

Effects of palmitoyl-KVK-L-ascorbic acid on skin wrinkles and pigmentation

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Received: 25 September 2016 / Revised: 23 February 2017 / Accepted: 1 March 2017
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Abstract Wrinkle formation and abnormal pigmentation are major clinical alterations associated with skin aging. As the aim of our study was to investigate the effects of palmitoyl-KVK-L-ascorbic acid on skin aging, the anti-wrinkle and depigmentation effects of palmitoyl-KVK-L-ascorbic acid were evaluated by measuring collagen expression in dermal fibroblast cells and inhibition of melanogenesis in B16F1 cells, respectively. The anti-aging effect of palmitoyl-KVK-L-ascorbic acid cream was also evaluated against a placebo cream in a clinical trial. Our results confirmed that the expression of type I collagen in dermal fibroblast cells treated with palmitoyl-KVK-L-ascorbic acid (0.1–4 µg/mL) increased in a dose-dependent manner. In B16F1 cells, treatment with 20 µg/mL palmitoyl-KVK-L-ascorbic acid reduced the melanin content by approximately 20% compared to alpha-melanocyte stimulating hormone treatment. In the clinical trial, application of palmitoyl-KVK-L-ascorbic acid cream led to an improvement in skin roughness and lightness in 12 and 8 weeks, respectively. Our data show that palmitoyl-KVK-L-ascorbic acid is an effective anti-aging agent that reduces wrinkles and abnormal skin pigmentation.

Keywords L-ascorbic acid · Palmitoyl-KVK · Skin wrinkles · Skin pigmentation

Introduction

The destruction of the structural integrity of the skin's extracellular matrix is the major cause of wrinkles in photo-aged skin. Type I collagen, which is the most abundant structural protein in skin and connective tissue, is synthesized and secreted in a soluble form by dermal fibroblasts. Type I collagen provides strength and elasticity to skin. UV exposure leads to the inhibition of type I procollagen production via the transforming growth factor-β1/Smad and mitogen-activated protein kinase signaling pathways, resulting in the degradation of type I collagen [2, 9].

Skin pigmentation is the result of melanin synthesis in the melanosomes of melanocytes followed by the progressive transfer of this melanin to keratinocytes. Melanin is the pigment responsible for skin color and plays normal physiological roles within the skin. However, UV exposure can cause an abnormal increase in the skin's melanin content, resulting in age spots and melasma.

The modulation of melanogenesis and collagen synthesis is an important objective in the development of cosmetics, and many cosmetic companies have developed skin whitening and anti-wrinkle agents. L-ascorbic acid (vitamin C), a water-soluble antioxidant in extracellular fluids is an important ingredient in these cosmetic products. It is involved in multiple cellular processes, including the stimulation of collagen biosynthesis and inhibition of skin damage by free radicals [3, 17, 18, 20]. However, the unstable structure and low skin penetration of L-ascorbic acid have limited its application in the cosmetic industry. The susceptibility of L-ascorbic acid to oxidative degradation has led

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to an interest in using derivatives of L-ascorbic acid that shows increased stability *in vitro*. In particular, studies have described the chemical modification of the hydroxyl group at the C-2 position to produce stable derivatives such as 2-O- α -D-glucopyranosyl-L-ascorbic acid (ascorbic acid 2-glucoside, AA-2G) [7, 12, 21].

To address this problem, we synthesized various L-ascorbic acid and peptide conjugates, and determined the effects of the conjugated form of palmitoyl-KVK and ascorbic acid on the stimulation of type I procollagen synthesis and inhibition of melanin content *in vitro*. These novel L-ascorbic acid derivatives, using palmitoyl-KVK, improve the stability and skin penetration of L-ascorbic acid [3, 13].

In this study, our aim was to investigate the effects of palmitoyl-KVK-L-ascorbic acid (palmitoyl-KVK aminopropyl ascorbyl phosphate, Palm-KVK-AA) on collagen synthesis in human dermal fibroblasts and on melanogenesis in B16F1 cells. Moreover, we wanted to confirm the anti-wrinkle and depigmentation effects of Palm-KVK-AA cream in an *in vivo* study.

Materials and methods

Preparation of palm-KVK-AA

Palm-KVK-AA was synthesized at Celltrion Chemical Research Institute (Yongin, Korea). Palm-KVK-AA was synthesized from commercially available palmitoyl tripeptide-5 (Pal-KVK) by coupling with an ascorbic acid derivative, Vitagen (3-aminopropyl [(2R)-2-[(1S)-1,2-dihydroxyethyl]-3-hydroxy-5-oxo-2H-furan-4-yl] hydrogen phosphate) (Fig. 1a). BOC-Pal-KVK (30.0 g, 36.9 mmol) was dissolved in N,N-dimethylformamide (400 mL), with N,N'-dicyclohexylcarbodiimide (7.98 g, 38.8 mmol) and

N-hydroxysuccinimide (4.45 g, 38.8 mmol) added to the solution. The solution was stirred at room temperature for 15 h. The solid byproduct, N,N'-dicyclohexylurea was removed after filtration of the solution. Subsequently, Vitagen (12.7 g, 40.6 mmol) and N,N-diisopropylethylamine (16 mL, 92 mmol) were added to the filtrate. The mixture was stirred at room temperature for 3 h and then concentrated. The residue was purified by recrystallization with methanol and isopropyl ether to yield compound (1) (36.5 g, 89%), which was a light pink solid with the following electrochemical/electrospray mass spectrometry (ES-MS) properties (ES-MS m/z = 1107 [M+H]⁺). The value of ES-MS m/z represents mass-to-charge ratio.

Compound (1) (36.5 g, 33.0 mmol) was then dissolved in 1,4-dioxane (200 mL) with the addition of 4 M HCl in 1,4-dioxane (99 mL, 396 mmol). The mixture was stirred at room temperature for 4 h. The mixture was then concentrated and the residue was purified by trituration using acetone and isopropyl alcohol to yield compound (2) (13.5 g, 43%), which had an off-white color and the following ultraviolet visible spectroscopy (methanol, λ_{\max} 238 nm) and ES-MS properties (ES-MS m/z = 907 [M+H]⁺ (free amine)) (Fig. 1b).

In vitro study

Human neonatal dermal fibroblasts (HDFn) and B16F1 cells were obtained from the Korean Cell Line Bank (Seoul, Korea) and the American Type Culture Collection (VA, USA), respectively. The cells were cultured at 37 °C in a humidified incubator containing 5% CO₂ and 95% air, in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% antibiotics (100 U/mL penicillin and 100 μ g/mL streptomycin). The aforementioned medium, serum, antibiotics and 0.5% trypsin-EDTA were purchased from Gibco (CA, USA).

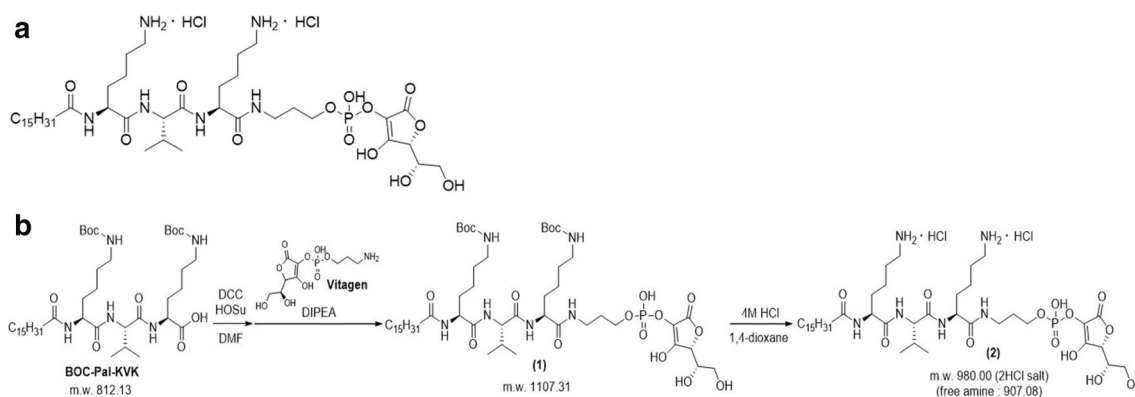


Fig. 1 Synthesis of palmitoyl-KVK-L-ascorbic acid. Palmitoyl-KVK-L-ascorbic acid (Palm-KVK-AA) was synthesized from commercially available Palmitoyl Tripeptide-5 (Pal-KVK) by coupling

with ascorbic acid derivative (Vitagen). Chemical structure (a); scheme for the synthesis of palmitoyl-KVK-L-ascorbic acid (b)

HDFn cells were seeded onto a 24-well plate at a density of 5×10^4 cells per well and cultured for 24 h, and then the medium was replaced with the serum-free medium. After 24h, HDFn cells were treated with Palm-KVK-AA at various concentrations (0.1, 0.5, 1, 2 and 4 $\mu\text{g/mL}$) and culture supernatants were collected at 48h. Type I procollagen concentration was determined using culture supernatants by Procollagen Type I C-Peptide EIA Kit (Takara, Shiga, Japan) according to the manufacturer's instructions.

B16F1 cells were seeded onto a 6-well plate at a density of 2×10^5 cells per well. Following overnight incubation, the medium was replaced with a medium containing only alpha-melanocyte stimulating hormone ($\alpha\text{-MSH}$; 10 nM) or $\alpha\text{-MSH}$ (10 nM) and Palm-KVK-AA at various concentrations (10, 20 and 50 $\mu\text{g/mL}$). The cells were incubated for a further 72 h. The cells were washed twice with phosphate-buffered saline and harvested by trypsinization using 0.0125% trypsin-ethylenediaminetetraacetic acid. The harvested cells were pelleted and dried in an oven. The pellet was extracted in 1 N sodium hydroxide with 10% dimethyl sulfoxide for 1 h at 60 °C. The extracts (200 μL) were then transferred to individual wells of a 96-well microplate and the optical density was measured at 490 nm using a microplate reader. The melanin content in each well was calculated and expressed as a percentage of the $\alpha\text{-MSH}$ concentration.

In vivo clinical study

To evaluate the anti-wrinkle effect of Palm-KVK-AA, 21 healthy Korean women aged between 41 and 55 years (50.1 ± 3.9 years) were enrolled in the study. The cream with 0.075% (0.75 mg/mL) of Palm-KVK-AA or without Palm-KVK-AA was applied to each half of the participant's face, twice daily for 12 weeks. The allocation of the cream was based on a double-blind randomized method. Skin replica images were evaluated using a visiometer (Skin-Visiometer SV 700; Courage-Khazaka Electronic, Germany). Visual assessment was performed using the global photodamage score (visual wrinkle assessment) [5], and dermal density was measured using Dermascan-C (Cortex Technology, Denmark) at baseline, 4, 8 and 12 weeks after the application of the cream.

To evaluate the depigmentation effects of Palm-KVK-AA, 23 healthy Korean women aged between 36 and 53 years (45.8 ± 5.2 years) were enrolled in the study. The cream with 0.075% (0.75 mg/mL) of Palm-KVK-AA or without Palm-KVK-AA was applied to each half of the participant's face, twice daily for 8 weeks. The allocation of the cream was based on a double-blind randomized method. Skin pigmentation was evaluated using a chromameter (CR-400; Minolta, Japan), and visual assessment was performed using Ellead Skin and Bio Research center SOP

at baseline, 4, 6 and 8 weeks after the application of the cream.

This clinical study was approved by the relevant institutional review boards of the Ellead Skin and Bio Research Center (Seongnam, Korea). The risks and benefits of the study were explained to the participants, and all participants provided informed consent and participated voluntarily.

Statistical analysis

All experiments were performed in triplicate. Descriptive statistics and means \pm standard deviation were calculated for all data. The Wilcoxon matched-pairs signed-ranks test and repeated measures analysis of variance were used for all between- and within-group comparisons. Depending on the dataset, *p*-values of $<0.05^*$, $<0.01^{**}$ or $<0.001^{***}$ were considered to be statistically significant.

Results

Anti-wrinkle effect of Palm-KVK-AA

The effects of Palm-KVK-AA (0.1, 0.5, 1, 2 and 4 $\mu\text{g/mL}$) on the expression of type I procollagen in HDFn cells are shown in Fig. 2a. A dose-dependent increase in type I procollagen secretion was observed. A concentration of 4.0 $\mu\text{g/mL}$ Palm-KVK-AA had a better effect on the expression of type I procollagen than transforming growth factor- $\beta 1$ (10 ng/mL) as a positive control. Cells treated with 1, 2 and 4 $\mu\text{g/mL}$ of Palm-KVK-AA exhibited significant increases in their Type I procollagen levels.

The in vivo assessment of the anti-wrinkle effects of the Palm-KVK-AA cream is shown in Fig. 2b–f. Data from visual assessment using the global photodamage score (visual wrinkle assessment), and visiometer and Dermascan-C analyses indicate that compared to the placebo cream, the application of Palm-KVK-AA cream for 12 weeks improved the global photodamage score (visual wrinkle assessment), skin roughness (R1), maximum roughness (R2), average roughness (R3), and dermal density (%). These results indicate that a cream containing 0.075% Palm-KVK-AA reduces facial wrinkles and has an anti-wrinkle effect.

Anti-pigmentation effects of Palm-KVK-AA

To determine the effects of Palm-KVK-AA on melanin synthesis, B16F1 cells were treated with Palm-KVK-AA at various concentrations (10, 20 and 50 $\mu\text{g/mL}$). Palm-KVK-AA, over a concentration range of 20 $\mu\text{g/mL}$, showed a significant decrease in melanin content by approximately 20% on $\alpha\text{-MSH}$ -induced melanogenesis

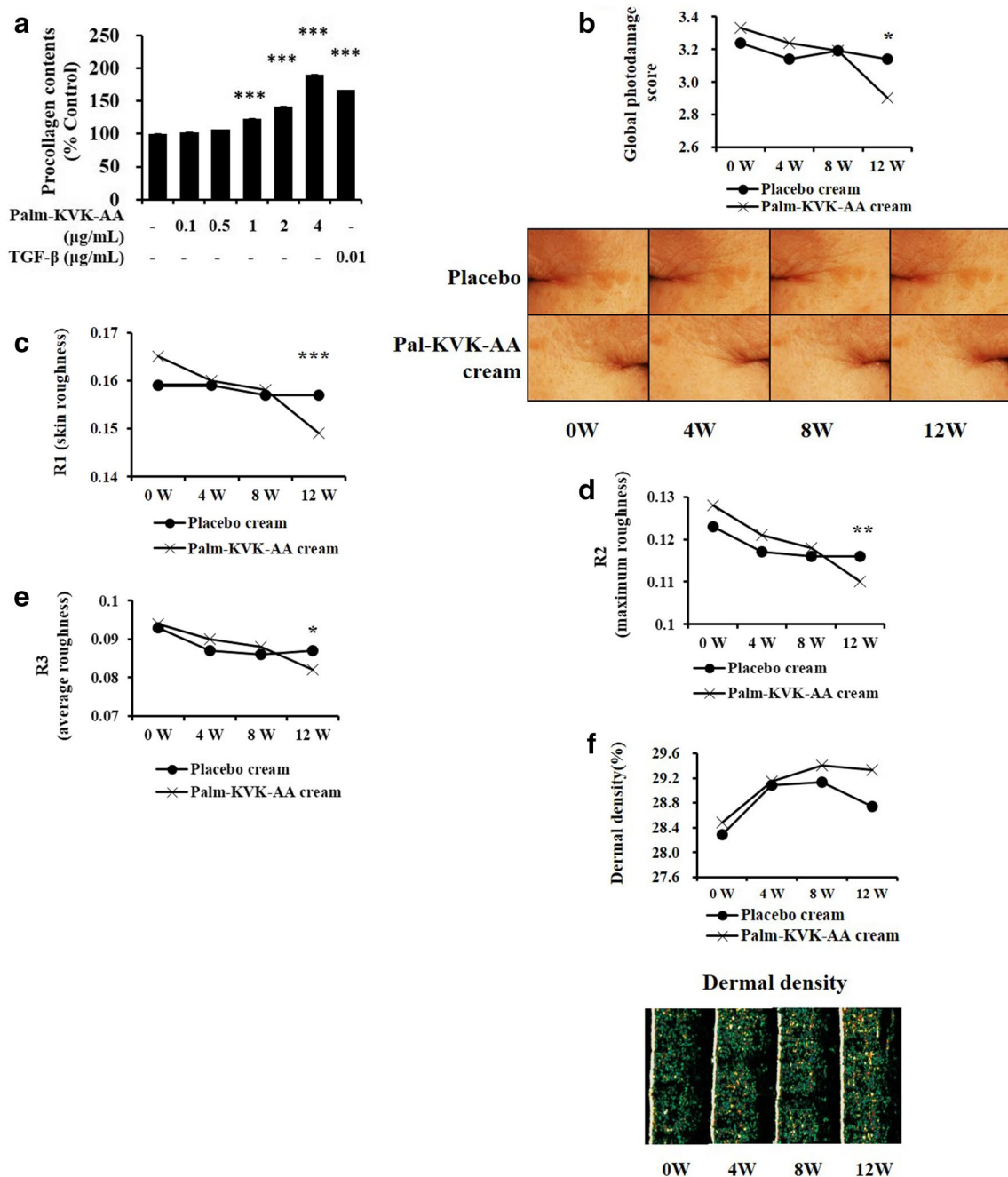


Fig. 2 Effect of palmitoyl-KVK-L-ascorbic acid (Palm-KVK-AA) on skin wrinkles. **a** Effect of Palm-KVK-AA on the stimulation of procollagen type I synthesis in human neonatal dermal fibroblasts measured using a Procollagen Type I C-Peptide EIA Kit is shown. The anti-wrinkle effects of Palm-KVK-AA cream were evaluated at 0, 4,

8 and 12 weeks by visual wrinkle assessment, visiometer, and Dermascan-C analysis. The average global photodamage score (**b**); skin roughness, R1 (**c**); maximum skin roughness, R2 (**d**); average roughness, R3 (**e**); dermal density (**f**)

in B16F1 cells compared to the α -MSH control group. Inhibitory effect of Palm-KVK-AA on melanin synthesis was showed at a much lower effective concentration for Palm-KVK-AA (20 μ g/mL) than for kojic acid (200 μ g/mL) as a positive control (Fig. 3a).

The depigmentation effects of Palm-KVK-AA cream are shown in Fig. 3b–c. The application of Palm-KVK-AA cream for 8 weeks resulted in an improvement in the clinical score (clinical pigmentation grades) and L*-values (brightness parameter) in the test group compared to values

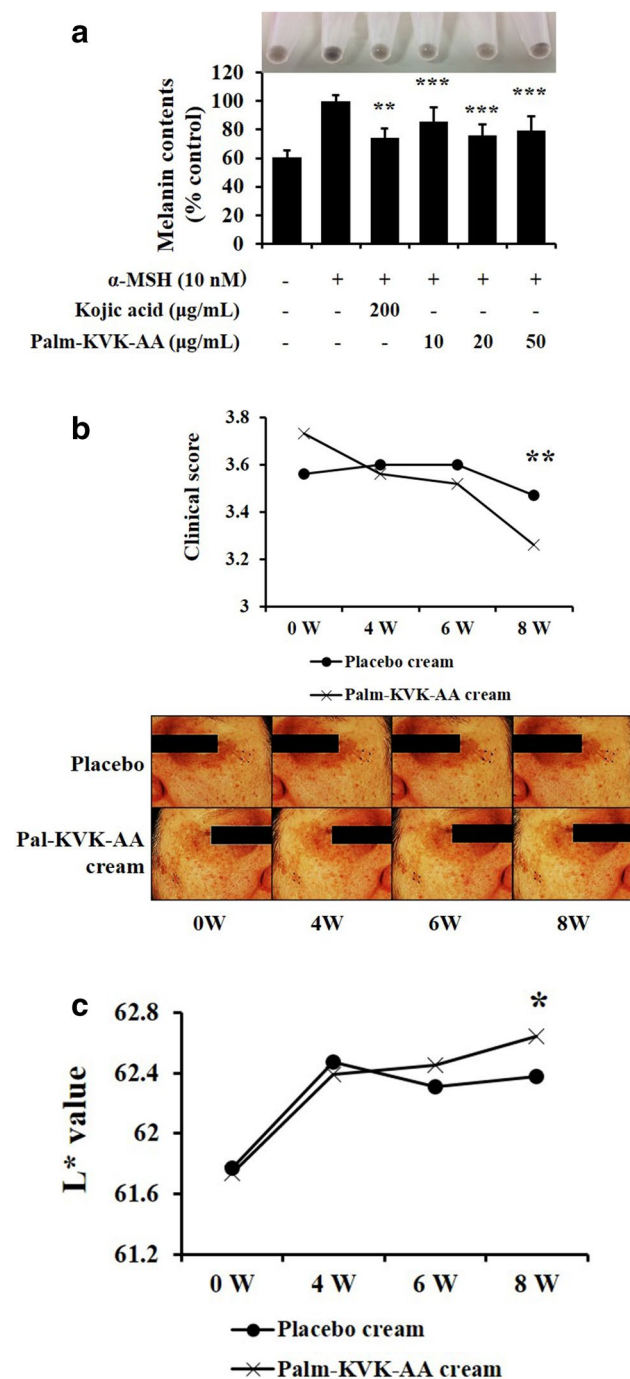


Fig. 3 Effect of palmitoyl-KVK-L-ascorbic acid (Palm-KVK-AA) on skin pigmentation. **(a)** Inhibitory effects of Palm-KVK-AA on melanin synthesis in α -MSH-induced B16F1 cells are shown. Melanin contents were measured from extracts of cells treated with α -MSH (10 nM) or a combination of α -MSH (10 nM) and Palm-KVK-AA at different concentrations for 72 h. The depigmentation effect of Palm-KVK-AA cream was evaluated at 0, 4, 6 and 8 weeks using the clinical score **(b)** and L value **(c)**

in the control group. These results indicate that a cream containing 0.075% Palm-KVK-AA improves skin lightness and has an anti-pigmentation effect.

Discussion

Palmitoyl-oligopeptides such as palmitoyl tripeptide-5 (palmitoyl-KVK) are among the most commonly used ingredients in cosmetics. Palmitoyl-KVK is a small peptide that mimics thrombospondin I and a peptide transduction domain (PTD) conjugate. Palmitoyl-KVK has been shown to increase skin penetration and to modulate various biological processes in the epidermis and dermis [6, 19]. As a mimic of thrombospondin I [4, 8, 14], palmitoyl-KVK promotes collagen formation through phosphorylation of Smad and MAPK molecules [1, 2, 9], and inhibits melanogenesis by reducing tyrosinase and TRP1 expression [10, 11, 15] by activation of TGF- β [16, 22].

In this study, we synthesized Palm-KVK-AA using palmitoyl-KVK to improve the stability and skin penetration of L-ascorbic acid. We confirmed that the expression of type I procollagen increased in a dose-dependent manner, and the expression of type I procollagen in HDFn cells at a concentration of 4 μ g/mL was two times higher than the expression at a concentration of 0.1 μ g/mL. We confirmed that the melanin content in B16F1 cells decreased by approximately 20% after treatment with Palm-KVK-AA, over a concentration of 20 μ g/mL, compared to the control treatment with α -MSH alone. The effective concentration for inhibition of melanin synthesis was much lower for Palm-KVK-AA (20 μ g/mL) than for kojic acid (200 μ g/mL), which is an established effective melanogenesis inhibitor and cosmetic agent. We also identified anti-aging effects of a cream containing 0.075% Palm-KVK-AA in an *in vivo* study that showed a reduction in facial wrinkles and improvement in skin lightness.

In conclusion, our data confirmed that Palm-KVK-AA has beneficial effects in stimulating collagen synthesis and inhibiting melanin synthesis *in vitro*. We also confirmed the anti-wrinkle and depigmentation effects of Palm-KVK-AA by *in vivo* testing of a Palm-KVK-AA cream. However, differences in the levels of stability and skin penetration of Palm-KVK-AA compared to those of L-ascorbic acid remain to be confirmed. We expect synergistic effects in stimulating collagen synthesis or inhibiting melanin synthesis by palmitoyl-KVK and L-ascorbic acid, respectively. Thus, Palm-KVK-AA is a potent anti-wrinkle and anti-melanogenic agent that will help in expanding the use of palmitoyl-oligopeptides in the cosmetic field.

Acknowledgements The authors are grateful to Dr. Beom Joon Kim (Department of Dermatology, Chung-Ang University College

of Medicine) for his critical reading of the manuscript and helpful comments.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures involving human participants were in accordance with the ethical standards of the national research committee and the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individuals who participated in the study.

References

1. Badenhorst T, Svirskis D, Wu Z (2014) Pharmaceutical strategies for the topical dermal delivery of peptides/proteins for cosmetic and therapeutic applications. *Aust J Pharmacol Ther* 2(6):1036
2. Cho JW, Il KJ, Lee KS (2013) Downregulation of type I collagen expression in silibinin-treated human skin fibroblasts by blocking the activation of Smad2/3-dependent signaling pathways: potential therapeutic use in the chemoprevention of keloids. *Int J Mol Med* 31(5):1148–1152
3. Choi HI, Park JI, Kim HJ et al (2009) A novel L-ascorbic acid and peptide conjugate with increased stability and collagen biosynthesis. *BMB Rep* 42(11):743–746
4. Chen H, Herndon ME, Lawler J (2000) The cell biology of thrombospondin-1. *Matrix Biol* 19(7):597–614
5. Chung JH, Lee SH, Youn CS et al (2001) Cutaneous photodamage in Koreans: influence of sex, sun exposure, smoking, and skin color. *Arch Dermatol* 137(8):1043–1051
6. Fields K, Falla TJ, Rodan K et al (2009) Bioactive peptides: signaling the future. *J Cosmetic Dermatol* 8(1):8–13
7. Fujinami Y, Tai A, Yamamoto I (2001) Radical Scavenging Activity against 1,1-Diphenyl-2-picrylhydrazyl of Ascorbic Acid 2-Glucoside (AA-2G) and 6-Acyl-AA-2G. *Chem Pharm Bull* 49(5):642–644
8. Gorouhi F, Maibach HI (2009) Role of topical peptides in preventing or treating aged skin. *Int J Cosmetic Sci* 31(5):327–345
9. Jin MH, Park SG, Hwang YL et al (2012) Cedrol enhances extracellular matrix production in dermal fibroblasts in a MAPK-dependent manner. *Ann Dermatol* 24(1):16–21
10. Kim DS, Park SH, Park KC (2004) Transforming growth factor- β 1 decreases melanin synthesis via delayed extracellular signal-regulated kinase activation. *Int J Biochem Cell B* 36(8):1482–1491
11. Kim WS, Park SH, Ahn SJ et al (2008) Whitening effect of adipose-derived stem cells: a critical role of TGF- β 1. *Biol Pharm Bull* 31(4):606–610
12. Kumano Y, Sakamoto T, Egawa M et al (1998) In vitro and In vivo Prolonged Biological Activities of Novel Vitamin C Derivative, 2-O- α -D-Glucopyranosyl-L-Ascorbic Acid (AA-2G), in Cosmetic Fields. *J Nutr Sci Vitaminol* 44(3):345–359
13. Lu C, Kim BM, Lee D et al (2013) Synthesis of lipoic acid-peptide conjugates and their effect on collagen and melanogenesis. *Eur J Med Chem* 69:449–454
14. Murphy-Ullrich JE, Poczatek M (2000) Activation of latent TGF- β by thrombospondin-1: mechanisms and physiology. *Cytokine Growth Factor Rev* 11(1–2):59–69
15. Murakami M, Matsuzaki F, Funaba M (2009) Regulation of melanin synthesis by the TGF- β family in B16 melanoma cells. *Mol Biol Rep* 36(6):1247–1250
16. Martínez-Esparza M, Jiménez-Cervantes C, Beermann F et al (1997) Transforming growth factor- β 1 inhibits basal melanogenesis in B16/F10 mouse melanoma cells by increasing the rate of degradation of tyrosinase and tyrosinase-related protein-1. *J Biol Chem* 272(7):3967–3972
17. Ochiai Y, Kaburagi S, Obayashi K et al (2006) A new lipophilic pro-vitamin C, tetra-isopalmitoyl ascorbic acid (VC-IP), prevents UV-induced skin pigmentation through its anti-oxidative properties. *J Dermatol Sci* 44(1):37–44
18. Panich U, Tangsupa-a-nan V, Onkoksoong T et al (2011) Inhibition of UVA-mediated melanogenesis by ascorbic acid through modulation of antioxidant defense and nitric oxide system. *Arch Pharm Res* 34(5):811–820
19. Reddy B, Jow T, Mantach BM (2012) Bioactive oligopeptides in dermatology: part I. *Exp Dermatol* 21(8):563–568
20. Shibayama H, Hisama M, Matsuda S et al (2008) Effect of a novel ascorbic derivative, disodium isostearyl 2-O-L-ascorbyl phosphate on human dermal fibroblasts: Increased collagen synthesis and inhibition of MMP-1. *Biol Pharm Bull* 31(4):563–568
21. Taniguchi M, Arai N, Kohno K et al (2012) Anti-oxidative and anti-aging activities of 2-O- α -glucopyranosyl-L-ascorbic acid on human dermal fibroblasts. *Eur J Pharmacol* 674(2–3):126–131
22. Zhang YE (2009) Non-Smad pathways in TGF- β signaling. *Cell Res* 19(1):128–139