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ORIGINAL ARTICLE

Potential role of the cell-penetrating peptide-conjugated soluble N-ethylmaleimide-sensitive factor attachment protein receptor motif of vesicle-associated membrane protein 2-patterned peptide in novel cosmeceutical skin product development

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Abstract

Aim: This study aimed to investigate and verify the effect of cell-penetrating peptide (CPP)-conjugated soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) motif of vesicle-associated membrane protein 2 (VAMP2)-patterned peptide (INCI name: Acetyl sh-Oligopeptide-26 sh-Oligopeptide-27 SP, trade name: M.Biome-BT) on improving skin function in vitro.

Methods: The cytotoxicity of CPP-conjugated SNARE motif of VAMP2-patterned peptide (CVP) was investigated using the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) assay against B16-F10 cells and human dermal fibroblasts (HDFs) and a reconstructed skin irritation test. The anti-wrinkle activity of M.Biome-BT was determined by assessing the release of norepinephrine and dopamine in PC-12 cells via ELISA. The skin-whitening effects of CVP were assessed in B16-F10 cells by measuring the intra- and extracellular melanin contents and expression levels of melanin production-related genes, such as microphthalmia-associated transcription factor (*MITF*), tyrosinase (*TYR*), tyrosinase-related protein-1 (*TRP-1*), and *TRP-2*.

Results: CVP is not cytotoxic to B16-F10 cells and HDFs, and no skin irritation was observed. CVP treatment considerably diminished K^+-induced norepinephrine and dopamine secretion compared with the non-treated control group (62% and 40%, respectively). Additionally, the inhibition ability of CVP on norepinephrine and dopamine release was comparable to that of botulinum neurotoxin type A (BoNT/A). CVP also increased intracellular melanin content in a dose-dependent manner, whereas extracellular melanin content decreased (76%–85%). However, CVP treatment did not

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affect the mRNA expression of *MITF*, *TYR*, *TRP-1*, and *TRP-2*. These results suggest that CVP does not inhibit melanin production; however, it may induce a whitening effect by inhibiting melanin transport.

Conclusions: Taken together, our findings indicate that CVP could be used as an active and safe cosmeceutical ingredient for antiaging applications.

KEYWORDS
cell-penetrating peptide, cosmetics, skin aging, soluble N-ethylmaleimide-sensitive factor attachment protein receptor, synthetic peptide

1 | INTRODUCTION

The skin is the largest organ of the human body, and it serves as a protective barrier that shields our body from external aggressions while maintaining water and temperature homeostasis.[1] Skin aging is a complicated biological process in living organisms, defined by a wide range of structural and physiological changes in the skin. Both intrinsic and extrinsic factors, such as metabolic processes, hormonal changes, genetics, poor nutrition, smoking, and excessive exposure to ultraviolet radiation, can cause wrinkling, rough-textured appearance, and tone loss in the skin.[2]

Wrinkles represent the most evident outcome of cutaneous aging. Their onset is associated with several events resulting from chrono- and photoaging, including dermo-epidermal junction thinning and the loss of collagen, glycosaminoglycans, and subcutaneous fat.[3] The formation of wrinkles is caused in part by excessive muscle fiber stimulation in the face, which pulls the skin inward, causing wrinkles.[4] Skin pigmentation is a major clinical alteration associated with skin aging caused by physiological and photoaging processes. Melanin in mammalian melanocytes is synthesized within melanosomes and plays a crucial role in protecting against the harmful effects of ultraviolet radiation. Melanin synthesis and distribution contribute to determining mammalian skin and hair color. Tyrosinase is a vital rate-limiting enzyme that catalyzes melanin synthesis in skin melanocytes, the mature melanin-forming cells in the skin. Skin hyperpigmentation is dependent on either the size, number, distribution, and composition of melanocytes or tyrosinase activity.[5] Increased epidermal pigmentation can cause various skin disorders, including melasma, age spots, freckling, and sites of actinic damage.[6] Thus, this problem has prompted the research of cosmetic and medical remedies into skin aging.

The use of botulinum neurotoxin type A (BoNT/A) has increased dramatically, particularly for eliminating facial wrinkles. BoNT/A is the most potent neurotoxin; it can cause muscle paralysis by inhibiting acetylcholine neurotransmitter release at nerve-muscle junctions. One or more of the three soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) proteins, vesicle-associated membrane protein 2 (VAMP2), synaptosome-associated protein of 25 kDa (SNAP-25), or syntaxin, form the SNARE complex, which is an essential step for neurotransmitter release in synapses. BoNT/A enzymatically cleaves the presynaptic SNARE protein SNAP-25 and blocks the formation of the SNARE complex and subsequent neurotransmitter release.[7] BoNT/A may also have a promising protective effect on skin pigmentation.[8] Jung et al.[9] investigated the mechanism of action of BoNT/A on skin pigmentation and hypothesized that there might be a connection between BoNT/A, tyrosinase activity, and melanin content. Even though the injection of BoNT/A is a simple and effective nonsurgical procedure, its usage is strictly prohibited due to its severe toxicity. To overcome this limitation, small peptide molecules that imitate the action of BoNT/A are gaining attention.

Peptides have higher commercial viability and stability than amino acids. Skin-permeable peptides can easily penetrate the skin barrier, whereas amino acids have low skin permeability. Thus, to improve skin conditions, many synthetic peptides have been developed and commercialized in functional cosmetic products with antioxidant, "Botox-like" wrinkle smoothing, collagen stimulation, and whitening effects.[10,11] Cell-penetrating peptides (CPPs), consisting of short peptides approximately 30 amino acids long, can carry various cargoes over cellular barriers and have low cellular toxicity.[12] CPPs, especially CPP–cargo conjugates, have shown a significant impact on improving drug delivery in vitro and in vivo, providing a commercial appeal, particularly as skin care products.[13] In the present study, we designed and synthesized seven peptides from the SNARE motifs of SNAP-25, syntaxin 4, and VAMP2 that mimic the action of BoNT/A. IMT-P8, a novel arginine-rich human protein-derived CPP, was conjugated to these peptides for higher skin permeability.[14] These peptides are expected to competitively suppress SNARE complex formation by interfering with parental SNARE protein interactions, blocking neurotransmitter release, and exhibiting higher skin permeability due to their transdermal delivery. Among seven synthetic peptides, the CPP-conjugated SNARE motif of VAMP2-patterned peptide (CVP; INCI name: Acetyl sh-Oligopeptide-26 sh-Oligopeptide-27 SP; CAS number: 2414071–80-8; trade name: M.Biome-BT) was shown to have the highest norepinephrine inhibitory activity.

In this study, we investigated the inhibitory activities of CVP on neurotransmitter release in PC-12 cells and melanin content and melanogenesis-related gene expression in B16-F10 cells as potential anti-wrinkle and skin-whitening ingredients for use in cosmetic products.

2 | MATERIALS AND METHODS

2.1 | Chemicals and reagents

Dimethyl sulfoxide (DMSO), α-melanocyte-stimulating hormone (MSH), NaOH, and 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma Aldrich. Fetal bovine serum (FBS), trypsin-ethylenediaminetetraacetic acid (EDTA), and horse serum (HS) were purchased from Gibco (Thermo Fisher Scientific, Inc.). Dulbecco's modified Eagle medium (DMEM), phosphate-buffered saline (PBS), and RPMI 1640 were purchased from Welgene.

2.2 | Synthesis of cell-penetrating peptides

Nine CPPs were synthesized using the 9-fluorenylmethoxycarbonyl (Fmoc)-based solid-phase method and characterized by Lugen Science. The CPP sequences are summarized in Table 1. Each CPP was labeled with fluorescein isothiocyanate (FITC) by reacting with its free N-terminal amino group. The purity of the peptides used in this study was greater than 95%, as determined via high-performance liquid chromatography. The peptides were delivered as lyophilized materials (acetate salts), dissolved in MQ-water, and stored as aliquots at −20°C until further analysis.

2.3 | Flow cytometry analysis of the cell-penetrating peptides

To determine the cell permeability of each CPP (CPP-1–CPP-9, Table 1), PC-12 cells obtained from the ATCC were grown in a six-well plate (3×10^5 cells/well) and cultured in RPMI 1640 supplemented with 10% HS for 1 day before analysis. The cells were treated with 10 μM of the FITC-labeled CPP candidates for 2 h. The cells were then harvested using 0.25% trypsin-EDTA for 3 min at 37°C and then centrifuged at 16,000 × *g* for 10 min. After washing twice with PBS, each CPP was analyzed with a BD FACS Canto II system using BD FACS Diva software 8.0 (BD Biosciences) for FITC fluorescence.

2.4 | Peptide (cell-penetrating peptide–cargo conjugate) synthesis

Seven peptides representing the various regions in the SNARE motifs of individual SNAREs were synthesized using the Fmoc-based solid-phase method and characterized by Lugen science. Seven peptide sequences were derived from each SNARE motif of SNAP-25, syntaxin 4, and VAMP2. The peptide sequences from each SNARE motif, representing the N-terminal, middle, and C-terminal regions, are summarized in Table 2. IMT-P8 (C2) was conjugated to the N-terminus of each peptide to enhance their skin permeability. The purity of the peptides used in this study was greater than 95%, as determined via high-performance liquid chromatography. The peptides were delivered as lyophilized materials (acetate salts), dissolved in MQ-water, and stored at −20°C as aliquots until further use.

2.5 | Norepinephrine release assay for peptide screening

To investigate the norepinephrine release of each peptide (C2-P1–C2-P7; Table 2), PC-12 cells were grown in a 24-well plate (1×10^5 cells/well) and cultured in RPMI 1640 medium supplemented with 10% HS for 2 days before analysis. Each peptide was added to each plate and incubated for 2 h. The spent medium was then replaced with RPMI 1640 medium supplemented with 20 μM of each respective peptide. After incubation for 2 h, the culture medium was removed, and the cells were washed twice with Krebs–Henseleit solution containing 118 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 2 mM KH_2PO_4, 24 mM $NaHCO_3$, and 11 mM dextrose. A Krebs–Henseleit solution with high potassium content (68 mM KCl) was then added to the cells to evaluate norepinephrine release. Norepinephrine concentration was measured using an ELISA kit (Abnova), following the manufacturer's recommended protocol.

TABLE 1 Information on the cell-penetrating peptides (CPPs) used in this study.

	Name	Sequence	Length	Charge	Origin
CPP-1	TD1	KAMININKFLNQC	13 aa	+2	BoNT/A HC (779–791 aa)
CPP-2	IMT-P8	RRWRRWNRFNRRRCR	15 aa	+9	Calcium channel alpha-1 subunit (503–517 aa)
CPP-3	Transkin	NGSLNTHLAPIL	12 aa	+1	Artificial
CPP-4	VP2-2	KYWWKNLKMMIIL	13 aa	+3	VAMP2 (87–99 aa)
CPP-5	RBD-1	YLNSSLYRGTKFIIK	15 aa	+3	BoNT/A HC (1149–1163 aa)
CPP-6	SN25-2	MAISGGFIRRV	11 aa	+2	SNAP-25 (127–137 aa)
CPP-7	STX-1	GIIMDSSISK	10 aa	+1	Syntaxin 1a (180–189 aa)
CPP-8	TDb-1	KYIVTNWLAKVNT	13 aa	+2	TD (711–723 aa)
CPP-9	Scramble	TALNYVGFIRMKWS	14 aa	+2	Artificial

TABLE 2 Amino acid sequences of synthetic peptides derived from the soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) motifs of SNAP-25, syntaxin 4, and VAMP2 origin.

Name	Sequence	Length	Charge	Peptide origin
C2-P1	RRWRRWNRFNRRRCR**EEMQRR**	21 aa	+9	Acetyl hexapeptide-3
C2-P2	RRWRRWNRFNRRRC R**DARENEMDEN LEQVSGI**	32 aa	+4	SNAP-25 (140–156 aa)
C2-P3	RRWRRWNRFNRRRCR**L SEIETRHSEIIKLENS**	32 aa	+7	Syntaxin 4 (192–208 aa)
C2-P4	RRWRRWNRFNRRRC R**RRLQQTQAQV DEVVDIM**	33 aa	+8	VAMP2 (30–46 aa)
C2-P5	RRWRRWNRFNRRRCR**D ARENEMDENLEQV**	29 aa	+4	SNAP-25 (140–153 aa)
C2-P6	RRWRRWNRFNRRRCR**L SEIETRHSEIIKL**	29 aa	+8	Syntaxin 4 (192–205 aa)
C2-P7	RRWRRWNRFNRRRCR**R RLQQTQAQVDEVV**	29 aa	+9	VAMP2 (30–43 aa)

Note: Seven peptides representing the N-terminal, middle, and C-terminal regions of the SNARE motifs of SNAP-25, syntaxin 4, and VAMP2 were synthesized. IMT-P8 (C2) was conjugated to the N-terminus of each peptide and its sequence is presented in bold typeface.

2.6 | 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl tetrazolium bromide cell viability assay

B16-F10 melanoma cells and human dermal fibroblasts (HDFs) obtained from ATCC were cultured in DMEM containing 10% FBS. The cytotoxicity of CVP against normal cells was determined using the MTT assay, as described by Mosmann.[15] Briefly, B16-F10 cells and HDFs were seeded in 96-well plates (1.5×10^4 cells/well) and cultured for 24 h. The cells were then treated with various concentrations of CVP (5–50 μM) for 48 h at 37°C in a 5% CO_2 incubator, centrifuged at 16,000 × *g* for 10 min, and the medium from each well was removed. Then, MTT solution (0.5 mg/mL) was added to each well, and the cells were incubated for another 3 h in the dark. The resulting colored formazan crystals were dissolved in 200 μL of DMSO, and the absorbance of the solution was measured at 570 nm with a microplate reader. The cell viability of the treated models was normalized to the negative control, which was set to 100%.

2.7 | Norepinephrine and dopamine release assay

PC-12 cells were grown and cultured in RPMI 1640 medium supplemented with 10% HS for 2 days. CVP and BoNT/A (Medytox) were then added to the cells and incubated for 2 h. Next, the spent medium was replaced with RPMI 1640 medium supplemented with 20 μM of CVP or BoNT/A. After incubation, the culture medium was removed, and the cells were washed twice with Krebs–Henseleit solution. A high-potassium Krebs–Henseleit solution was then added to the cells to evaluate norepinephrine and dopamine release. Norepinephrine and dopamine concentrations were measured using ELISA kits (Abnova), following the manufacturer's recommended protocol.

2.8 | Skin irritation test

The 3D reconstructed human epidermis tissue model Neoderm® (TEGO Science) was used to evaluate whether CVP causes skin irritation, following the OECD guidelines for chemicals testing.[16] Next, 20 μM of the following substances were applied to the Neoderm® model and maintained for 15 min at room temperature (23–27°C): PBS (negative control), 5% (w/v) sodium dodecyl sulfate (SDS; positive control), and CVP. Afterward, each tissue model was transferred to a new 12-well plate and incubated at 37°C for 15 min in a 5% CO_2 incubator. Each substance was then removed from the model skin surface by washing with PBS, and the skin was further incubated in an incubator (37°C, 5% CO_2) for 42 h. Afterward, the spent growth medium was replaced with a maintenance medium containing the MTT agent and incubated for an additional 3 h. The formazan crystals in the tissues were extracted with 0.04 N HCl in isopropanol, and the optical densities (OD) was measured at 580 nm. Finally, the cell viability was calculated as follows:

Cell viability (%) = A_{580} of the tested cells/A_{580} of the control × 100%.

2.9 | Melanin content and melanogenesis-related gene expression in b16-f10 cells

2.9.1 | Treatment and lysis of cells

B16-F10 murine melanoma cells were grown at a density of 1×10^5 cells/well in a six-well plate and cultured in DMEM for 24 h. Then, the cells were treated with CVP at 5 or 10 μM for 48 h in the presence (for melanin content experiments) or absence (for gene expression analysis) of 200 nM α-MSH. Thereafter, the spent medium was collected for extracellular melanin content determination. Briefly, the cells were washed twice with PBS and harvested. The harvested

cells were then centrifuged at 16,000×*g* for 10 min to obtain the pellet for intracellular melanin content and gene expression assessments.

2.9.2 | Measurement of cellular melanin content

Melanin content was measured as described previously[17] with minor modifications. After lysis, B16-F10 cell pellets were dissolved in 1 N sodium hydroxide containing 10% DMSO for 1 h at 95°C. The absorbance of the solution was measured at 495 nm using a microplate reader (EPOCH2, BioTek).

2.9.3 | Real-time quantitative reverse transcriptase PCR

The mRNA expressions of *TYR*, *TRP-1*, *TRP-2*, and *MITF* were determined using real-time quantitative reverse transcriptase PCR (qRT-PCR) with glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*; Bionics) as an internal control. Total cellular RNA was extracted with Qiazol (Qiagen) according to the manufacturer's instructions. The RNA concentration and overall quality were determined using a NanoDrop ONE system (ThermoFisher) and electrophoresis on a 1% agarose gel. Complementary DNA was synthesized using a GoScript™ Reverse Transcriptase kit (Promega) following the manufacturer's instructions. A TB Green® Premix Ex Taq™ II (Takara) was used for all samples, and the reactions were performed in a final volume of 15 μL. Real-time qRT-PCRs for the target genes and internal controls were performed in triplicate using 10 μL of the primers listed in Table 3. Gene expression values were calculated using the ΔΔCt method,[18] where a corresponding non-treated sample was designated as a calibrator for each sample.

2.10 | Statistical analyses

All data were expressed as the mean ± standard deviation (SD). Statistical analyses were performed using the statistical software package of the GraphPad Prism 5.0 software. Statistical differences were evaluated using one-way analysis of variance (ANOVA), followed by Dunnett's post hoc test for multiple comparisons with controls. A p-value < 0.05 was considered statistically significant.

TABLE 3 Sequences of the primer pairs used to amplify target genes in this study.

Target gene	Sequence (5′–3′)	
TYR	Forward	ACACCTGAGGGACCACTAT
	Reverse	CATTGGCTTCTGGGTAAACT
TRP-1	Forward	GCCACAAGGAGGTTAGAAGACA
	Reverse	CCAGTAAGGAAGGGAGAAAGAG
TRP-2	Forward	AGAAGTTTGACAGCCCTCC
	Reverse	CAAGTTGCTCTGCGGTTAG
MITF	Forward	AACGGGAACAGCAACGAGC
	Reverse	TCACCAGATCAGGCGAGCA

3 | RESULTS

3.1 | Selection of cell-penetrating peptides

To select the CPP that can best penetrate PC-12 cells, FACS analysis was carried out to quantify the cellular uptake of the CPPs. We observed cellular uptake efficiencies of the nine CPP candidates in PC-12 cells. The results showed that CPP-2 displayed the highest cellular uptake at 99.8% (Figure 1). Considering this result, IMT-P8 was selected and conjugated to each peptide.

3.2 | Synthetic peptide selection

To investigate the inhibitory effect of the seven synthetic peptides on neurotransmitter release, norepinephrine release was quantified via FACS analysis. While most of the tested peptides examined were ineffective, C2-P4 reduced norepinephrine release by 30%–40% (Figure 2). As a result, we confirmed the neurotransmitter release inhibitory effect of C2-P4, herein designated as CVP.

3.3 | Effect of cell-penetrating peptide-conjugated SNARE motif of VAMP2-patterned peptide on cell viability

The cell viability of B16-F10 cells and HDFs after treatment for 48 h with CVP at concentrations of 0–50 μM was assessed using the MTT assay. B16-F10 melanoma cells treated with 20 μg/mL CVP demonstrated 85.6% viability, suggesting that 20 μg/mL CVP was slightly cytotoxic (Figure 3A). Therefore, the intracellular and extracellular melanin contents and expression levels of melanin production-related genes in B16-F10 melanoma cells were analyzed after treatment with 0–10 μg/mL CVP. CVP showed no significant cytotoxicity at doses ranging from 6.25 to 50 μM in HDFs (Figure 3B). Next, an in vitro skin irritation test for CVP was performed using a reconstructed human epidermis model. As shown in Figure 3B, the relative cell viability of the negative control (NC, PBS), positive control (PC, 5% SDS), and CVP were 100.0%, 8.2%, and 87.2%, respectively. This result suggests that CVP does not cause skin irritation according to the OECD 439 classification.

3.4 | Effects of cell-penetrating peptide-conjugated SNARE motif of VAMP2-patterned peptide on neurotransmitter release

To elucidate whether CVP improved the appearance of wrinkles, the release of norepinephrine and dopamine was determined in PC-12

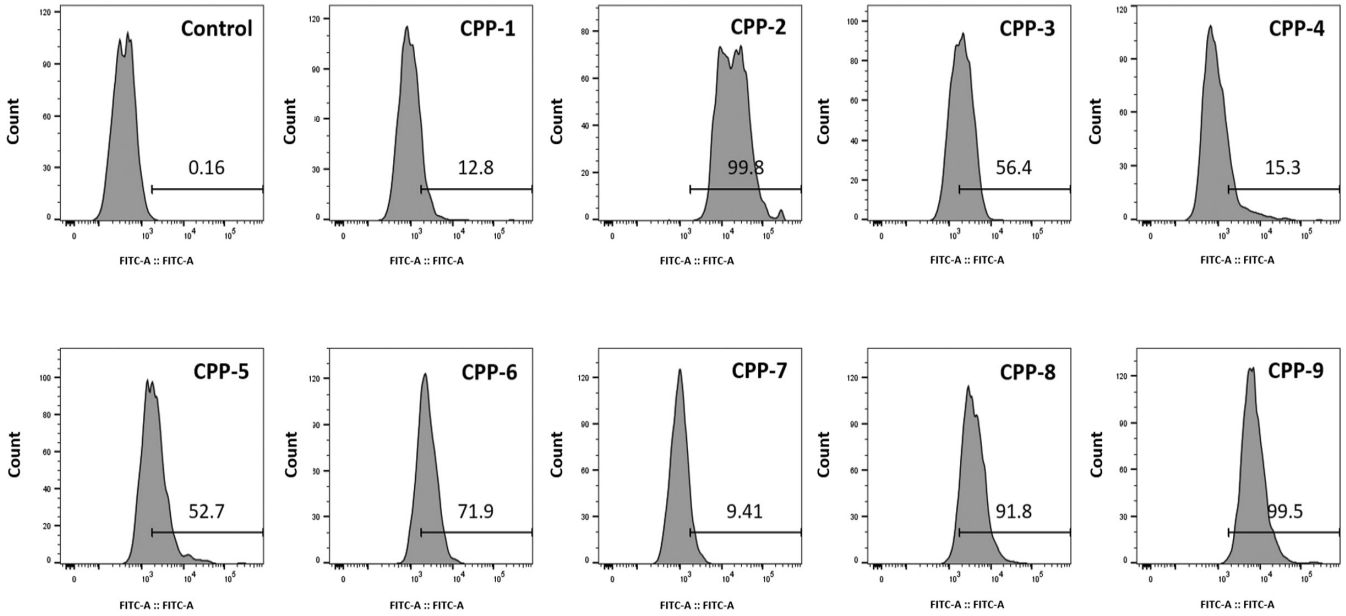

FIGURE 1 The cell membrane permeabilities of the nine cell-penetrating peptides were determined using flow cytometry. The horizontal line in each histogram indicates the percentage of FITC-positive cells after a 2-h exposure to each cell-penetrating peptide. FITC, fluorescein isothiocyanate.

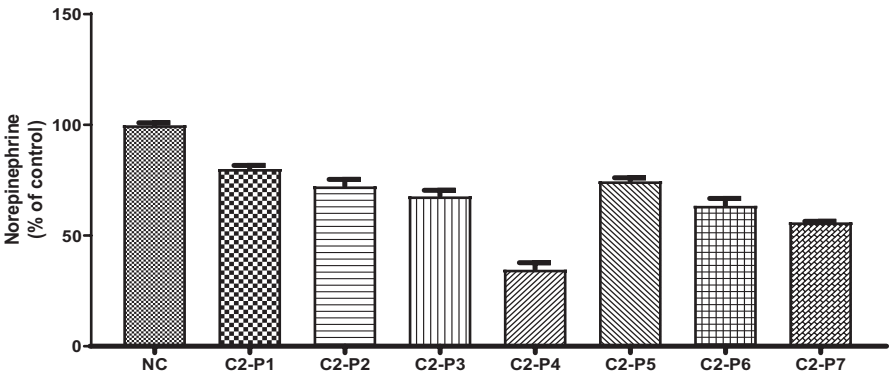

FIGURE 2 Inhibitory effects of the seven synthetic peptides derived from the SNARE motifs of SNAP-25, syntaxin 4, and VAMP2 on norepinephrine release in PC-12 cells. C2 = IMT-P8. The results are presented as a percentage relative to the control. Values are represented as the mean ± standard deviation (SD) of triplicate values.

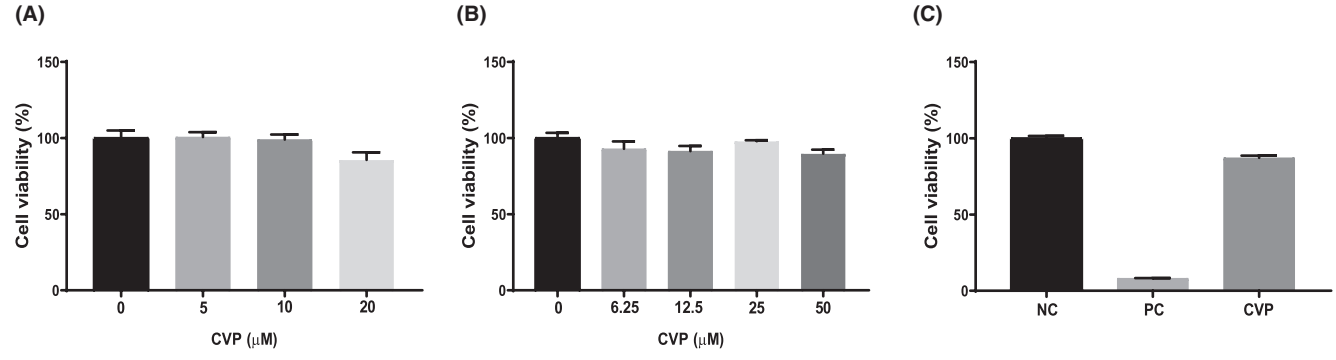

FIGURE 3 Viability of (A) B16-F10 cells and (B) human dermal fibroblasts exposed to different concentrations of cell-penetrating peptide-conjugated soluble N-ethylmaleimide-sensitive factor attachment protein receptor motif of vesicle-associated membrane protein 2-patterned peptide (CVP) for 48 h. (C) In vitro skin irritation test for CVP in the human epidermis tissue model Neoderm®. The results are presented as a percentage relative to the control. Values are represented as the mean ± standard deviation (SD) of triplicate values.

cells via ELISA. The depolarization of PC-12 cells by high K^+ concentrations caused the release of neurotransmitters such as norepinephrine and dopamine.[19] Incubating PC-12 cells with CVP significantly attenuated the K^+-induced norepinephrine secretion compared with that in the non-treated control group (approximately 62%; $p < 0.001$; Figure 4A). In addition, CVP also significantly diminished

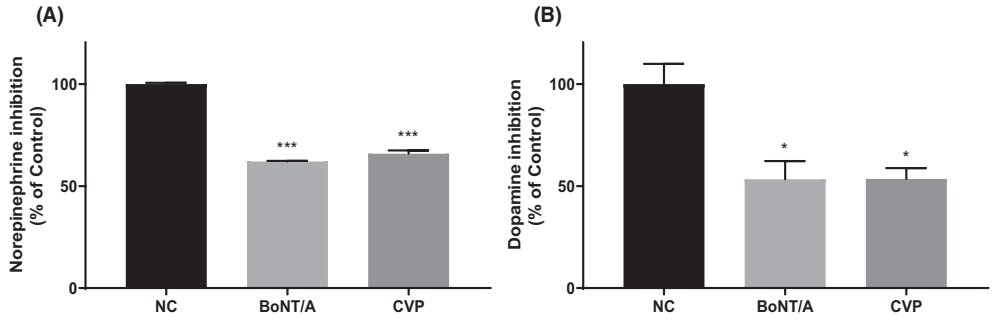

FIGURE 4 Inhibitory effects of cell-penetrating peptide-conjugated soluble N-ethylmaleimide-sensitive factor attachment protein receptor motif of vesicle-associated membrane protein 2-patterned peptide (CVP) on (A) norepinephrine and (B) dopamine release in PC-12 cells. The results are presented as percent inhibition relative to the control group. Values are represented as the mean ± standard deviation (SD) of triplicate values. $^{*}p < 0.05$ and $^{***}p < 0.001$ compared with the vehicle-treated control group.

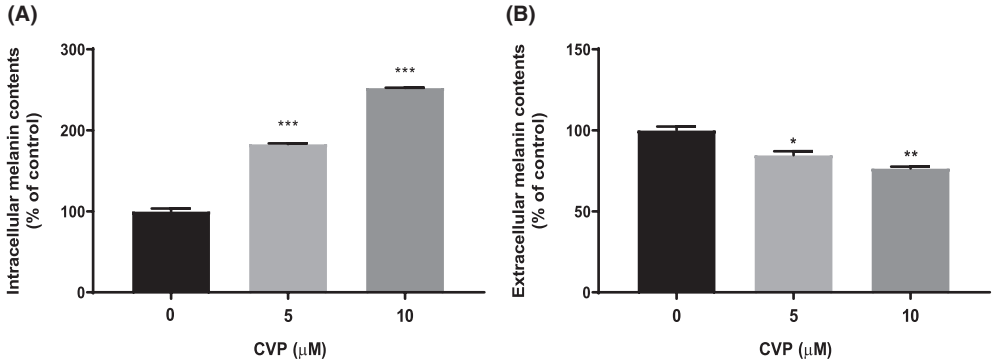

FIGURE 5 Effects of cell-penetrating peptide-conjugated soluble N-ethylmaleimide-sensitive factor attachment protein receptor motif of vesicle-associated membrane protein 2-patterned peptide (CVP) on melanogenesis in B16-F10 cells. B16-F10 cells were exposed to 200 nM α-MSH in the presence of 5 or 10 μM CVP. The results are presented as a percentage relative to the control. Values are represented as the mean ± standard deviation (SD) of triplicate values. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ compared with the vehicle-treated control group.

the K^+-induced release of dopamine compared with that in the negative control group (approximately 40%, $p = 0.046$; Figure 4B). The considerable modulation of both neurotransmitters by CVP in PC-12 cells suggests that CVP has the potential to be an anti-wrinkle agent for cosmetic skin products as it emulates the action of BoNT/A.

3.5 | Effects of cell-penetrating peptide-conjugated SNARE motif of VAMP2-patterned peptide on melanin synthesis

To assess the inhibitory effect of CVP on melanin synthesis, we determined the quantity of intracellular and extracellular melanin in B16-F10 cells 48 h after treatment with CVP at concentrations of 5 and 10 μM. As melanogenesis is stimulated by α-MSH, we evaluated the influence of CVP treatment on melanin synthesis in the presence of α-MSH in the culture media (Figure 5). CVP substantially increased intracellular melanin content in a dose-dependent manner as compared to cells treated with α-MSH alone (Figure 5A), whereas the extracellular melanin content of the α-MSH-stimulated B16-F10 cells significantly decreased to levels that were 85% and 76% in a dose-dependent manner (Figure 5B). These findings suggest that CVP may decrease the extracellular melanin content by hindering melanosome transport from melanocytes to keratinocytes. To confirm whether the increase in intracellular melanin content detected in this experiment was related to melanogenesis, the expression of melanogenesis-related genes in the B16-F10 cells was further investigated.

3.6 | Effects of cell-penetrating peptide-conjugated SNARE motif of VAMP2-patterned peptide on melanogenesis-related gene expression

The effect of CVP on the expression of melanogenesis-related genes, such as *MITF*, *TYR*, *TRP-1*, and *TRP-2*, was examined in B16-F10 cells after treatment with CVP in the presence or absence of α-MSH using real-time qPCR. In general, α-MSH induces *MITF* expression, and the *MITF* downstream genes, such as *TYR* and *TRP-1*, induce melanogenesis. In this study, we confirmed that *MITF*, *TYR*, *TRP-1*, and *TRP-2* expression levels were significantly increased by α-MSH stimulation, whereas CVP treatment did not affect the mRNA expression of these genes. Therefore, we confirmed that CVP had no effect on melanogenesis in melanocytes.

4 | DISCUSSION

Our research team has been interested in the overwhelming impact of wrinkle development and hyperpigmentation on skin aging. Hence, we developed a functional antiaging peptide "CVP" containing IMT-P8 CPP using the solid-phase method to provide a suitable solution for wrinkle development and hyperpigmentation. Furthermore, we verified the potential of CVP as an anti-wrinkle and skin-whitening ingredient for cosmetics using various in vitro assay systems.

First, the MTT assay and skin irritation test showed that CVP had no visible cytotoxicity effect. According to the ISO 10993-5 International Standard, a 30% or greater decrease in cell viability is regarded as cytotoxic. CVP has no potential cytotoxic effect on B16-F10 cells and HDFs and does not induce skin irritation; therefore, it has been suggested as an effective and safe active ingredient without any associated cytotoxicity in the cosmetic industry.

Secondly, norepinephrine and dopamine release following the depolarization of PC-12 cells with a short pulse of a high concentration of K^+ was significantly inhibited in the CVP-treated group compared to that in the non-treated control group. Moreover, CVP showed an effect similar to that of BoNT/A, the most potent neurotoxin. BoNT/A induces muscle paralysis by enzymatically cleaving the presynaptic SNARE protein SNAP-25 and blocking the formation of the SNARE complex, which results in a lasting inhibition of acetylcholine release in motor neurons.[20,21] Furthermore, CVP showed a 2-fold higher inhibitory activity of norepinephrine release than Argireline® (Figure S1). Argireline® is patterned from the N-terminal end of the SNAP-25 protein and is a topical alternative to Botox. It has been scientifically proven to have Botox-like effects, such as reduced fine lines and wrinkles and improved skin elasticity and firmness by suppressing the release of these catecholamines.[22] This result suggests the potential use of CVP as an anti-wrinkle agent in cosmetic applications.

Thirdly, focusing on the anti-pigmentation effect of CVP, the extracellular melanin content of B16-F10 melanoma cells was effectively decreased in a CVP concentration-dependent manner (Figure 5B). However, the concentration of intracellular melanin considerably increased compared to the control (Figure 5A), suggesting that CVP can influence the accumulation of melanin in cells. In addition, it was shown that CVP also inhibits the release of melanosomes from cells.

Finally, we found that CVP did not affect the expression of specific melanogenesis-related genes (Figure 6); therefore, it may be involved in releasing melanin from melanocytes and transporting it to keratinocytes. In skin pigmentation, melanocytes are located in the spinous layer and transfer melanin to keratinocytes. Melanin-containing keratinocytes that have been taken up by melanocytes then progressively migrate to the epidermis.[23] As a consequence, spots and darkening emerge on the skin.[24] A recent study demonstrated that SNARE and Rab3a on melanosomes and SNARE complexes in melanocytic cell membranes target melanosomes to the plasma membrane and transfer them to keratinocytes. Rab3a, a GTP-binding protein involved in exocytosis in neuronal cells, is present in melanosome-containing fractions with several SNAREs, including VAMP2 and SNAP-25. The co-fractionation of SNAREs and Rab3a from mature melanosomes plays a crucial role in melanin exocytosis. It was also stated that Rab3a regulates trans-SNARE complex assembly, which facilitates vesicle fusion with the plasma membrane.[25] In this study, CVP did not exert an inhibitory effect on melanin production; however, it may induce a whitening effect by inhibiting melanin transport. Based on our recent understanding of skin melanogenesis, the binding of VAMP2 to Rab3A was displaced by CVP, which might be responsible for inhibiting

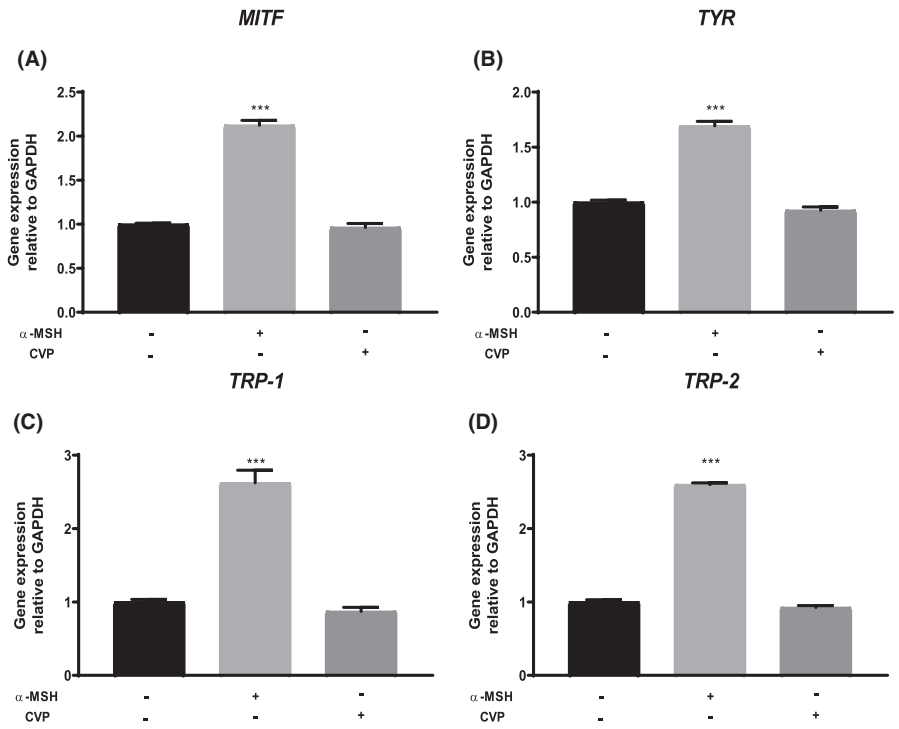

FIGURE 6 Effects of cell-penetrating peptide-conjugated soluble N-ethylmaleimide-sensitive factor attachment protein receptor motif of vesicle-associated membrane protein 2-patterned peptide (CVP) on the mRNA expression of *MITF*, *TYR*, *TRP-1*, and *TRP-2* in B16-F10 cells. The mRNA expression of (A) *MITF*, (B) *TYR*, (C) *TRP-1*, and (D) *TRP-2* was measured and normalized to *GAPDH* expression. $^{***}p < 0.001$ compared with the vehicle-treated control group.

melanin-containing vesicle fusion with the cell membrane, melanin transport, and transfer to keratinocytes. Further research on this transport mechanism induced by treatment with CVP is strongly recommended.

To our knowledge, the present study is the first to identify the potential of CVP as an anti-wrinkle and skin-whitening ingredient for use in cosmetic products. However, it has several limitations. Further studies on the inhibitory effects of CVP on SNARE complex formation are necessary to determine the underlying molecular mechanism of its anti-wrinkle effects. Additionally, further efforts to identify the mechanism by which CVP inhibits melanosome transport in melanocytes are needed. Furthermore, a series of in vivo trials is necessary to determine its safety and effectiveness in intact skin systems.

In conclusion, CVP demonstrated the ability to inhibit melanin transfer to keratinocytes and neurotransmitters release such as norepinephrine and dopamine, proving promise as a cosmetic ingredient and an antiaging agent.

AUTHOR CONTRIBUTIONS

H.J.L. and D.K. designed the research study, performed the research, analyzed the data, and wrote the paper. H.J.C., S.K., and M.S. designed the research study and performed the research. S.K. analyzed the data. D-K.L and W-h.K designed the research study and wrote the paper. All authors have read and approved the final manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICS STATEMENT

The study "Potential role of the CPP-conjugated SNARE motif of VAMP2-patterned peptide in novel cosmeceutical skin product development" was reviewed and approved by Institutional Review Board (IRB) of Medytox Inc.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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