Effects of Centella asiatica Ethanolic Extract Encapsulated in Chitosan Nanoparticles on Proliferation Activity of Skin Fibroblasts and Keratinocytes, Type I and III Collagen Synthesis and Aquaporin 3 Expression In vitro

ABSTRACT

Background: The activity of skin cell proliferation, collagen synthesis and skin hydration decrease with the process of aging; therefore, the skin looks dull, dry and sagging. Aquaporin 3 (AQP3) is a key protein that plays a major role in skin cell proliferation and skin hydration. Retinoic acid (RA) is still considered for anti-aging treatment, but it frequently shows side effects, such as skin irritation. Centella asiatica (CA) formulation in chitosan nanoparticles has a promising potential as anti-aging cosmetic.

Aim: The aim of this study was to evaluate the effects of CA ethanolic extract encapsulated in chitosan nanoparticles (CAEE + CNP) on skin cell proliferation, collagen synthesis and AQP3 expression in vitro, when compared to RA.

Methods: Microculture tetrazolium assay was conducted to analyse the proliferation of normal human dermal fibroblasts (NHDF) and normal human epidermal keratinocytes (NHEK) at 24, 48 and 72 h. Type I and III collagen synthesis was evaluated at the same time points using ELISA. Aquaporin 3 expression at 24 h was evaluated using immunocytochemistry and measured quantitatively using ImageJ software. All treatments involved several concentrations of CAEE + CNP and RA through serial dilution.

Results: Collagen type I and III synthesis of NHDF and NHEK was neither significantly different from untreated controls nor from RA-treated cells. Nevertheless, CAEE + CNP stimulated the proliferation of both NHDF and NHEK. Additionally, AQP3 expression in both cell types was upregulated by CAEE + CNP.

Conclusion: CAEE + CNP is a promising formulation for anti-aging activity by inducing skin cell proliferation and AQP3 expression. The clinical trials are still needed to evaluate skin hydration in vivo.

KEYWORDS aquaporin 3, Centella asiatica, chitosan nanoparticles, collagen, fibroblasts, hydration, keratinocytes, proliferation

INTRODUCTION

Skin aging is a complex process controlled by genetic determination (chronologic aging) and under the influence of external factors (extrinsic aging). Skin aging leads to various modifications in cells and tissue. A number of studies have shown that aging process results in reduced proliferation activity in both keratinocytes and fibroblasts. Furthermore, cell renewal rate and the synthesis of type I and type III collagen in skin fibroblasts decreased naturally in skin as the years go by. This loss of regenerating power is both, cause and a measure of skin aging.

Aging skin is manifested by the diminished water content of stratum corneum (SC). Age-related trans-epidermal water loss (TEWL) can be evaluated by removing the superficial epidermal layer by tape stripping. In aged skin,
the barrier repair capacity is significantly impaired\(^2\). Hydration of the SC determines the appearance and the physical properties of the skin. The aging process reduces skin surface hydration, which results in dry, dull, coarse and saggy appearance with loss of elasticity. Other visible signs include pigmentation and appearance of benign skin tumours. Moreover, the epidermis becomes thinner with age and as a result wrinkles develop\(^3\)–\(^4\).

Cosmeceutical anti-aging formulations contain active ingredients to achieve local therapeutic effects, and should not have harmful systemic side effects\(^5\). Well known anti-aging ingredients like vitamin A or retinoic acid (RA) and its derivatives (retinoids), have harmful side effects by irritating the skin and causing dry, flaky skin, which is sensitive to light\(^6\). Moreover, usage of these retinoids containing formulations is limited, as they may cause contact RA dermatitis and have teratogenic effects\(^7\)–\(^8\). Therefore, it is necessary to develop innovative cosmeceutical anti-aging formulations with active ingredients which are effective and stable, yet safe and nontoxic. Additionally, they should be compatible with other active ingredients and be delivered efficiently to the target cells\(^9\)–\(^10\).

Indonesian biodiversity promises great potential for selection and development of herbal cosmetics and cosmeceuticals, which are effective and safe. Natural products contain a wealth of interesting and possibly beneficial cosmeopharmaceutically active compounds. For example, Pereda et al.\(^9\) reported that hydroglycolic \textit{Piptadenia colubrina} extract can improve skin hydration.

\textit{Centella asiatica} (CA) (gotu kola, \textit{Hydrocotyle asiatica}) is often found in Indonesia and has been used for various medicinal purposes since 1,000 years ago, CA has been applied for wound healing and the treatment of asthma, ulcers, leprosy, lupus erythematosus, psoriasis, vein disease and cancer\(^10\)–\(^13\). CA stimulates the synthesis of collagen for skin tissue regeneration\(^14\)–\(^16\). The biologically active ingredients in CA are triterpenes namely \textit{asiatic acid}, \textit{madecassic acid}, \textit{asiatoside} and \textit{madecassoside}\(^10\)–\(^13\). Although CA extracts (CAE) possess high potential of biological activities, their clinical usage is limited due to poor physical stability, with CA extracts having high hygroscopic index. Powder extracts are promptly liquefied within a few minutes when exposed to normal environment. Therefore, nanoparticles which entrap the extract and protect it from external moisture should be developed for stabilisation\(^17\).

Chitosan is a biopolymer produced from chitin by partial deacetylation. The polysaccharide chitin is the main compound of the exoskeletons in crustaceans and fungal cell walls. Chitin nanofibrils are applied in cosmetics as carriers to increase penetration\(^18\)–\(^19\), as well as active ingredients for anti-aging cosmetics as it helps to maintain the integrity of skin barrier and increases the capacity of cornocytes to store water\(^20\). Chitosan consists of acetylated and deacetylated units. Later, they are composed of \(\beta\)-(1,4)-\(\delta\)-glucosamine, whereas, the former units consist of \(N\)-acetyl-\(\delta\)-glucosamine. Chitosan is hypoallergenic and has natural antibacterial properties, it is biocompatible and biodegradable and its low cost warrants biomedical application in wound healing\(^21\)–\(^22\). Furthermore, chitosan is used in anti-aging skin cosmetics, both as penetration enhancer and as active anti-aging compound\(^19\).

Nanocarrier systems as innovative drug and cosmetic delivery technology have been widely used\(^23\)–\(^24\). This technology is commonly based on lipid carriers, such as liposomes and solid lipid nanoparticles with 100–300 nm in diameter\(^23\)–\(^25\). Nanoparticles have unique physical properties which make them ideal for various skin care products\(^26\). For example, a lipid nanocarrier (LNC) system loaded with tocopheryl acetate (TA) exhibited the ability to enhance skin hydration\(^26\). Apart from lipid-based carriers, polymeric nanoparticles are being developed as novel delivery systems, including those based on chitin/chitosan\(^27\)–\(^28\).

Aquaporins (AQPs) as water channels are the key factors in skin hydration mechanisms\(^29\). The most abundant AQP in the skin is AQP3, a water/glycerol transporting protein. However, its expression in normal human dermal fibroblasts (NHDF) and normal human epidermal keratinocytes (NHEK) decreases with the aging process. Non-expression of AQP3, particularly in the basal layer leads to reduced SC hydration and skin elasticity\(^10\). Therefore, AQP3 is a key protein target for future anti-aging treatment to improve durability, texture and quality of the skin surface\(^11\). Naturally active compounds that can stimulate AQP3 expression would be effective hydrating agents as emollients in anti-aging cosmetics\(^11\).

RA influences keratinocyte differentiation, reduces epidermal cell adhesion and facilitates cornocyte exfoliation from the surface of epidermis. In the dermis, retinoids regulate fibroblast proliferation, induce angiogenesis and play an essential role in the synthesis of collagen and elastin fibrils\(^33\). Moreover, it was reported that RA stimulates AQP3 expression in NHEK\(^12\).

In this study, we evaluated the effect of CA ethanolic extract (CAEE) encapsulated into chitosan nanoparticles (CAEE + CNP) on the proliferative activity of NHDF and NHEK, on the synthesis of type I and type III collagen in NHDF and determined the ability of CAEE + CNP to increase AQP3 expression in NHDF and NHEK.

**MATERIALS AND METHODS**

**Materials**

RA was purchased from BASF, Germany and chitosan with a deacetylation degree > 75% from Sigma-Aldrich, USA. All other chemicals were obtained from Merck, Germany; the assay kits used were purchased from Sigma-Aldrich, via their subsidiaries in Indonesia or Singapore. CA, from Tawangmangu, was used to prepare the ethanolic extract in the Centre for Pharmaceutical and Medical Technology, Agency for the Assessment and Application of Technology (BPPT PUSPITEK), Serpong.

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CAEE was encapsulated into chitosan nanoparticles by ionic gelation method using Sigma-Aldrich chitosan with a deacetylation degree of >70%.

Serial concentrations of all compounds used were obtained by 1 + 1 dilution steps with the respective solvent: CAEE + CNP (100, 50, 25, 12.5, 6.25 and 3.125 mg/mL); RA (10, 5, 2.5, 1.25, 0.625 and 0.3125 mg/mL) and (1, 0.5, and 0.25 mg/mL).

Serial concentrations of all compounds used were obtained by 1 + 1 dilution steps with the respective solvent: CAEE + CNP from 100, 50, 25, 12.5, 6.25 mg/mL down to 3.125 mg/mL; RA from 10, 5, 2.5, 1.25, 0.625 mg/mL down to 0.3125 mg/mL to determine proliferation activity of NHDF and NHEK. RA dilution steps from 1, 0.5 mg/mL to 0.25 mg/mL were used to determine type I and III collagen synthesis and in immunocytochemistry.

**Cell cultures of human fibroblasts and keratinocytes**

In this study, primary NHDF and NHEK cells were derived from male children’s foreskin and cultured in Roswell Park Memorial Institute (RPMI) medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin from the Cell Culture Laboratory Faculty of Medicine, University of Gajah Mada, Yogyakarta. NHDF cell culture from 7th passage was used to test the synthesis of type I and III collagen as well as for the immunocytochemical test in the Integrated Laboratory of Yarsi University, Jakarta. Meanwhile, NHEK cell culture was used in the same laboratory to test keratinocyte proliferation and immunocytochemistry. Early, the cell passages between 2 and 7 were used in all experiments.

**MTT assay of fibroblast and keratinocyte proliferation**

MTT assay (MTT = 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) was adapted to measure fibroblast and keratinocyte cell proliferation. Cells were seeded at a ratio of 1:3 and 1 × 10⁶ cells/mL density, before subjected to treatment with 0.3125, 0.625, 1.25, 2.5, 5, and 10 mg/mL of RA and 6 concentration (3.125, 6.25, 12.5, 25, 50, 100 mg/mL) of CAEE + CNP. The MTT assay was repeated three times. The number of viable cells was evaluated at 24, 48, and 72 h after treatment, as an indicator of fibroblast proliferation, furthermore at 24 and 48 h for keratinocytes proliferation. Subsequently, obtained values were normalised against untreated control group (100%).

**Evaluation on type I and III collagen synthesis using ELISA**

Type I and III collagen synthesis was examined by using designated ELISA kit Alpha 1 from CUSABIO Life science, US. Fibroblast collagen synthesis activity was evaluated at 24 and 48 h after treatment and compared against untreated NHDF as negative control. Results for collagen synthesis were reported in mg/mL.

**Immunocytochemistry test**

Immunocytochemistry is a specific protein detection technique using a specific antibody (Ab) to recognise the antigen presented on cells. Immunocytochemical staining was done by using anti-aquaporin 3 Ab, Ab125219. Then, the expression of AQP3 in both NHDF and NHEK was quantitatively analysed by using ImageJ.

**STATISTICAL ANALYSIS**

The data obtained from this study are presented as images, tables or graphs. To assess significance across the different treatment groups, unpaired t-test and one-way ANOVA test were used, followed by post-hoc Tukey test. Significance level was set at \( P < 0.05 \) using SPSS software.

**RESULTS**

Figure 1 shows the transmission electron micrographs (TEM) of CNP and CAEE + CNP. Particle size distribution of CAEE + CNP analysed with Beckman Particle Size Analyzer CV. Main peak was obtained at 96.3 ± 26.6 nm with a polydispersity index (PI) of 0.308. The cumulative percentage of this peak amounts to 100%, which means that there is only one size population with uniform distribution.

Figure 2 compares cell proliferative activity of cultured NHDF after exposure with serial dilutions of CAEE + CNP and RA at 24, 48 and 72 h. At 24 h stimulation, fibroblast proliferation is high for 6.25 mg/mL CAEE + CNP with ~160% of control. This is significantly higher as compared to untreated control and RA-treated cells. At 48 h, both compounds demonstrate similar results with maximum effects seen on 3.125 mg/mL concentration. After 72 h, RA shows dominant effects on fibroblast proliferation. The statistical analysis with unpaired t-test revealed no significant difference between the two groups.

Figure 3 compares type I collagen synthesis in cultured NHDF after 24, 48 and 72 h of exposure with CAEE + CNP and RA. The different concentrations of CAEE + CNP were obtained by serial dilution from 12.5 mg/mL down to 3.125 mg/mL and with RA by serial dilution from 0.1 mg/mL down to 0.025 mg/mL.

In general, CAEE + CNP and RA exerted similarly positive effects as compared to negative control. After 24 and 48 h of exposure, CAEE + CNP and RA did not differ from control. At 72 h, however, both CAEE + CNP and RA showed greater type I collagen synthesis, as compared to control group. Comparing for all time periods (24, 48 and 72 h) through one-way ANOVA test, there were no statistically significant differences between CAEE + CNP, RA and control at all concentrations.
Fig. 1 Chitosan nanoparticles (CNP) (a) and *Centella asiatica* ethanolic extract (CAEE) encapsulated into chitosan nanoparticles (CAEE + CNP) (b); magnification ×100,000; black bars = 50.0 nm.

Fig. 2 Comparison of cell proliferative activity of NHDF without treatment (control), after exposure to *Centella asiatica* ethanolic extract encapsulated into chitosan nanoparticles (CAEE + CNP) and retinoic acid (RA) at various concentrations at 24, 48 and 72 h. The concentrations of CAEE + CNP were obtained by serial dilution from 100 mg/mL down to 3.125 mg/mL and RA from 10 mg/mL down to 0.3125 mg/mL.

Figure 4 compares type III collagen synthesis in cultured human fibroblasts after 24, 48 and 72 h of exposure to CAEE + CNP and RA. The different concentrations of CAEE + CNP were obtained by serial dilution from 12.5 mg/mL down to 3.125 mg/mL and with RA by serial dilution from 1 mg/mL down to 0.25 mg/mL. Type III collagen synthesis generally increases over time (with its highest value at 48 h) and fluctuates slightly more than type I collagen (compare with controls in Figs. 3, 4).

After 24 h, results from CAEE + CNP, RA and controls were not significantly different at 3.125 mg/mL ($P = 0.151$) using one-way ANOVA test.

After 48 h of exposure to CAEE + CNP and RA, the lowest concentration, synthesis of type III collagen provided a higher yield than control. At 72 h, control had the highest value. Overall, it appears that the amount of synthesised collagen does not strictly depend on the time exposure and concentrations of the test compounds.
Effects of CA ethanolic extract in chitosan nanoparticles

**Fig. 3** Comparison of type I collagen synthesis in NHDF without treatment (control) and after 24, 48 and 72 h of exposure to *Centella asiatica* ethanolic extract encapsulated into chitosan nanoparticles (CAEE + CNP) and retinoic acid (RA) at various concentrations for 24, 48 and 72 h. The concentrations of CAEE + CNP were obtained by serial dilution from 12.5 mg/mL down to 3.125 mg/mL and RA from 1 mg/mL down to 0.25 mg/mL.

**Fig. 4** Comparison of type III collagen synthesis in NHDF without treatment (control) and after 24, 48 and 72 h of exposure to *Centella asiatica* ethanolic extract (CAEE) encapsulated into chitosan nanoparticles (CAEE + CNP) and retinoic acid (RA) at various concentrations for 24, 48 and 72 h. The concentrations of CAEE + CNP were obtained by serial dilution from 12.5 mg/mL down to 3.125 mg/mL and with RA by serial dilution from 1 mg/mL down to 0.25 mg/mL.

After 48 h, there is no statistically significant difference at all the concentrations tested between CAEE + CNP, RA and control (P-value >0.05) using one-way ANOVA test. These results are not significantly different when compared to 24 h exposure, though there is an increase in collagen synthesis with CAEE + CNP and RA.

After 72 h, CAEE + CNP and RA showed no statistically significant differences using one-way ANOVA test,
although there was a decrease in type III collagen synthesis activity with CAEE + CNP and RA compared with 24 and 48 h of exposure.

Figure 5 compares cell proliferative activity of cultured NHEK after exposure with CAEE + CNP and RA at serial dilutions for 24 and 48 h.

After 24 h of treatment, RA showed maximum result with 155% of control at a concentration of 5 mg/mL, while CAEE + CNP gave 145% at 3.125 mg/mL. Overall, CAEE + CNP stimulated lower proliferative effect than RA. Unpaired t-test statistical analysis showed no significant difference.

Keratinocyte proliferation at 48 h looked similar for the two compounds tested. CAEE + CNP at all concentrations (between 3.125 and 25 mg/mL) and RA (between 0.3125 and 5 mg/mL) stimulated proliferation to 110–120% of control (100%). Interestingly, the two highest concentrations of CAEE + CNP and the highest RA concentration showed lower proliferative activity than control (<100%).

Figure 6 shows AQP3 expression in NHDF without and with exposure to CAEE + CNP for 24 h, as detected by immunocytochemistry. The results showed an increase in protein AQP3 expression in NHDF after exposure for 24 h to CAEE + CNP at 3.125, 6.25 and 12.5 mg/mL. Semi-quantitatively, we were able to obtain optimal results at the highest concentration of 12.5 mg/mL.

Figure 7 shows protein expression of AQP3 in cultured NHDF without and with exposure to RA for 24 h, as detected by immunocytochemistry. Similar AQP3 expression was seen at all concentration, although with a reverse trend as compared to CAEE + CNP.

The results showed an increase in AQP3 protein expression in NHDF after exposed to RA at concentration of 0.25, 0.5 and 1 mg/mL. Semi-quantitatively, optimal results were gained at 0.25 mg/mL concentration of RA.

Figure 8 shows AQP3 expression in cultured NHDF before and after treatment with CAEE + CNP and RA for 24 h, as quantitatively analysed with computer software ImageJ.

Quantified particle density from ImageJ analysis demonstrated that CAEE + CNP stimulated the expression of AQP3 protein in a concentration-dependent manner, with optimal concentration at 12.5 mg/mL. In contrast, the expression of AQP3 in dermal fibroblast after treated with RA decreased with increasing concentration (0.25–0.5 and 1 mg/mL).

By using one-way ANOVA test, we obtained a statistically significant difference on AQP3 expression in cultured NHDF ($P < 0.001$) after being exposed with CAEE + CNP and RA at concentration of 3.125 and 0.25 mg/mL, respectively, compared with control. Furthermore, with post-hoc Tukey test, there is also a significant difference between CAEE + CNP groups and RA groups ($P < 0.001$).

Another significant difference on AQP3 expression in NHDF was also obtained by exposing 6.25 mg/mL of CAEE + CNP and 0.5 mg/mL of RA compared with control ($P < 0.05$). The following post-hoc Tukey test showed a significant difference between CAEE + CNP and RA ($P = 0.006$).

This study revealed a significant difference of AQP3 expression in NHDF after being exposed to CAEE + CNP and RA at concentration of 12.5 and 1 mg/mL, respectively, compared with control ($P < 0.05$).
statistical analysis was conducted by using post-hoc Tukey test, and there is no significant difference between CAEE + CNP and RA.

Figure 9 shows protein expression of AQP3 in cultured NHEK without and with exposure to CAEE + CNP for 24 h, as detected by immunocytochemistry. After 24 h of exposure to CAEE + CNP concentration at 3.125 and 6.25 mg/mL, there was an increased expression of AQP3 protein in the cytoplasm of keratinocytes compared to control, while the exposure of 12.5 mg/mL actually decreased AQP3 expression. These results indicate that AQP3 expression in cultured NHEK showed inverted concentration dependence upon exposure with CAEE + CNP. Similarly, quantitative analysis was done using ImageJ software.

Figure 10 shows protein expression of AQP3 in cultured NHEK without and with exposure to RA for 24 h, as detected by immunocytochemistry. AQP3 expression on the keratinocytes increased after exposure to RA at concentration of 0.025, 0.05 and 0.1 mg/mL. The intensity of protein AQP3 expression in keratinocytes increases proportionally with the increasing of RA concentration.

Figure 11 shows AQP3 expression in cultured NHEK as quantitatively analysed using computer software ImageJ. Qualitative results of immunocytochemistry were analysed by using ImageJ software to obtain quantitative data for further statistical evaluation.

From the immunocytochemistry results on keratinocytes, the quantitative analysis based on colour density showed that CAEE + CNP at concentration of 3.125 and 6.25 mg/mL stimulated AQP3 protein expression slightly higher than control. For RA stimulation, however, there is a linear correlation between AQP3 protein expression and the treatment concentration.

Statistical analysis by using one-way ANOVA test showed no significant difference on AQP3 expression in cultured NHEK after being exposed with CAEE + CNP and RA at concentration of 3.125 and 0.25 mg/mL, respectively, compared with control.

After exposure with 6.25 mg/mL of CAEE + CNP and 0.5 mg/mL of RA showed a significant difference on AQP3 expression in NHEK (P < 0.05) compared with control, as statistical analysis was conducted by one-way ANOVA test. Further statistical analysis using post-hoc Tukey test showed no significant difference between CAEE + CNP and RA.

Using one-way ANOVA test, we obtained another significant difference on AQP3 expression in NHEK after
Fig. 7 Aquaporin 3 (AQP3) expression (brown colour, pointed by arrows) in NHDF: (a) without treatment (control); and after 24 h exposed to retinoic acid (RA) at concentrations of: (b) 0.25 mg/mL, (c) 0.5 mg/mL, and (d) 1 mg/mL; immunochemical staining with anti-aquaporin 3 antibody Ab125219.

Fig. 8 Quantitative analysis of AQP3 expression in cultured NHDF by computer software ImageJ. *Statistically significant difference found between 3.125 mg/mL of CAEE + CNP and RA 0.25 mg/mL using one-way ANOVA and post-hoc Tukey tests (P < 0.05). **Statistically significant difference found between 6.25 mg/mL of CAEE + CNP and RA 0.5 mg/mL using post-hoc Tukey test (P < 0.05). ***Statistically significant difference found between 0.25 mg/mL of RA and control using post-hoc Tukey test (P < 0.05). ****Statistically significant difference found between 0.5 mg/mL of RA and control using post-hoc Tukey test (P < 0.05).
Fig. 9 Aquaporin 3 (AQP3) expression (brown colour, pointed by arrows) in NHEK: (a) without treatment (control); and after 24 h exposed to CAEE + CNP at concentration of: (b) 3.125 mg/mL; (c) 6.25 mg/mL and (d) 12.5 mg/mL; immunochemical staining with anti-aquaporin 3 antibody Ab125219.

Fig. 10 Aquaporin 3 (AQP3) expression (brown colour, pointed by arrows) in NHEK: (a) without treatment (control) and after 24 h exposed to retinoic acid (RA) at concentration of: (b) 0.25 mg/mL; (c) 0.5 mg/mL and (d) 1 mg/mL; immunochemical staining with anti-aquaporin 3 antibody Ab125219.
**DISCUSSION**

RA is a potent stimulator of keratinocyte proliferation and regulator of cell differentiation. It increases the thickness of stratum granulosum and is widely used in cosmetic anti-aging treatment of the skin\(^3\). Since RA has harmful side effects, it is interesting to search for alternatives, i.e., other active substances with better pharmacological profile (bioavailability, toxicity) as compared to RA.

In this study, we used CAEE, which had shown better proliferative activity in preliminary experiments than the aqueous extract (data not shown). A study in Malaysia had also proven that ethanolic extracts have better biological effects than aqueous extract of CA\(^3\). Chitosan–alginate nanoparticles developed as carriers for CA extracts were considered as promising systems to stabilise the extracts\(^17\). Hence, we used our own CNP to encapsulate our CAEE (CAEE + CNP).

Leonida (2012) investigated the activity of chitosan as antimicrobial, wound-healing and anti-aging compounds. The authors reported that ‘nano-sizing’ enhanced its effectiveness\(^3\). Moreover, when well-known substances are prepared into nanoparticles, they may display completely different properties and can behave in an unpredictable manner. This means that nanoparticles could re-invent the properties of substances currently used in cosmetic dermatology to create novel chemical entities from the old ones\(^8\).

In this study, we used chitosan with a degree of deacetylation more than 85% (not consistent with ‘Materials and Methods’ Section). A study by Howling et al. (2001) demonstrated that chitosan with a relatively high degree of deacetylation strongly stimulated fibroblast proliferation while chitosan with lower levels of deacetylation showed less activity\(^22\).

**Proliferation of cultured NHDF**

From our results, it can be concluded that the proliferative effect of CAEE + CNP is not strictly concentration-dependent but rather time-dependent (Fig. 3). After 24 h of exposure, CAEE + CNP showed a generally better proliferative effect on NHDF (with a peak at 6.25 mg/mL = 160% of controls) than RA. After 48 h, there are no significant differences between CAEE + CNP and RA. After 72 h, the effect of RA dominated the picture with a peak of about 180% of controls (Fig. 3).

A study by Wu et al.\(^37\) investigated the effect of four triterpenes from CA on the proliferation of NHDF. At low concentrations (1, 3 and 10 µM), all compounds tested did not enhance proliferation compared with the control group. Meanwhile, Song et al.\(^38\) showed that madecassoside, a bioactive compound of CA-inhibited proliferation of keloid fibroblasts in a time and concentration-dependent manner. A study by Lu et al. (2004)\(^39\) found that asiaticoside, another compound of CA, strongly induced cell-cycle progression, proliferation and collagen synthesis in dermal fibroblasts. Asiaticoside also increased migration of keratinocytes by ~20% in wound healing process\(^40\).

In the dermis, RA was found to regulate fibroblast proliferation and play a role in aging prevention because...
of its inhibitory effects on the expression of metalloproteinases. Varani et al. demonstrated that the ability of RA to stimulate NHDF proliferation depends on the concentration of Ca²⁺ in the extracellular environment. Although RA exerts the above effects and has also such clinical activity in dermal tissue, all-trans-RA (ATRA) reduced proliferation in NHDF cultures. Moreover, retinoids generally decreased NHDF proliferation after 48 h of incubation.

**Collagen expression in cultured NHDF**

Type I and III collagens are the major structural and functional components of skin connective tissue. Hence, we were interested to investigate the efficacy of CAEE + CNP on the biosynthesis of these collagens in NHDF compared to RA. We found that the effect of CAEE + CNP on type I collagen synthesis at 24 and 48 h of exposure was not significantly different from RA and controls. At 72 h, control values were slightly decreased, but CAEE + CNP and RA maintained type I collagen synthesis on the same level.

Type III collagen synthesis fluctuated and generally increased over time with its highest control value at 48 h; exposure to CAEE + CNP and RA at their lowest concentrations slightly stimulated biosynthesis at 48 h. At 72 h, control value was higher than all other values; in other words, all the treatment had inhibitory effects on type III collagen synthesis.

In a study from Malaysia, CA was shown to have stimulatory effect on collagen synthesis in a concentration-dependent manner. At 50 mg/mL, their CA extract enhanced 3-fold collagen production, compared to the control. Their data indicated higher proliferative stimulation by CA than by vitamin C which was used as a positive control and enhanced collagen synthesis of 2-fold over negative controls.

**Proliferative activity of cultured NHEK**

Our findings revealed that CAEE + CNP and RA increased proliferation activity at certain concentrations; however, the effect is not strictly concentration-dependent. The picture differed between 24 and 48 h of incubation. The highest results were obtained with each of the two compounds at 24 h: RA (5 mg/mL, 155%) > CAEE + CNP (3.125 mg/mL, 145%). All other results including controls were not significantly different from each other.

In literature, exposure of NHEK to RA increases proliferative activity; RA has regulatory effects on epidermal keratinisation, keratinocyte differentiation and inflammation. In keratinocytes, RA activates peroxisome proliferator-activated receptor (PPAR) β/γ in parallel to the activation of RA receptor (RAR); consequently, PPAR β/γ is central to the maintenance of skin permeability, barrier integrity and keratinocyte survival during inflammation and wound healing. RA regulates keratinocytes proliferative activity and differentiation through retinoid receptors of RAR and RXR.

**The expression of AQP3 in NHDF**

Aquaporin 3 (AQP3) is the most common aquaporin in skin. AQP3 is a membrane transporter of water and glycerol expressed in plasma membrane in the basal layer of human epidermis. The function of AQP3 in NHDF is not totally clear yet, but it may be involved in cell migration which occurs in wound healing process. AQP3 expression in human skin increased as a response to stress in diseases such as atopic eczema and to various agents such as RA.

The expression level of AQP3 in NHDF decreases significantly beyond the age of 60 years. This finding might explain why wound healing process is slower in old age than in youngsters, suggesting that AQP3 may play an important role in the aging of human skin. Li et al. found that the expression of AQP3 protein in sun-protected human skin decreased with skin aging.

The research by Cao et al. showed that the expression of AQP3 in NHDF increased after treatment with EGF in concentration- and time-dependent patterns. Ryu et al. investigated the effect of AQP3 overexpression in HDF after incubated with TGF caused fibrosis, but the same effect have not yet found in HDF fibroblast. Our study demonstrates that CAEE + CNP formulation increases the expression of AQP3 in NHDF after 24 h of incubation in a concentration-dependent pattern. RA also influenced the expression of AQP3 in NHDF, but the result decreased with increasing concentrations (Fig. 8).

**The expression of AQP3 in NHEK**

Hydration of the SC is an important determinant of skin appearance. Reduced SC hydration was found in aged skin, making it looked dry, dull and saggy. The expression of AQP3 in NHEK decreases with aging.

In skin epidermis, as a water channel, AQP3 facilitates cell migration in wound healing and as a glycerol transporter enhances keratinocyte proliferation and differentiation. Therefore, AQP3 is also a key target protein for anti-aging treatment. The correlation was found among AQP3 expression, epidermal ATP concentration and cell proliferation as AQP3-facilitated glycerol transport and lipid synthesis in the epidermis.

ATRA increases AQP3 expression in NHEK through RAR-γ. ATRA of 0.05% increased AQP3 expression in human skin explants after application for 24 h. This is consistent with our finding that RA increased AQP3 expression in keratinocytes at concentrations of 0.25 and 0.5 mg/mL, with optimal concentration at 1 mg/mL. A study by Song et al. showed the AQP3 overexpression induced by ATRA, in which time and dose dependent will be followed by increased water transport, keratinocyte, skin permeability and TEWL, and caused dry skin. Nicotinamide attenuates AQP3 overexpression induced...
by RA through inhibitor of EGFR in cultured human skin keratinocytes. AQP3 is the abundant skin aquaglyceroporin functioning as a channel for both water and glycerol. Thus, AQP3 has an important role in skin hydration. The recent discovery of AQP3 functions has led to several studies of other novel compounds, motivated by the relation between AQP3 expression, water content and moisturising effect on skin. Several cosmetic companies have marketed cosmetics-containing ingredients that are claimed to increase AQP3 expression in skin. The increase of AQP3 expression is claimed to have anti-ageing, anti-wrinkle and other beneficial effects on skin appearance.

Currently, research is under way to investigate compounds with the ability to stimulate AQP3 expression, thereby increasing skin hydration endogenously. Recent studies of herbal AQP3 stimulants included P. colubrina green C. arabia and extract of A. turkestanica (a plant from Central Asia). The increase of AQP3 expression in NHEK was claimed to have moisturising effect on skin. Our research indicates that after 24 h of exposure, CAEE + CNP can induce AQP3 expression in keratinocytes at concentrations of 3.125 and 6.25 mg/mL.

Our study demonstrates that CAEE + CNP increased the expression of AQP3 in NHEK ~1.6-fold, compared to negative control at an optimal concentration of 3.125 mg/mL after incubation for 24 h. This result suggests that CAEE + CNP increases skin hydration and that CAEE + CNP can be used as an active compound for cosmetic skin care/moisturiser.

SUMMARY AND CONCLUSION

CAEE + CNP can be used as an effective formulation to stimulate the proliferation of fibroblasts and keratinocytes during short 24 h exposure time. The best effect of CAEE + CNP for the proliferation of fibroblasts and keratinocytes was also obtained at low concentrations (6.25 and 3.125 mg/mL, respectively).

Efficacy of CAEE + CNP on stimulating type I and III collagen synthesis in fibroblasts was not significantly different from RA, after exposure to all concentrations and time periods tested. CAEE + CNP increase AQP3 expression in fibroblasts and keratinocytes after 24 h of exposure at optimal concentrations of 12.5 and 3.125 mg/mL, respectively.

Thus, CAEE + CNP can be used as an active formulation for novel anti-ageing cosmetic that regulates skin hydration by increasing skin cell proliferation and AQP3 expression. In the future, pre-clinical and clinical studies are needed for further validation.

REFERENCES