant and anti-inflammatory and phytochemicals, is an efficient system for bioactive protein and peptide production [5, 6]. Base on the advantages of plant cell suspension cultures, rice callus suspension culture (RCSC) is an ideal bioreactor for cosmetic applications, offering a safe, genetically tractable, and scalable system free from animal-derived contaminants, prions, and human pathogens[1, 3, 4]. Rice callus-based system provides a sustainable and cost-effective alternative to traditional agriculture, requiring no cultivable land [1, 3]. In addition, Plant callus serves as a versatile platform for producing both recombinant proteins and plant-derived extracellular vesicles (PDEVs), which emerged as bioactive delivery carrier due to their stability, low immunogenicity, and ability to encapsulate diverse bioactive molecules [7, 8]. Recent studies have further demonstrated the ability of PDEVs to encapsulate bioactive compounds, including peptides and antioxidant molecules, enhancing the functional efficacy and promoting cellular uptake in cosmetic applications [9].

Currently, no studies have reported the application of human type III collagen (rhCol3) -containing rice callus extract hydrolysates and rice (*Oryza sativa*) callus-derived extracellular vesicles (OsEVs) loaded with pentapeptide-48. RhCol3 is a critical structural protein for tissue elasticity, decreases significantly with age, contributing to skin aging and impaired function [10]. In addition, pentapeptide-48, a synthetic peptide with amino acid sequence Thr-Arg-Ser-Glu-Leu, which is designed to mimic the functional domain of Royalactin, a protein found in

royal jelly, known for promoting dermal cell proliferation, extracellular matrix (ECM) synthesis, and improving skin elasticity [11].

This study established a dual-functional RCSC-based platform for both recombinant protein production and extracellular vesicles-mediated delivery. (1) rhCol3-expressing rice callus cells were generated through genetic engineering, and enzymatic hydrolysis was applied to obtain rhCol3-containing rice callus extract hydrolysates. These hydrolysates demonstrated anti-aging potential by modulation of ECM-related genes. (2) OsEVs were successfully isolated, characterized and functionally validated, followed by the encapsulation of pentapeptide-48. The cellular uptake efficiency of both free pentapeptide-48 and OsEVs-loaded pentapeptide-48 were evaluated. Furthermore, quantitative analysis of ECM-related gene and skin barrier-associated gene expression revealed the regulation of critical genes involved in skin regeneration and anti-aging processes. These findings suggest that these two components demonstrate synergistic anti-aging and skin-protective effects, suggesting the potential as an innovative and sustainable materials for next-generation green cosmetics. The schematic overview of the study design is shown in Figure 1.

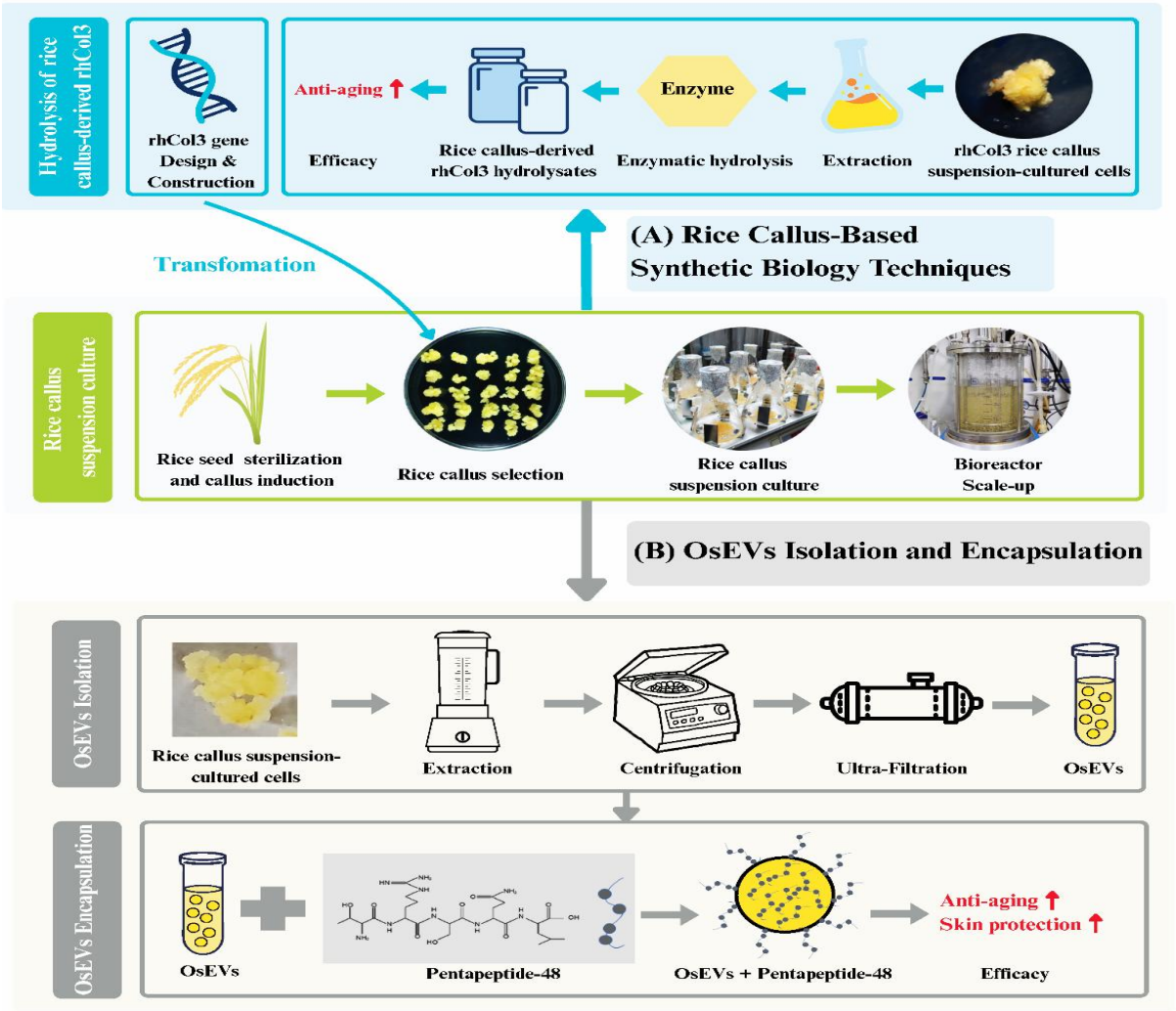


**Figure 1.** Schematic illustration of the preparation and application of rice callus suspension-culture cells and its derived recombinant proteins and extracellular vesicles.

## 2. Materials and Methods

### Materials

The *Oryza sativa* rice callus was induced from Zhonghua 11 seeds. FITC conjugated pentapeptide-48 was obtain from NJPeptide (NJPeptide, China). Murashige & Skoog Basal Medium with Vitamins was purchased from HiMedia (HiMedia, India).

### Preparation of rhCol3-containing rice callus extract hydrolysates

The rhCol3 gene was rationally designed, chemically synthesized and cloned into a binary vector, then introduced into rice callus via *Agrobacterium*-mediated transformation. The transformed callus was selected on antibiotic-containing plates and then transferred to suspension culture for further confirmation of protein expression. RhCol3-containing rice callus were expanded in a bioreactor, harvested, and centrifuged (15,000 × g, 20 minutes, 4 °C). The supernatant was collected, and protein content was quantified using the bicinchoninic acid (BCA) assay. Enzymatic hydrolysis with Alcalase was performed, followed by centrifugation (15,000 × g, 10 minutes) and filtration (0.22 μm) to obtain rhCol3-containing rice callus extract hydrolysates.

### Extraction and isolation of OsEVs from rice callus suspension-cultured cells

Rice callus suspension-cultured cells were cultured and separated from the culture medium. The cells were homogenized in ice-cold distilled water using a mechanical blender. The homogenate was filtered through a mesh sieve to remove gross debris. Solid fraction was separated by sequential centrifugation: first at 3,000 × *g* for 30 minutes, followed by 6,000 × *g* for 30 minutes, and finally at 15,000 × *g* for 60 minutes. The supernatant was sequentially filtered through sterile membranes. OsEVs were subsequently isolated and concentrated using ultrafiltration. The purified OsEVs were lyophilized and dispensed into glass vials.

### Characterization of OsEVs

The particle concentration and size distribution of OsEVs were determined by nanoparticle tracking analysis (NTA) and dynamic light scattering (DLS). The morphology of OsEVs was performed using transmission electron microscopy (TEM).

### Encapsulation of pentapeptide-48 in OsEVs

Pentapeptide-48 (or FITC-conjugated) was dissolved in ice-cold sodium citrate buffer and mixed with reconstituted OsEVs. To improve encapsulation efficiency, the mixture of peptide and OsEVs was sonicated (5 minutes, 4-8°C) and then incubated (30 minutes, 4 °C). Free peptides were removed by ultrafiltration.

### Cell culture

Human splenic fibroblasts (HSF) and human immortalized keratinocytes (HaCaT) were cultured in DMEM medium supplemented with 10 % fetal bovine serum (FBS) and 1 % P/S. The cells were maintained at 37 °C in a humidified incubator with 5 % $CO_2$.

### Cell proliferation assay

HaCaT cells were seeded at 5 × $10^3$ cells/well in 96-well plates and incubated for 24 hours to allow attachment. Cells were treated with various concentrations of OsEVs for 24 hours. After treatment, 10 μL of CCK-8 solution was added to each well and incubated at 37 °C for 1 hour. Absorbance was measured at 450 nm using a microplate reader.

### Wound-healing assay

HaCaT cells were cultured in 6-well plates and allowed to adhere for 24 hours. After incubation, the inserts were carefully removed, and cells were washed twice with PBS. The cells were treated with or without OsEVs (1 x $10^{10}$ particles/mL) for 24 hours. The culture medium was then removed, and the cells were washed twice with PBS. The wound-healing area was imaged and quantified by ImageJ software.

**Cell permeation studies**.

HaCaT cells were seeded in 6-well culture plates at a density of 5 × $10^3$ cells per well and incubated at 37 °C in a humidified atmosphere containing 5 % $CO_2$. After 24 hours, the cells were treated with pentapeptide-48-FITC (20 µg/mL), OsEVs (1 x $10^{10}$ particles/mL) and OsEV + pentapeptide-48-FITC (1 x $10^{10}$ particles/mL OsEVs containing 20 µg/mL pentapeptide-48-FITC) for 24 hours. The culture medium was then removed, and the cells were washed twice with PBS. Fluorescence imaging was performed by fluorescence microscope. The average fluorescence intensity was quantified using ImageJ software.

**Gene expression analysis of OsEVs encapsulating pentapeptide-48 and rhCol3-containing rice callus extract hydrolysates**

HSF cells were seed in 6-well culture plate and incubated at 37 °C in a humidified incubator containing 5 % $CO_2$ for 24 hours to allow cell attachment. After incubation, the cells were treated with PBS, pentapeptide-48 (20 µg/mL) and OsEVs + pentapeptide-48 (1 x $10^{10}$ particles/mL OsEVs containing 20 µg/mL pentapeptide-48) for 24 hours. The culture medium was then removed, and the cells were washed twice with PBS. Total RNA was extracted with TRIzol reagent according to the instructions. The extracted total RNA was reverse-transcribed into complementary DNA (cDNA) using the reverse transcription kit. Gene expression levels of *Matrix metalloproteinase-3 (MMP-3), Tissue inhibitor of metalloproteinases-1 (TIMP-1), Hyaluronidase-1 (Hyal-1)* and *Transglutaminase-1 (TSM-1)* were evaluated using real-time quantitative PCR (RT-qPCR).

In addition, the cells were treated with rhCol3-containing rice callus extract hydrolysates at final concentrations of 0 %, 0.25 %, 0.5 %, and 1 % (v/v) for 24 hours. Total RNA was extracted and reverse-transcribed as previously described. The gene expression levels of ECM-related genes, including *Elastase, Elastin, MMP-3, TIMP-1, Hyal-1,* and *Collagen I*, were analyzed. Gene expression levels were calculated using the ΔΔCt method, and *GAPDH* was used as the internal control gene.

**Statistical Analysis**

All data in this study were statistically analyzed using one-way ANOVA and presented as the mean ± S.D. Statistical significance was indicated as $p<0.05$ (*), $p<0.01$ (**), and $p<0.001$ (***). ImageJ was used for image quantification, and GraphPad Prism 8.0 was used for statistical analysis.

## 3. Results

**Physicochemical characterization and morphological analysis of OsEVs and OsEVs + Pentapeptide-48**

The OsEVs were isolated from rice callus suspension-cultured cells and loaded with pentapeptide-48 via sonication, which improved fluid dynamics and molecular permeation efficiency (Figure 2a). NTA confirmed that the purified OsEVs exhibited high purity with an average particle size of 112.5 ± 1.2 nm (Figure 2b). DLS analysis revealed the size distribution ranging from approximately 30-230 nm, with a zeta potential of -17 mV. After encapsulation of pentapeptide-48 (OsEVs + Pentapeptide-48), NTA analysis showed the average particle size increased to 128 ± 7.0 nm, representing a 14 % increase compared with native OsEVs (Figure 2b). This increase is likely attributed to the incorporation of the peptide. TEM further confirmed the characteristic morphology of both OsEVs and OsEVs + Pentapeptide-48, revealing an average diameter in the range of 80-130 nm, with a typical vesicle morphology consisting of bilayer membrane and saucer-like shaped structure (Figure 2c).

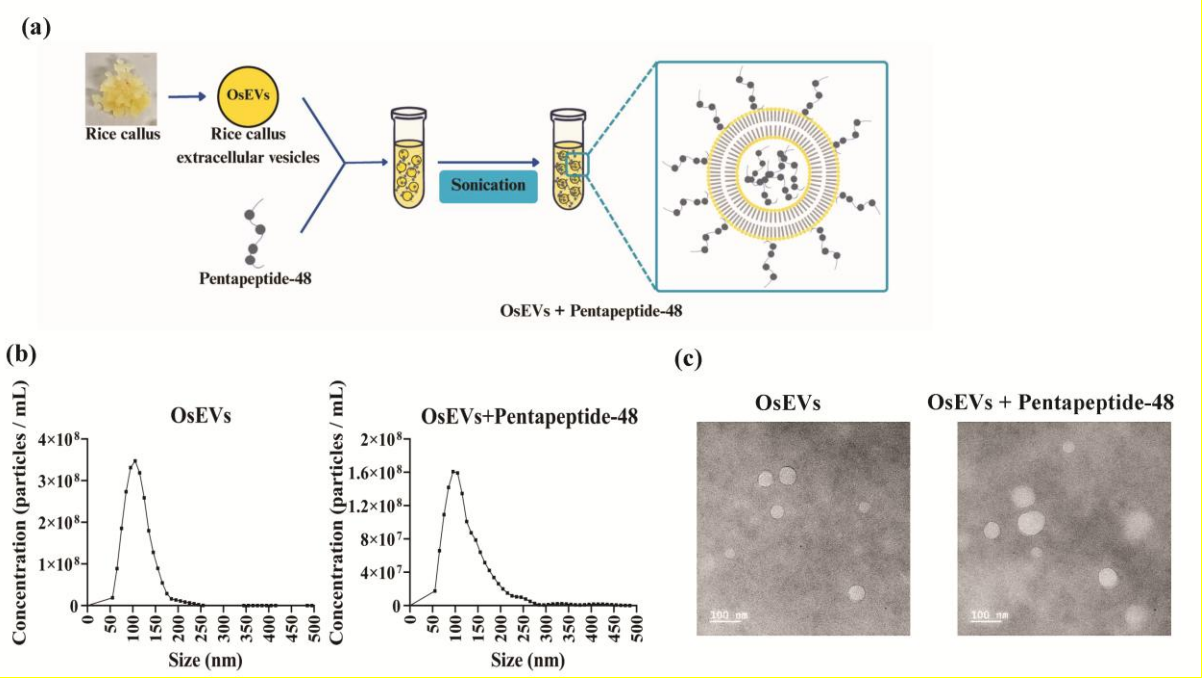


**Figure 2.** Characterization of OsEVs and OsEVs+Pentapeptide-48. (a) Schematic illustration of the encapsulation process of pentapeptide-48 into OsEVs. (b) NTA showing size distribution and particle concentration of OsEVs and OsEVs+Pentapeptide-48. (c) TEM images displaying the morphology of OsEVs and OsEVs+Pentapeptide-48. Scale bar is 100 nm.

## Functional evaluation of OsEVs

Cell proliferation of OsEVs was evaluated using the cell counting kit-8 (CCK-8) assay. As shown in Figure 3a, OsEVs-treated cells exhibited a significant dose-dependent increase in cell proliferation, achieving a maximum of 142.4 %, which was 19.0 % higher than the proliferation rate observed in the vitamin C-treated group. Additionally, the wound-healing rate in the OsEVs-treated group was approximately 60.9 %, representing a 38.3 % increase compared with the control group (Figure 3b and 3c). These findings suggest that OsEVs exhibit excellent regenerative effects on human keratinocytes.

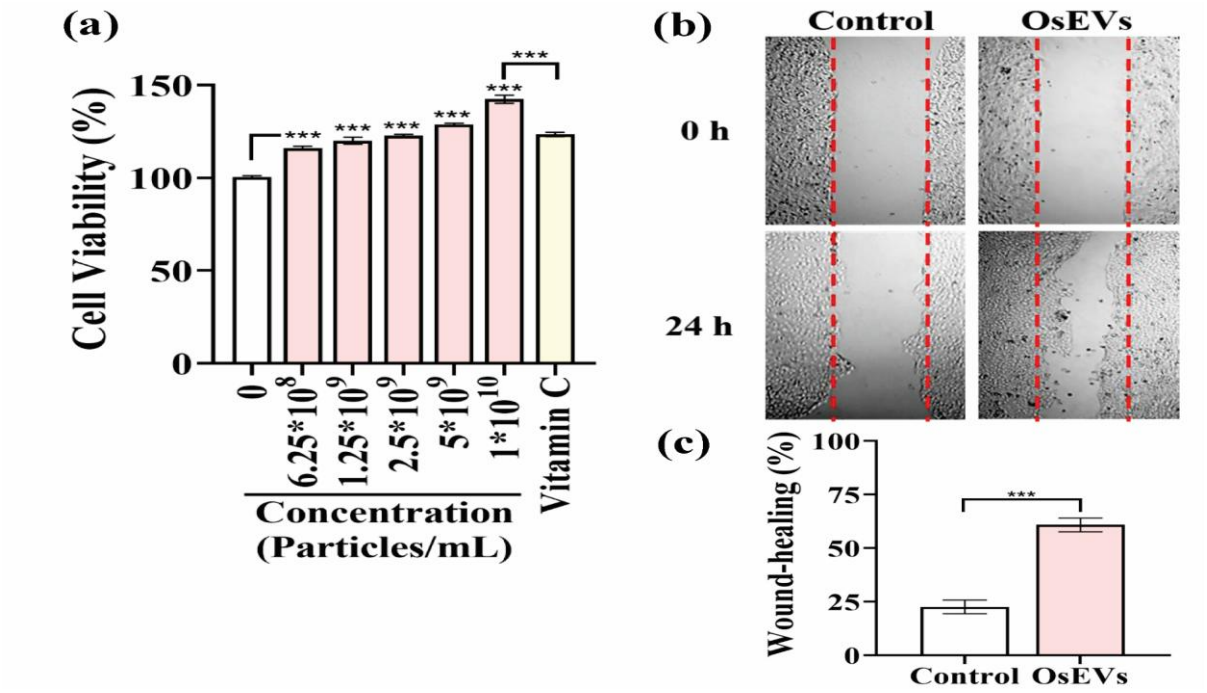


**Figure 3.** Cell proliferation and wound-healing assay of OsEVs. (a) For cell proliferation assays, HaCaT cells were treated with vitamin C (0.25 mg/mL) and various concentrations of OsEVs for 24 h. Imaging (b) and quantification (c) of the wound-healing assay were performed in cells treated with or without OsEVs (1 x $10^{10}$ particles/mL) for 24 h. Data are shown as the mean ± S.D. *(***p<0.001)*.

## OsEVs as intracellular delivery nanocarriers

To assess the cellular uptake efficiency of free peptides versus OsEVs-loaded peptides. FITC-conjugated pentapeptide-48 was used for fluorescent tracking. Pentapeptide-48-FITC was encapsulated into OsEVs and applied to HaCaT cells for 24 h. Intracellular fluorescence was visualized using fluorescence microscopy (Figure 4a), and quantitative analysis showed that OsEVs+Pentapeptide-48-FITC exhibited the highest fluorescence intensity among all treatment groups (Figure 4b), exceeding both free pentapeptide-48-FITC and OsEVs alone. These results suggest that OsEVs exhibit excellent permeability potential as a natural nanocarrier for peptide delivery.

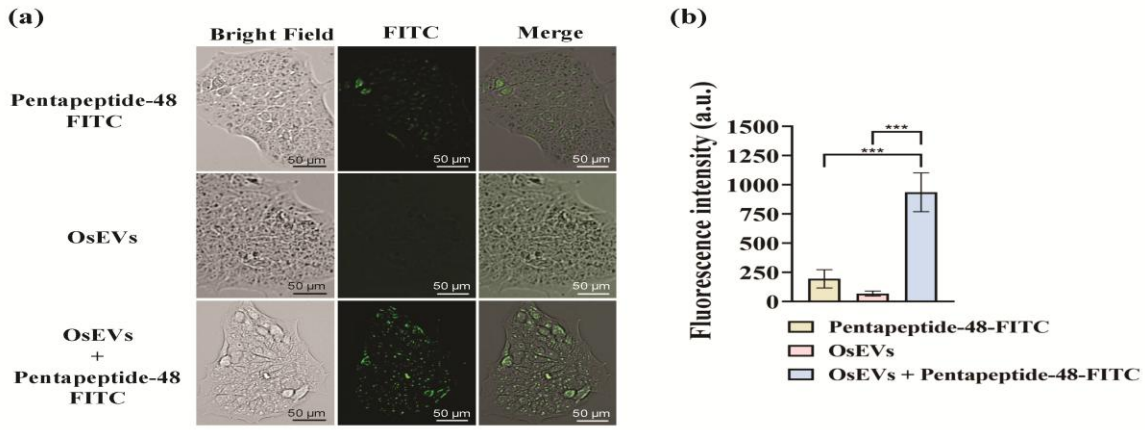

**Figure 4. Cell permeation ability of pentapeptide-48, OsEVs, and OsEVs+Pentapeptide-48 in human keratinocytes.** (a) Fluorescence images of HaCaT cells showing intracellular uptake of pentapeptide-48, OsEVs, and OsEVs+Pentapeptide-48. Scale bar is 50 μm. (b) Analysis of FITC fluorescence intensity in HaCaT cells indicating intracellular uptake of pentapeptide-48, OsEVs, and OsEVs+Pentapeptide-48. Data are represented as the mean ± S.D. *(***p<0.001)*.

## OsEVs-mediated delivery improves the bioactivity of pentapeptide-48 in skin anti-aging and barrier protection

To evaluate the functional efficiency of OsEVs+Pentapeptide-48, the expression levels of genes associated with anti-aging and skin barrier protection were analyzed in HSF cells. Three ECM–related genes (*MMP-3, TIMP-1, and Hyal-1*) and one skin barrier–associated gene (*TGM-1*) were examined (Figure 5). Treatment with OsEVs-loaded pentapeptide-48 significantly modulated the expression of genes involved in ECM remodeling and skin barrier function. Compared with peptide treatment alone, *MMP-3* and *Hyal-1* were downregulated by 10.4 % and 8.6 %, respectively. In contrast, the expression of *TIMP-1* and *TGM-1* (encoding transglutaminase-1, critical for skin barrier integrity) was upregulated by 18.9 % and 7.0 %, respectively. MMP-3, TIMP-1, and Hyal-1 are invovled in ECM remodeling , while TGM-1 is associated with skin protective function. These results demonstrate that OsEVs-mediated delivery of pentapeptide-48 enhances both its anti-aging and skin barrier-supporting effects at the molecular level, indicating the potential of OsEVs as a natural and effective nanocarrier for bioactives delivery. These findings suggest that OsEVs-loaded pentapeptide-48 represents a potential approach for the development of innovative and effective cosmetic products.

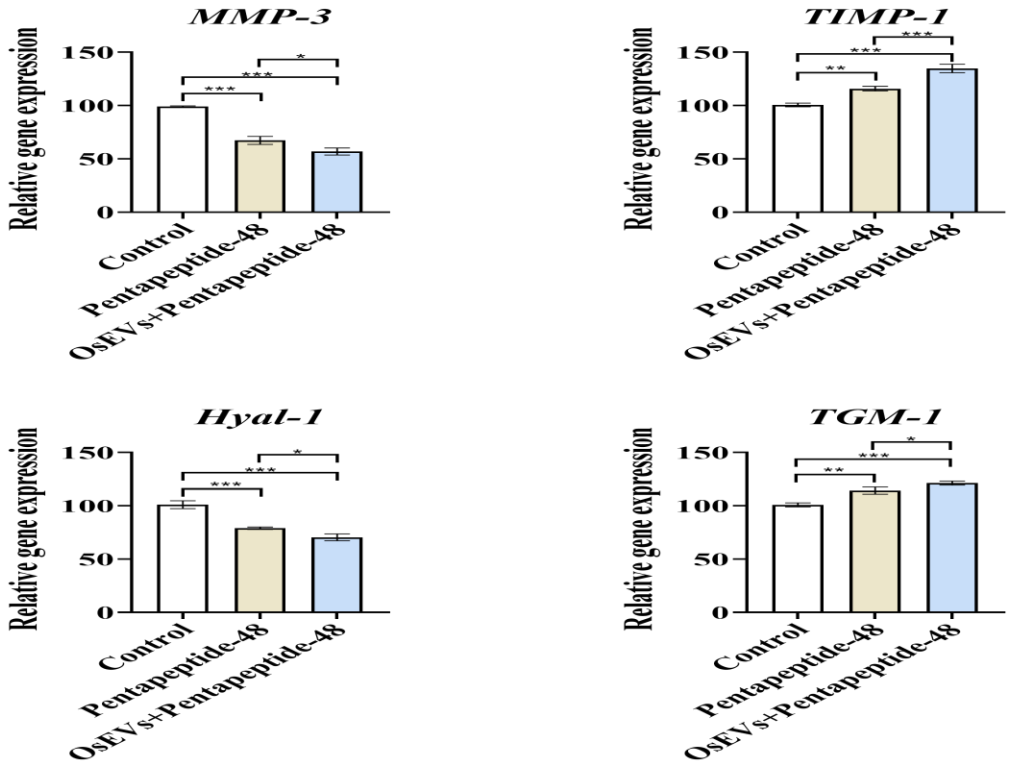

**Figure 5. Anti-aging and skin protective effects of pentapeptide-48 and OsEVs+Pentapeptide-48.** Gene expression levels of *MMP-3*, *TIMP-1*, *Hyal-1* and *TGM-1* were quantified by RT-qPCR. Data are presented as the mean ± S.D. *(* p<0.05, ** p<0.01, ***p<0.001).*

## RhCol3-containing rice callus extract hydrolysates enhance anti-aging activity in human skin cells

The anti-aging potential of rhCol3-containing rice callus extract hydrolysates were evaluated by analyzing the expression of six ECM-related genes in HSF cells treated with 0.25 %, 0.5%, and 1 % hydrolysate (Figure 6). The results showed that the gene expression levels of *Elastase* (87.4 ± 2.8 %, 78.0 ± 3.8 %, and 61.7 ± 3.8 %), *MMP-3* (a matrix metalloproteinase) (79.5 ± 4.0 %, 59.8 ± 3.6 %, and 47.4 ± 2.9 %), and *Hyal-1* (a lysosomal hyaluronidase) (86.3 ± 3.9 %, 75.3 ± 4.6 %, and 65.3 ± 1.6 %) were significantly downregulated in a dose-dependent manner. In contrast, the gene expression levels of *Elastin* (111.9 ± 2.2 %, 123.4 ± 3.5 %, and 132.5 ± 2.3 %), *TIMP-1* (an endogenous inhibitor of MMPs) (108.4 ± 2.7 %, 126.3 ± 5.8 %, and 138.7 ± 1.7 %), and *Collagen I* (98.7 ± 1.7 %, 112.5 ± 2.6 %, and 115.9 ± 0.8 %) were progressively upregulated. These findings suggest that hydrolysates from rhCol3-expressing rice callus suspension culture cells exhibit anti-aging potential by modulating ECM-related genes. The suppression of *Elastase, MMP-3 and Hyal-1*, accompanied by the upregulation of *Elastin, TIMP-1, and Collagen I*, suggests enhanced ECM stability, reduced matrix degradation, and improved skin elasticity [12, 13]. These results reveal the critical role of rhCol3-containing rice callus extract hydrolysates in promoting ECM remodeling and cellular regeneration.

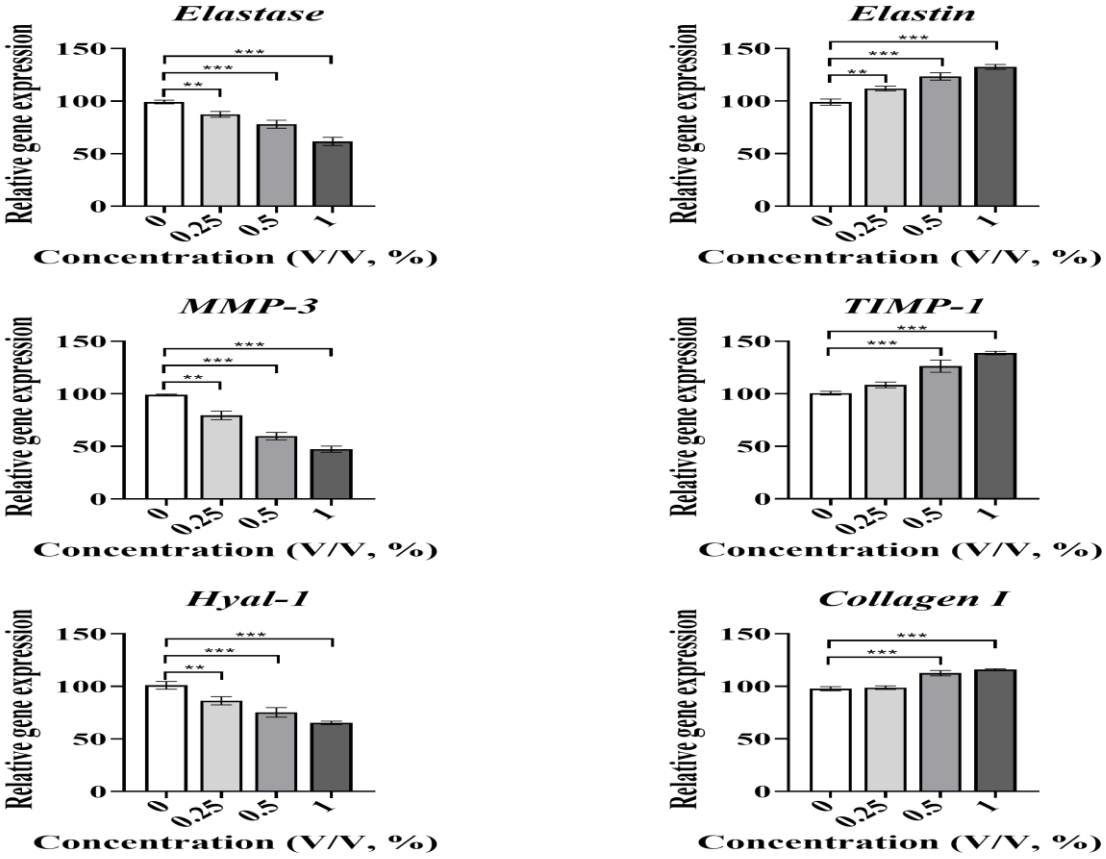

**Figure 6.** Anti-aging effects of hydrolysates derived from rhCol3 expressed in rice callus suspension-cultured cells. The gene expression levels of *Elastase*, *Elastin*, *MMP-3*, *TIMP-1*, *Hyal-1*, *Collagen I*. The ECM-related genes were quantified by RT-qPCR. Data are presented as the mean ± S.D. *(* p<0.05, ** p<0.01, ***p<0.001).*

## 4. Discussion

This study presents an RCSC-based platform for the dual production of recombinant proteins and extracellular vesicle-mediated peptide delivery. The rhCol3-containing rice callus extract hydrolysates effectively modulated the expression of ECM-related genes associated with dermal remodeling, supporting the anti-aging potential of plant-derived biomolecules [6]. Futhermore, OsEVs demonstrated efficient cellular permeation ability and enhanced the bioactivity of encapsulated pentapeptide-48, consistent with the regenerative application of PDEVs [8, 9]. The observed synergistic modulation of ECM stability and skin barrier protection suggests the potential of RCSC-derived materials to address various challenges of skin aging. The RCSC-based platform offers a safe, sustainable, and biocompatible alternative to animal-derived or synthetic approaches for next-generation cosmetic bioactives.

Future research should focus on investigating the molecular mechanisms of EV-mediated cargo delivery, optimizing large-scale production processes, and validating the functional efficacy in advanced 3D skin models and preclinical in vivo systems. Expanding its application to include more bioactives and macromolecules could further establish RCSC-derived materials as versatile, animal-free alternatives for regenerative cosmetic and biomedical uses.

## 5. Conclusion

This study successfully established a dual functional RCSC-based platform for cosmetic bioactive production. The generation of rhCol3-containing rice callus extract hydrolysates, which demonstrated significant anti-aging effects. Functionally engineered OsEVs encapsulating pentapeptide-48 exhibited enhanced cellular uptake efficiency and bioactivity, contributing to improved anti-aging and skin protective effects. These findings highlighting the potential as innovative and sustainable alternatives to animal-derived ingredients and synthetic carriers for the development of next-generation green cosmetics.

## 6. References

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