IFSCC 2025 full paper (IFSCC2025-767)

"Development of a novel capsule formulation containing two amphiphilic vitamin derivatives as shell components and evaluation of its skin physiological effects for application to cosmetics for sensitive skin"

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1. Introduction

Sensitive skin refers to a state of hyper-reactivity of the skin under physiological or pathological conditions [1], and developed by a complex process involving barrier function, neurological factors, and inflammatory response [2] wherein it is shown to be associated with high reactivity, poor tolerance, and. susceptibility to allergy. Therefore, in the approach to cosmetics for sensitive skin, it is necessary to penetrate the active ingredient into the skin without burdening the skin barrier function with the formulation, leading to improvement of skin tissue. Especially, impaired skin barrier function is an important cause of skin sensitivity [3].

Filaggrin (FLG) connects keratin fibers to maintain the flattened shape of keratinocytes and to form the keratin envelope. The above protein is important for maintaining skin barrier homeostasis. Prostaglandin E2 (PGE2) is a member of the PGE series that is well known as an inflammatory mediator that causes vasodilatation, hyperalgesia, and fever [4]. Numerous reports have appeared describing the relation between PGE2 and UV inflammation, and that cutaneous UV-induced inflammation is mediated by PGE2 [5]. PGE2 is released in keratinocytes in vitro in response to UV irradiation [6]. Cyclooxygenase (Cox) converts arachidonic acid to PGE2, a precursor of PGE2 [7]. There are two isoforms of Cox: one is constitutively expressed (Cox-1) and the other is inducible (Cox-2). Cox-2 is an immediate early gene that is induced by tumor promoters, growth factors, carcinogens, and inflammatory cytokines [8]. UV exposure increases the levels of Cox-2 expression in human skin [9].

We have recently synthesized an amphiphilic vitamin C (VC) derivative, disodium isostearyl ascorbyl phosphate (VCP-IS-2Na), which exhibited high stability in various aqueous solutions at a wide range of pH values and satisfactory thermal stability [10]. VCP-IS-2Na has skin permeability superior to that of VC and exhibits VC activity in vitro and in vivo after enzymatic hydrolysis to free VC by phosphatase and/or esterase. So far, we have shown that VCP-IS-2Na has anti-melanogenesis effects [11] and stimulation on collagen synthesis [12].

In this study, we investigated whether the combination of four vitamin derivatives exerted a synergistic effect on the suppression of released PGE2, mRNA expression of Cox-2 under UVB, H2O2, t-BHP, and IL-1β, and the stimulation of filaggrin synthesis in normal human epidermal keratinocytes (NHEKs). We also investigated the suppression of collagen synthesis, MMP-1 activity, mRNA expression of microfibril-relating genes, and hyaluronic acid synthesis in normal human dermal fibroblasts (NHDFs) under UVA irradiation. Furthermore, we developed a novel nano-capsule (NC-4VT), whose shell was made of amphiphilic vitamin E (VE) derivative, sodium tocopheryl phosphate (TFP-Na) and vitamin C derivative, disodium isostearyl ascorbyl phosphate (VCP-IS-2Na), containing vitamin A derivative, hydrogenated retinol (HR) and vitamin B6 derivative, pyridoxine tris-hexyldecanoate (PTH), and also examined.

2. Materials and Methods

Preparation of complex of 4VT (CP-4VT) and nano-capsule 4VT (NC-4VT)

We developed a novel nano-capsules containing HR and PTH, whose outer shells are composed with combination of amphiphilic vitamin derivatives, TFP-Na and VCP-IS-2Na.

CS-4VT were mixed with HR, PHH, and hydrogenated polydecene, and then VCP-IS-2Na, glycerin, pentylene glycol, and water were added to the solution.NC-4VT was prepared with nano-particulating CP-4VT, which was mixed with polyols, oil and four vitamin derivatives by high pressure water jet device.

Skin permeation and retention assay with reconstructed human skin model

The skin penetration assay of CP-4VT and NC-4VT was performed by using epidermal human skin model, LabCyte (J-TEC Co., JAPAN). Sample solution (50 µl) was added to the interior of skin model with 1.0 ml medium. After a fixed period of time, the collected mediums and extract, which is homogenated portion and 1.0 ml 50% ethanol, were subjected to HPLC analysis of HR.

Cells culture

Normal human epidermal keratinocytes (NHEKs; Kurabo Co., Osaka, Japan) were maintained in Humedia-KG2 (Kurabo Co. , Osaka, Japan) supplemented with insulin, human recombinant epidermal growth factor (EGF), hydrocortisone, gentamicin, amphotericin B and bovine pituitary extract (BPE), as instructed by the supplier.

Normal human dermal fibroblasts (NHDFs; Kurabo Co., Osaka, Japan) were maintained in FibroLife® basal medium (BM; Lifeline® Cell Technology, Maryland, USA) supplemented with L-glutamine, hFGF-β, insulin, ascorbic acid, hydrocortisone, and 2% fetal bovine serum (FBS), as instructed by the supplier.

UVB irradiation and stimulation with IL-1β or peroxides of NHEKs

NHEKs (3.0×10^5 cells/ml) were incubated with various concentration of samples for 24 h, and washed with PBS(-). UVB: Cells were irradiated with UVB at an intensity of 60 mJ/cm^2, and then washed with PBS(-) and placed in fresh medium prior to incubating at 37℃ for 24 h. IL-1β: Cells placed in fresh medium were treated with 10 ng/ml IL-1β for 24 h at 37℃. Peroxide: Cells placed in fresh medium were treated with 0.5 mM *t*-BHP for 0.5 h or 1 mM H_2O_2 for 0.5 h at 37℃, washed with PBS(-), suspended in fresh medium, and then incubated at 37℃ for 24 h.

Suppression of released PGE_2 on NHEKs

PGE_2 levels in medium were determined by an enzymatic immunoassay kit (Cayman Chemicals, MI, USA) according to the manufacturer's instructions.

Suppression of the mRNA expression of Cox-2 and FLG on NHEKs

After treatment of NHEKs with samples, NKEKs were incubated RNAcell protect reagent (QIAGEN, Valencia, CA, USA). Total RNAs of NHEKs were extracted using NucleoSpin® RNA Plus (Machery-Nagel GmbH & Co. KG, Germany) and reverse-transcribed to cDNA with Oligo

dT Primers and Random 6 mers using a PrimeScript® RT reagent Kit (TaKaRa Bio Inc., Japan). Real-time PCR was performed with SYBR® Premix Ex TaqTM II (TaKaRa Bio Inc.) and the products were analyzed using a Thermal Cycler Dice® Real Time System TP800 (TaKaRa Bio Inc.). Primers used for quantitative PCR are provided in Table 1.

Table 1. Primers used for quantitative PCR

	Sense	Anti-sense
Cox-2	5'-CCTGTGTTCCACCAGGAGAT-3'	5'-CCCTGGCTAGTGCTTCAGAC-3'
FLG	5'-TCAAACAGGAGGGACAGACC-3'	5'-TATCCTCCCTGACCACTTGC-3'
FBN-1	5'-CTTCCACCTAACAGGCCATTAACA-3'	5'-CTATCACATGGTTCCATAGGTGCAG-3'
MFAP-4	5'-CCTATGCCAAGTACGCTGACTTCT-3'	5'-AACTTCTGGCCACTGTGGTAGGAC-3'
EMILIN-1	5'-CCTTCLACAGAGTCCTGCTCAA-3'	5'-CGCTCAGCAAGTAGCGTCCA-3'
LTBP-4	5'-TTCACLACTGTCAGCTGTGCTC-3'	5'-TTTCAAAGCCGGTTGGACAAG-3'

Stimulation of filaggrin synthesis on NHEKs

NHEKs were inoculated to 96-well microplate (3.0×105 cells/well) and cultivated for 24 h. After the cultivation with samples for 72 h, the cells were pelleted and sonicated in RIPA buffer. Amounts of filaggrin were measured by using ELISA system.

Suppression of collagen and hyaluronic acid decrease, and MMP-1 production stimulated by UVA irradiation on NHDFs

NHDFs were inoculated into 6-well plates at a density of 1.0 × 106 cells/well for 24 hr. The cells were changed to fresh medium containing samples and then cultured for 24 h. Control cells were cultivated without samples. They were exposed to 20 J/cm2 UVA irradiation. The supernatants of each well were collected after 24 h cultivation, and the amount of collagen type I, hyaluronic acid and interstitial MMP-1 was measured by using ELISA system.

Suppression of the mRNA expression of microfibril-related genes induced by UV irradiation in NDHFs

NHDFs were inoculated into 6-well plates at a density of 1.0 × 106 cells/well for 24 hr. The cells were changed to fresh medium containing samples then cultured for 24 h. They were exposed to 20 J/cm2 UVA irradiation, the medium was changed to fresh medium, and the cells were cultured for a further 6 h before total RNAs were extracted. Total RNAs of NHDFs were extracted and analyzed using former methods.

Skin permeation and retention assay with reconstructed human skin model

The skin penetration assay of CP-4VT and NC-4VT was performed by using epidermal human skin model, LabCyte (J-TEC Co., JAPAN). Sample solution (50 µl) was added to the interior of skin model with 1.0 ml medium. After a fixed period of time, the collected mediums were subjected to HPLC analysis of HR.

Statistical analysis

Test results are expressed as a mean ± standard deviation (SD). Analysis of covariance between groups was calculated for all variables. The level of significance was set at < 5% (two-tailed test).

3. Results

Suppressive effect on the increase of released PGE_2 in NHEKs exposed to UVB irradiation and stimulation with IL-1β or peroxides

The combination with TFP-Na and VCP-IS-2Na showed the suppression of released PGE_2, induced by UVB, H_2O_2, t-BHP, and IL-1β by 59%, 62%, 76%, 43%, and especially synergistic effect of showed stronger suppression that induced by UVB and t-BHP than other exposes

(**Figure 1**). CP-4VT showed the stronger suppression of released PGE_2 than four vitamin derivatives and their combination. NC-4VT suppressed stronger released PGE_2, induced by UVB, H_2O_2, *t*-BHP, and IL-1β than CP-4VT by 112%~150%.

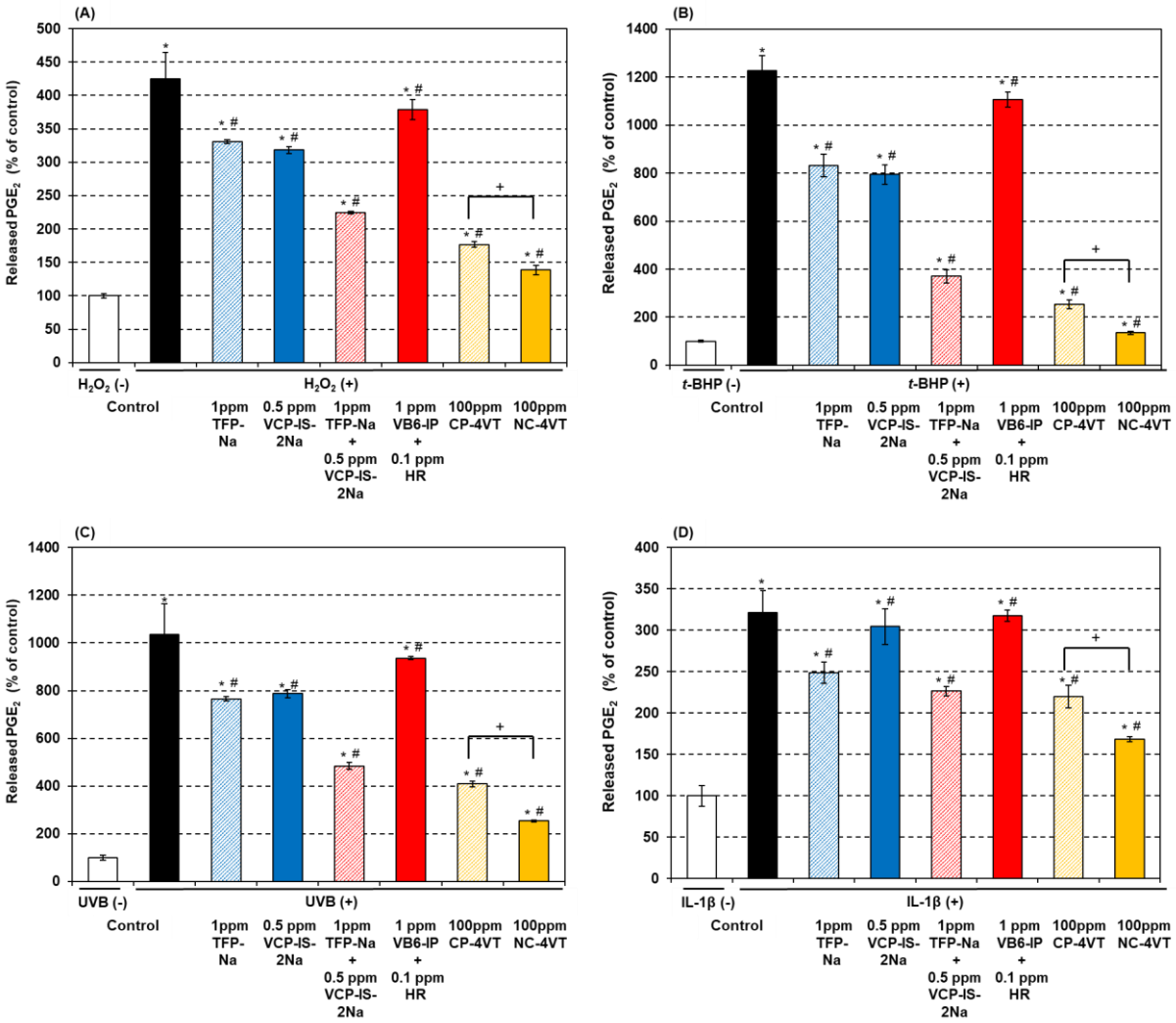

Figure 1. Suppressive effect on the increase of released PGE2 in NHEKs exposed to H_2O_2 (**A**) , *t*-BHP (**B**), UVB irradiation (**C**) and IL-1β (**D**).

Each value reported represent means ± SD. Values were significantly different from the non-exposed group, (-), at $p < 0.05$ (*). Values were significantly different from the exposed group, (+), at $p < 0.05$ (#). Values were significantly different in two groups at $p < 0.05$ (+).

<u>Suppression of the mRNA expression of Cox-2 induced by UVB irradiation and stimulation with IL-1β or peroxides in NHEKs</u>

The combination with TFP-Na and VCP-IS-2Na showed the suppression of upregulation of Cox-2 mRNA expression, induced by UVB, H2O2, t-BHP, and IL-1β by 72%, 47%, 41%, 85%, and especially synergistic effect of showed stronger suppression that induced by $_t$-BHP than other exposes (**Figure 2**). CP-4VT showed the stronger suppression of upregulation of Cox-2 mRNA expression than four vitamin derivatives and their combination. NC-4VT suppressed stronger upregulation of Cox-2 mRNA expression induced by UVB, H_2O_2, *t*-BHP, and IL-1β than CP-4VT by 114%~155%.

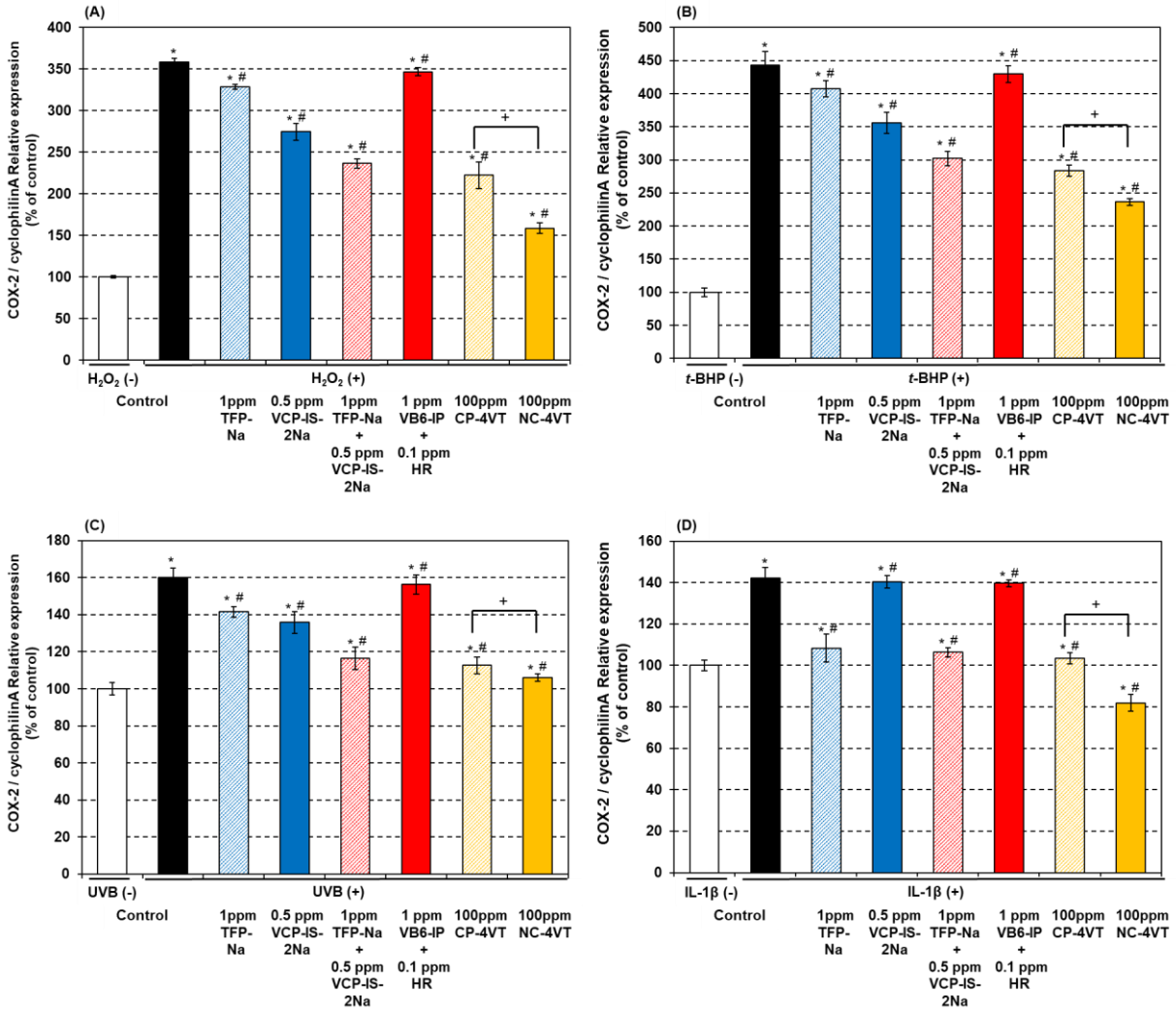

Figure 2. Suppressive effect on Cox-2 mRNA expression in NHEKs exposed to H_2O_2 (**A**) , *t*-BHP (**B**), UVB irradiation (**C**) and IL-1β (**D**).

Each value reported represent means ± SD. Values were significantly different from the non-exposed group, (-), at $p < 0.05$ (*). Values were significantly different from the exposed group, (+), at $p < 0.05$ (#). Values were significantly different in two groups at $p < 0.05$ (+).

Stimulation of filaggrin synthesis and the FLG mRNA expression in HKEKs

Pretreatment of NHEKs with four vitamin derivatives showed the significant stimulation of the FLG mRNA expression (**Figure 3A**). However, combination with HR and PTH only showed the stimulation of the FLG mRNA expression and filaggrin synthesis (**Figure 3B**) by 257% and 212 % in four vitamin derivatives. CP-4VT showed a little stronger stimulation of filaggrin synthesis than combination with HR and PTH. Furthermore, NC-4VT stimulated filaggrin synthesis than CP-4VT by 144% and 112%.

Suppression of excess MMP-1 production and decrease in collagen stimulated by UVA irradiation in NHDFs

NC-4VT showed a significant 74% suppression of the excess production of MMP-1 induced by UVA irradiation (Figure 4A). The suppression rate of excess production of MMP-1 was the highest when NC-4VT, reaching up to 96%. Pretreatment of NHDFs with combination with

TFP-Na and VCP-IS-2Na showed the significant suppression of the decrease in collagen irradiated with UVA by 47%, respectively (**Figure 4B**). The suppression rate of decrease in collagen was the highest when combining CP-4VT, reaching up to 55%.

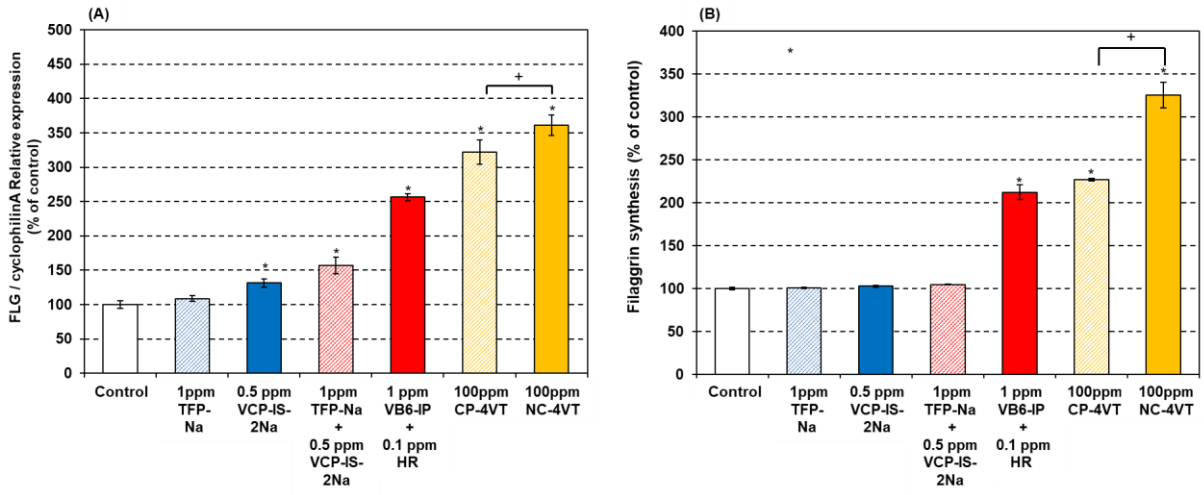

Figure 3. Suppressive effect on FLG mRNA expression (**A**) and filaggrin synthesis (**B**) in NHEKs.

Each value reported represent means ± SD. Values were significantly different from control group, (-), at $p < 0.05$ (*). Values were significantly different in two groups at $p < 0.05$.(+).

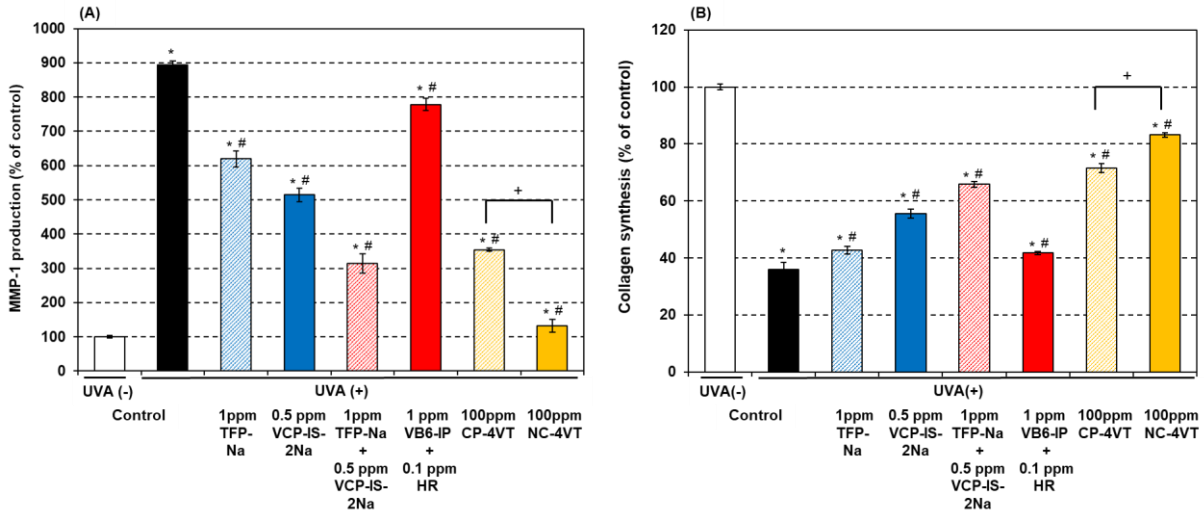

Figure 4. Suppressive effect on the increase of MMP-1 production (**A)** and the decrease of collagen synthesis (**B**) induced by UVA irradiation.

Each value represents the mean ± S.E. of three experiments. Values were significantly different from the non-irradiated group, UVA(-), at $p < 0.05$ (*). Values were significantly different from the irradiated group, UVA(+), at $p < 0.05$ (#). Values were significantly different in two groups at $p < 0.05$ (+).

<u>Suppressive effect of the mRNA expression of microfibril-related genes induced by UV irradiation in NDHFs</u>

The combination with TFP-Na and VCP-IS-2Na suppressed the downregulation of FBN-1, MFAP-4, EMILIN-1, and LTBP-4 at 30 J/cm2 UVA irradiation by 38%, 28%, 42%, and 34%,

respectively (**Figure 5**). CP-4VT suppressed the downregulation of FBN-1, MFAP-4, EMILIN-1, and LTBP-4 at 30 J/cm^2 UVA irradiation by 42%, 31%, 48%, and 45%, respectively. CP-4VT suppressed to about the same extent on the downregulation of mRNA expression in the case of combination with TFP-Na and VCP-IS-2Na. The suppressive effects were showed by 63%, 58%, 88%, and 66% in the case of NC-4VT. These results indicated that the synergistic effect of TFP-Na and VCP-IS-2Na and nano-capsulation suppressed the downregulation of FBN-1, MFAP-4, EMILIN-1, and LTBP-4 showed reaching up to 147%, 188%, 187%, and 148%.

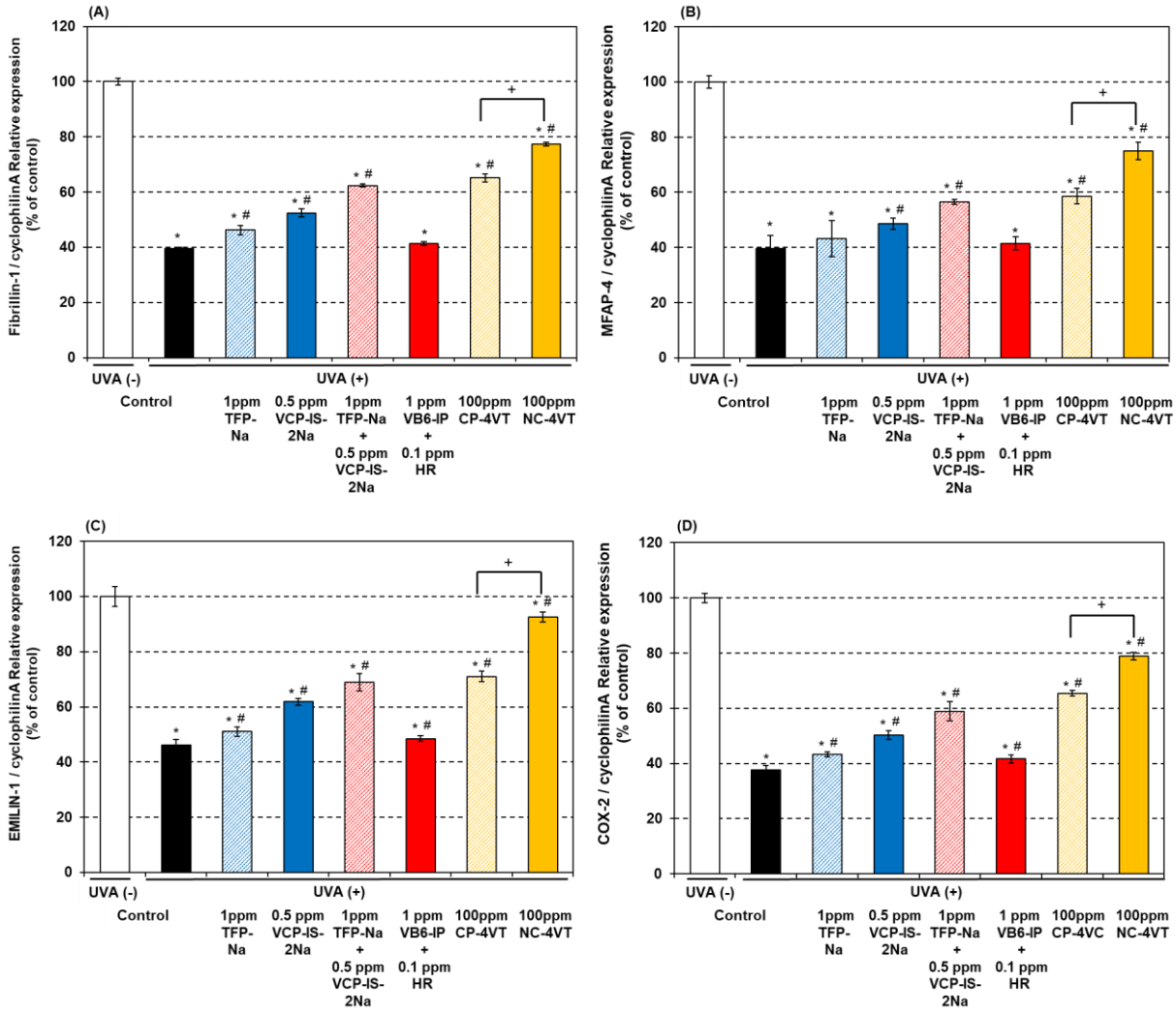

Figure 5. Suppressive effect on the downregulation of the mRNA expression of Fibrillin-1 (**A**), MFAP-4 (**B**), EMILIN-1 (**C**) and LTBP-4 (**D**) in NHDFs exposed to UVA irradiation.

Each value reported represent means ± SD. Values were significantly different from the non-irradiated group, UVA(-), at $p < 0.05$ (*). Values were significantly different from the irradiated group, UVA(+), at $p < 0.05$ (#). Values were significantly different in two groups at $p < 0.05$ (+).

<u>Suppression of decrease in hyaluronic acid stimulated by UVA irradiation in NHDFs</u>

Pretreatment of NHDFs with the combination with HR and PTH showed the significant suppression of the decrease in hyaluronic acid irradiated with UVA by 23%, respectively (**Figure 6**). The suppression rate of decrease in hyaluronic acid was the highest when

combining CP-4VT, reaching up to 49%. NC-4VT showed a significant highest 70% suppression of the decrease in hyaluronic acid induced by UVA irradiation. The suppression rate of the decrease in hyaluronic acid was the highest when NC-4VT, compared with CP-4VT reaching up to 146%.

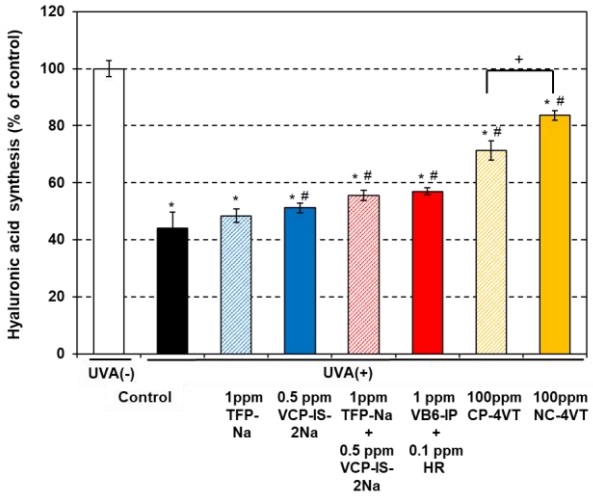

Figure 6. Suppressive effect on the decrease of hyaluronic acid synthesis induced by UVA irradiation.

Each value represents the mean ± S.E. of three experiments. Values were significantly different from the non-irradiated group, UVA(-), at $p < 0.05$ (*). Values were significantly different from the irradiated group, UVA(+), at $p < 0.05$ (#). Values were significantly different in two groups at $p < 0.05$ (+).

Skin permeation and accumulation assay with reconstructed human skin model

The maximum penetration dose (ratio) of HR was ~3.89 nmol after 10 h treatment with CP-4VT (**Figure 7**). Although, the maximum permeation dose (ratio) of caffeic acid was ~11.89 nmol after 10 h treatment with NC-4VT. The permeation of NC-4VT is higher than that of CP-4VT by 3.1 times.

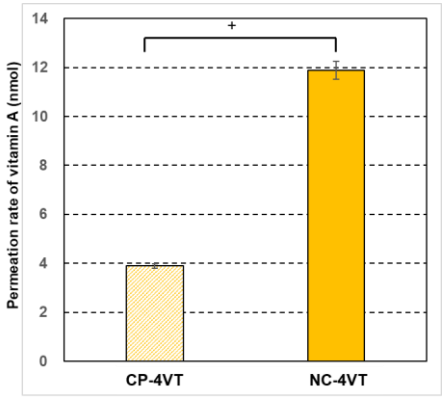

Figure 7. Skin permeation of CP-4VT and NC-4VT on encapsuled HR with epidermal skin model Values were significantly different in two groups at $p < 0.05$ (+).

3. Discussion

The present study demonstrated that the combination of TFP-Na and VCP-IS-2Na showed a synergistic effect on the suppression of released PGE_2 (**Figure 1**) and mRNA expression of Cox-2 (**Figure 2**) under UVB, H_2O_2, *t*-BHP, and IL-1β in NHEKs. These results suggested that

one of reasons for these protective effects against inflammation is the inhibition of released PGE_2 that accompanies the suppression of mRNA expression of Cox-2.

Suppression of lipid peroxidation in cell membranes is a major function of VE and VC in the cytosol directly scavenges free radicals species that could otherwise destroy VE, and it recycles Toc from *α*-tocopheroxyl radicals [13, 14]. In primary cultures of hepatocytes or NHDFs, initial concentrations of both VE and VC progressively decrease with time [15, 16]. The cause of this reduction has been attributed to oxidative stress [17]. In cultured H411E rat liver cells, VC supplements preserve VE, and both VC and VE supplements decrease lipid peroxidation in cell membranes [18]. These results indicated that ascorbate loading of cells spares cellular VE either directly or through recycling of VE by preventing lipid peroxidative damage due to oxidative stress. In the current study, VCP-IS-2Na synergistically enhanced the inhibition of PGE_2 synthesis by TFP-Na. These effects may either be due to recycling of VE derived for TFP-Na directly or by VCP-IS-2Na.

Furthermore, we also investigated the synergistic effect of TFP-Na and VCP-IS-2Na on suppression of decrease in collagen, the excess production of MMP-1, and mRNA expression of microfibril-relating genes in NHDFs under UVA irradiation.

We developed a novel capsule NC-4VT containing two amphiphilic vitamin derivatives TFP-Na and VCP-IS-2Na as shell components and two vitamin derivatives HR and PTH as intended components. NC-4VT showed more excellent on former all effects than CP-4VT.

On the other hands, we also clarified that these synergistic protective effect of NC-4VT showed on the stimulation of filaggrin synthesis in NHEKs (**Figure 3**) and hyaluronic acid synthesis in NHDFs (Figure 6), which is caused by HR and PTH. Figure 7 showed nanoencupsilation enhanced permeability of NC-4VT by 3 times in human skin model. Therefore, it is considered that the increase of these synergistic protective effect on NC-4VT is brought by not only the improvement of the skin permeability of the active reagents and the protection from the environments in which the activity of these materials are easily lost, but also the achieve of an effective ordered approach of TFP-Na, VCP-IS-2Na, HR and PTH to human skin by nanoencapsulation.

NC-4VT is significantly suppressed excess production of MMP-1 (**Figure 4A**), the excess decrease in type I collagen production (**Figure 4B**), and the decreases of the mRNA expression of FBN-1 and EMILIN-1 (**Figure 5**), and the excess decrease in hyaluronic acid (**Figure 6**) in NHDFs exposed to UVA irradiation. Therefore, NC-4VT is effective to reconstruct of forming dermis layer. These results indicated that NC-4VT not only suppresses external irritating factors but also stimulates filaggrin synthesis, which acts on the skin barrier function at epidermal layer, and reconstructs collagen and elastin fibers caused against skin aging at dermal layer for sensitive skin.

5. Conclusion

We clarified the synergistic effect of TFP-Na and VCP-IS-2Na on suppression of the release of PGE_2 induced by various stimulating factors, which is mainly caused by TFP-Na, and suppression of the decrease in collagen, the excess MMP-1 production, mRNA expression of microfibril-relating genes, which is mainly caused by VCP-IS-2Na, in NHDFs under UVA irradiation.

Next, we developed a novel capsule NC-4VT containing two amphiphilic vitamin derivatives TFP-Na and VCP-IS-2Na as shell components and two vitamin derivatives HR and PTH that show synergistic effects. NC-4VT was found to have a higher inhibition of inflammatory mediators in NHEKs and that it serves as a protective agent against exogenous stimulants, and reconstructive effect of forming collagen and elastin fibers than CP-4VT. Furthermore, NC-4VT

stimulated the synthesis of filaggrin, which is effective in improving barrier function, and is caused by PTH, in NHEKs, and suppressed the decrease of hyaluronic acid synthesis, which is effective in improving water retention function and is caused by HR, induced UVA irradiation. These results suggest that NC-4VT is an effective capsule ingredient that suppresses external irritating factors at epidermal layer while effectively penetrating into the skin, improving the skin barrier function and reconstructing skin fibers caused against skin aging at dermal layer for sensitive skin.

6. References

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