Evaluation of the anti-aging and skin rejuvenation effect of a novel facial cream containing small synthetic peptide which to unlock TGF-β potential

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Abstract

Background:

In recent years bioactive peptides have established themselves as cornerstone ingredients in skin care, mainly for anti-aging cosmetics. Transforming growth factor beta (TGF-β) as a role model for peptide design and the gateway to skin rejuvenation. This study is about a design a new peptide to unlock TGF-β potential with skin rejuvenation and evaluation the anti-aging effect of the facial cream containing the designed peptide.

Methods:

In-vitro LAP-TGF-β activation, the samples were tested with TGF-β Emax immunoassay system (promega). TGF-β and the peptide suppress pro-inflammatory factors (TNF-α, MMP-1, and MMP-3 and IL-8) in human normal keratinocytes. Keratinocyte culture and treatment phorbol 12-myristate 13-acetate (PMA) experiments were test. Quantitation of cytokines and matrix metalloproteinases (MMPs) were quantified from supernatant of cell cultures with a multiplex bead array system (Luminex 100 xMAP) using the Procarta Cytokine Assay Kit (Panomics, Fremont USA). Clinical evaluation with 36 female subjects is to evaluate the anti-wrinkle effect of Sample B with Pal-KVK. Study of the variations, directly in vivo, of the cutaneous relief parameters on crow's feet (average roughness Ra, maximum amplitude Rt and average relief Rz) using DermaTOP (EOTECH - France).

Illustration of the visual expected effect by realization of macrophotographs

Results:

The result of the in-vitro shows a significant higher time-dependent increase release of TGF-β from LAP-TGF-β complex after incubation with the peptide than control group. TGF-β and the peptide Pal-KVK strongly in habited up-regulation of TNF-α, MMP-1, and MMP-3 and IL-8 expression. The clinical evaluation result shows a significantly anti-wrinkle effect within 7 days, and smooth fine lines effect within 14 days.

Conclusion:

The small synthetic peptide Pal-KVK showed an unlock TGF-β potential function, and suppress pro-inflammatory factors (TNF-α, MMP-1, MMP-3, and IL-8) in human normal keratinocytes. In clinical testing, the cream containing the peptide showed a significant smoothing and anti-wrinkle efficacy on crow's feet and underneath eye after 28 days twice-daily application.

Keywords: peptide; skin-aging; anti-wrinkle; TGF-β.

Introduction.

How can one discover or design a new peptide with anti-aging and skin rejuvenation effect in skin care application? First, we need to clear the trigger of biological

activities for anti-aging and skin rejuvenation, and then to identify the structures of the peptide. General method could undertake random screening with a large and diverse library of different peptide structures. However, due to the great number of structural possibilities, this method would be an inefficient approach.

Nowadays, as a deep understanding of skin biology to identify biological regulators, which might be appropriate for skin health and integrity. The transforming growth factor beta (TGF-β) pathway, which is known to play a pivotal role in the pathogenesis of skin anti-aging [1]. TGF-β is one of these cellular master switches. with a molecular weight of approximately 25kDa [1], TGF-β is the master regulator of the ECM, and itself acts as a multifunctional cytokine to regulate growth, differentiation, and other functions in various cell types [2]. And TGF-β is known to stimulate the fibroblasts in the dermis to synthesize several proteins such as collagen, the main constituent of dermal ECM [2]. collagen I (80–85%) and collagen III (10–15%) which provide strength and resilience to the skin are the most prominent [3,10]. Fibulin-5, which is important for elastogenesis, and hyaluronan is a novel TGF-β-inducible protein[4] and other proteoglycans essential for electrolyte control, water retention and many other processes[5]. TGF-β triggers synthesis of some components of the dermal-epidermal junction such as laminin V [7], collagen IV [8] and collagen VII [9]. TGF-β inhibits the expression of protein-degrading matrix metalloproteases (MMP) such as MMP-1, -2, -3 and -13 [10].

There is an opportunity to find a peptide that could mimic the natural activity of TSP-1 and Trigger TGF-β activation. In this study, we choose the best performers peptide in the screening Palm-Lys-Val-Lys-OH (Pal-KVK), as an anti-aging and skin rejuvenation effect active ingredient. We design in-vitro LAP-TGF-β activation testing and the quantitation of cytokines and MMPs testing to clear activation of TGF-β and inhibition of TNF-α, IL-8, MMP-1, and MMP-3 expression of the peptide Pal-KVK. And the in-vivo 28 Days clinical testing, we study the smooth and wrinkles of crow's feet after 28 days treatment of sample with Pal-KVK using DermaTOP [11-12]. Those Several in-vitro activity tests and in-vivo application studies were performed to demonstrate the effectiveness of Pal-KVK peptide as an anti-aging and skin

rejuvenation active.

Materials and Methods.

Materials

The peptide **(Pal-KVK)** was provided by R&D center of Mageline Biology Tech Co., Ltd. The resulting peptide was diluted in 30 ml of dioxane: water = 4:6, treated overnight with 2.0 g of Bio-Rad resin (acetate form), filtered, rotated and lyophilized. The control **sample A** without Pal-KVK and the **sample B** with 100ppm Pal-KVK, were provided by Mageline Biology Tech Co., Ltd.

TGF-β and antibodies: Recombinant human latent TGF-β and monoclonal antibody againsthuman LAP (TGF-β) were purchased from R&D Systems (Abingdon, UK). TGF-β Emax ® ImmunoAssay was from Promega (Madison, USA).
Monoclonal antibody against laminin V (P3H9-2) was from Santa Cruz Biotechnology Inc (Santa Cruz, USA) and monoclonal antibody against Collagen I was from Chemicon (Temecula, USA).

Methods

Activation of LAP-TGF-β:

Plates were coated with an anti-LAP monoclonal antibody (R&D Systems) overnight at 4°C. After washing, LAP-TGF-β (R&D Systems) was added at 5.25 ng/well. Peptides Pal-KVK (25μM) were added and incubated for 1 h–24 h (37°C). Samples were tested with TGF-β Emax ® ImmunoAssay System (Promega), following the manufacturer's instructions. This ELISA is for specific detection of biologically active TGF-β and LAP-TGF-β is not detected. Absorbance was measured at 450 nm in the plate reader Samples were assayed in duplicate. Dosages were performed in three independent experiments.

Quantitation of cytokines and MMPs:

Normal Human Keratinocytes from foreskin grew in CnT-07 Medium (CELLnTEC,

Switzerland) at 37ºC for two days, after which 0.1 μM PMA was added and then the test substances TGF-β 12.5 ng/ml and palm-KVK 10 uM were added immediately after PMA stimulation. Cytokines and matrix metalloproteinases (MMPs) were quantified from supernatant of cell cultures with a multiplex bead array system (Luminex 100 xMAP) using the Procarta Cytokine Assay Kit (Panomics, Fremont USA).

Clinical evaluation with human subjects:

The study was performed with 36 female healthy subjects, Age between 35 and 55, average age 48 years, Subject with wrinkles / fine lines on crow's feet, wrinkles / fine lines on wrinkles underneath eyes, dark shadows and bags under eyes and lids sagging. Subject having given her informed, written consent. Subject that is willing to cooperate and aware of the necessity and duration of controls so that perfect adhesion to the protocol established by the clinical trial center could have been expected.

The primary objective of this study was to evaluate the anti-wrinkle effect of sample A versus sample B, after 7, 14 and 28 days of twice-daily use.

The secondary objectives of this study were, for the studied products:

- to evaluate their effect on lids sagging,
- to illustrate the visual expected effect.
- to evaluate the subjective appreciation of their properties, their efficacy and their future use.

Table 1 The experimental design schedule of 28-Days Clinical testing

	D0	D7	D14	D28
Information of the subject about study conditions and collection of hier informed consent.	•			
Verification of inclusion and non-inclusion criteria.	•			
Acquisition of a 3D-picture of each crow's foot and each under eyes area using Dermatop®	•	•	•	•
Acquisition of a photograph of each hemi-face and of the entire face for: - illustrations and further analysis by NEWTONE Technologies® - - scoring of lids sagging	•	•	•	•
Distribution of a daily log.	•	•	•	•
Distribution of the study products.	•			•
Subjective evaluation questionnaire.				•

The study was conducted according to Helsinki Declaration (1964) and its successive update. Data were obtained using the study protocol, current internal procedures and as closely as possible to the guidance on Good Clinical Practice CPMP/ICH/135/95, January 1997.

Anti-wrinkle effect evaluation (DermaTOP)

Study of the variations, directly in vivo, of the cutaneous relief parameters on crow's feet and under eyes wrinkles (average roughness Ra, maximum amplitude Rt and average relief Rz) using DermaTOP® (EOTECH - France). Acquisitions were also sent to NEWTONE Technologies® for further analysis (under eyes wrinkles and bags).

Table 2 The Primary criterion of DermaTOP evaluation

	Acquisition with DermaTop®
DERMSCAN	Crow's feet wrinkles Under eyes wrinkles
NEWTONE® Technologies	Wrinkles around eyes Bags under eyes

Study of the variations of lids sagging using macrophotographs and 10-point structured scale. Illustration of the visual expected effect by realization of macrophotographs some pictures were sent to NEWTONE Technologies® for further analysis: dark circles.

Table 3 The Secondary criteria of DermaTOP evaluation

	Photographs
DERMSCAN	Scoring of lids sagging
NEWTONE® Technologies	Dark circles analysis

Measurements were done directly in vivo, on crow's feet and under eyes wrinkles using the fringe projection system DermaTOP. This technique consists in calculating a phase image from images with interference fringe projection. This image then allows to determine the height of each point.

The acquisition software allows to obtain 2D and 3D measurements and to determine parameters of the cutaneous relief on 50 vertical profiles distributed along the zone of interest. An automatic repositioning system allows the precise re-identification of the zone of measurement.

Ra: the average roughness (in µm):

$$Ra = \frac{1}{l} * \int_{0}^{lr} |R(x)| dx$$

a **decrease** in this parameter characterizes a **<u>smoothing effect.</u>** (Ratio between the integrated surface around the mean value and the length of the skin evaluated).

Rt: the **average relief** (in µm): average of all picks-to-valley heights. maximum difference between the highest peak and the deepest furrows registered over the entire profile.

Rz: the relief amplitude (in µm): average of the 5 maximum picks-to-valley height.

mean value of these different maxima obtained on five successive regions of the profile. The Pal-KVK-containing sample B was tested against Sample A and the treatment regime was twice per day for 28 days.

A decrease in one of these parameters (**Rt and Rz**) characterizes an anti-wrinkle effect.

Subjective-self evaluation

This study aims to make objective a type of experience that until now has been characterized by subjective evaluations.

The subjects evaluated the efficacy of the product according to the facial condition.

Remarks 1: Evaluate standard: 0 score means dissatisfy, 1 score means quite dissatisfy, 2 score means general, 3 score means quite satisfy, 4 score means satisfy.

Remarks 2: Satisfaction=number of subjects whose score>2 ÷ total number of subjects × 100%

Results.

In-vitro LAP-TGF-β activation

The transforming growth factor beta (TGF-β) pathway, which is known to play a pivotal role in skin aging, is one of these cellular master switches. We design the in-vitro LAP-TGF-β activation testing to find the peptide which can reactive the TGF-β from LAP-TGF-β. **Figure 1** shows the result of the in-vitro release of TGF-β from LAP-TGF-β complex after incubation with Placebo and Pal-KVK (25μM). Pal-KVK induced a time-dependent release of active TGF-β which was not the case for the negative control condition in which no tripeptides were added.

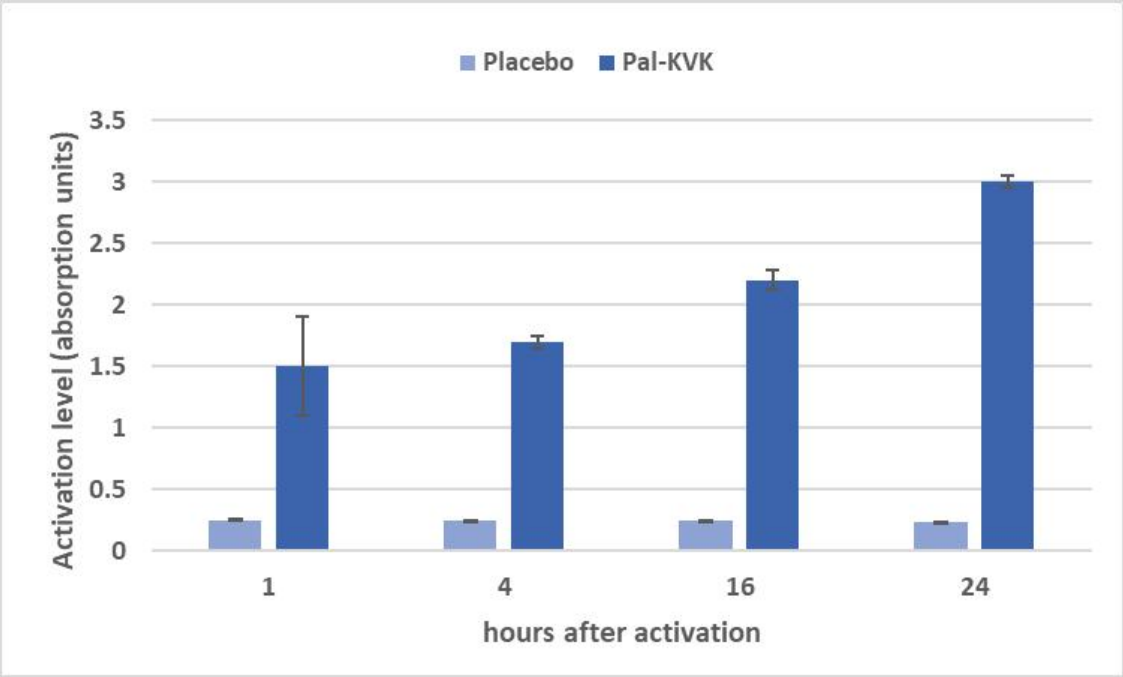

Figure 1 ELISA quantification of free TGF-β released from LAP-TGF-β.

In-vitro pro-inflammatory factors inhibition

As peptide Pal-KVK show a time-dependent TGF-β activation, we need to evaluation the pro-inflammatory factors inhibition function of the Pal-KVK. So, we designed the in vitro testing to compare the TGF-β and the peptide suppress pro-inflammatory factors in human normal keratinocytes. The keratinocytes were exposed to 0.1μM phorbol 12-myristate 13-acetate (PMA) as a chemical stress signal to induce a pro-inflammatory reaction. TGF-β and the palm-KVK peptide always strongly inhibited up-regulation of MMP-1, MMP-3, and pro-inflammatory cytokine expression (TNF-α and IL-8) (**Figure 2**).

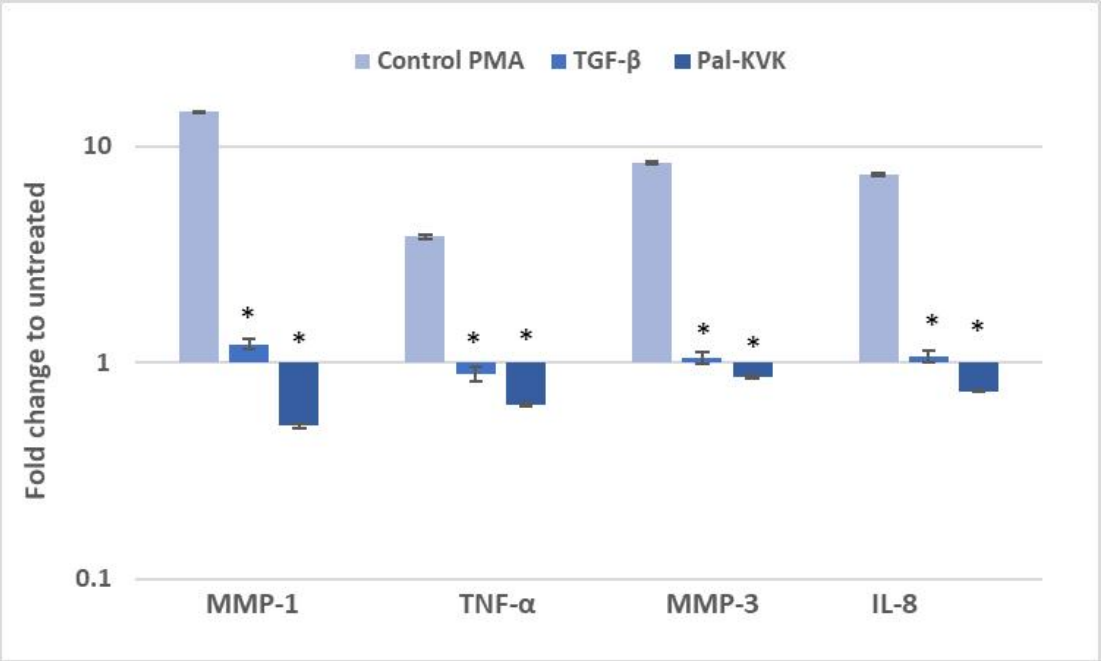

Figure 2 TNF-α, IL-8, MMP-1 and MMP-3 expression detected on protein level 24 hours after PMA (0.1 μM) induced stress signal with normal human keratinocytes (NHK) in passage 10. The fold increase in fluorescence intensity is compared to untreated control cells (=1.00). TGF-β at 12.5 ng/ml; palm-KVK at 10 μM. (*P<0.01).

In-vivo Anti-wrinkle effect evaluation (DermaTOP)

Under these study conditions, after 28 days of twice-daily use, the different studied parameters evidenced the following effects: a smoothing and anti-wrinkle effect on crow's feet, in comparison with the initial state and with the placebo at each kinetics time.

When comparing to the initial state Sample B (100ppm Pal-KVK) induced: a significant decrease in the average roughness (Ra) of -6% on D7, -7% on D14 and D28. A smoothing effect was observed in respectively 63%, 69% and 69% of the subjects. when comparing sample B (100ppm Pal-KVK) to the placebo (sample A), it induced: a significant decrease in the average roughness (Ra) of -5% on D7 and D14 and -8% on D28. A smoothing effect was observed in respectively 65%, 71% and 65% of the subjects (**Figure 3**).

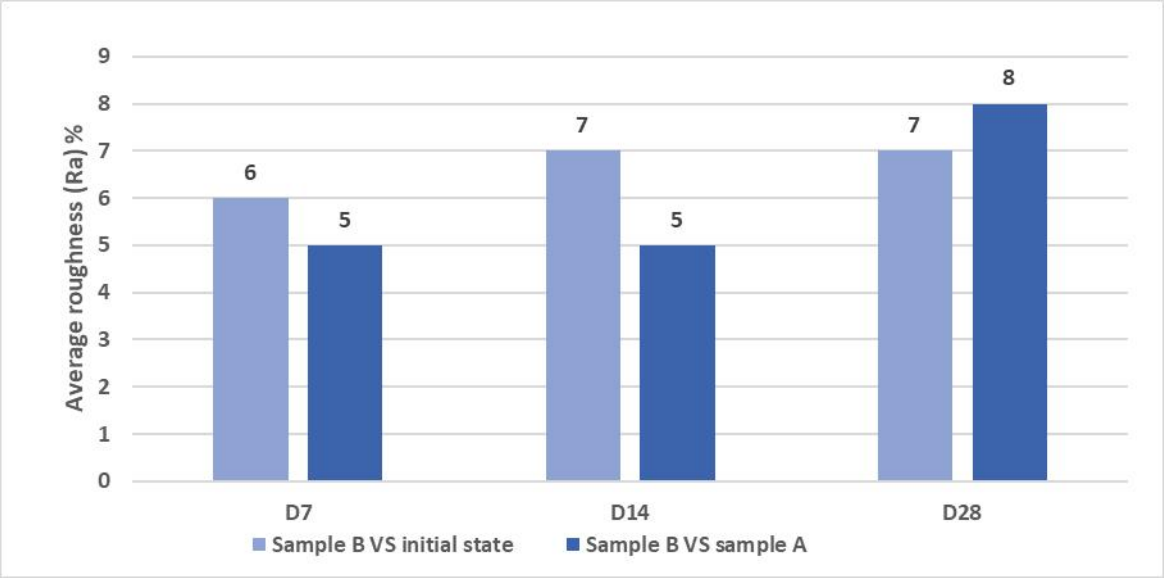

Figure 3 The smoothing effect (Ra) of formulation with Pal-KVK

As Figure 4 showed, when comparing sample B (100ppm Pal-KVK) to the initial state, it induced: a significant decrease in the average relief (Rz) of -6% on D7, -7% on D14 and -9% on D28. An anti-wrinkle effect was observed in respectively 86%, 77% and 69% of the subjects. When comparing sample B with sample A, a significant decrease in the average relief (Rz) of -6% on D7, -4% on D14 and -8% on D28. An anti-wrinkle effect was observed in respectively 74%, 71% and 65% of the subjects.

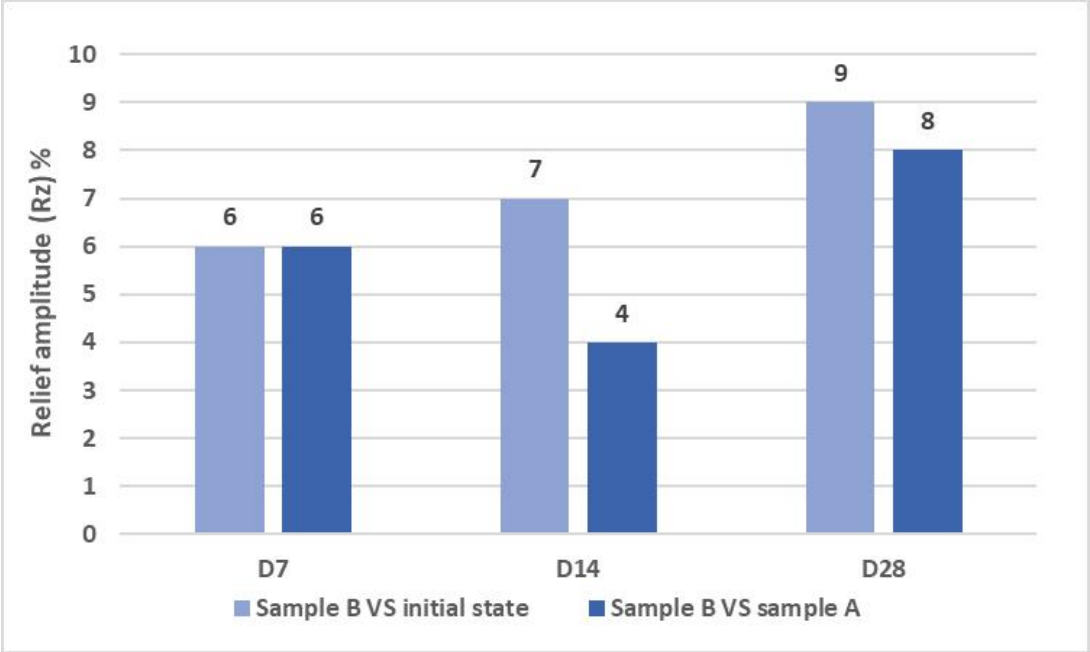

Figure 4 the anti-wrinkle effect (Rz) of formulation with Pal-KVK

As Figure 5 showed, when comparing sample B (100ppm Pal-KVK) to the initial state, a significant decrease in the relief amplitude (Rt) of -6% on D7, -8% on D14 and D28. An anti-wrinkle effect was observed in respectively 63%, 69% and 63% of the subjects.

Sample A did not induce any noticeable variation in the cutaneous relief parameters.

When comparing sample B with sample A, a significant decrease in the relief amplitude (Rt) of -5% on D7 and -8% on D28 and a limit significant decrease of -4% on D14. An anti-wrinkle effect was observed in respectively 68% and 62% of the subjects.

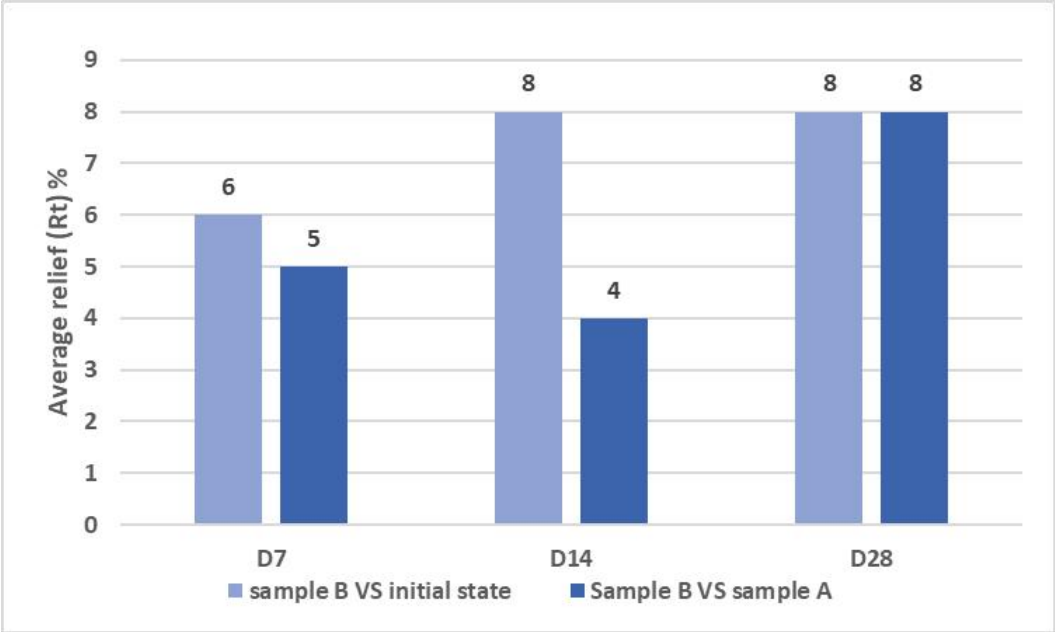

Figure 4 the anti-wrinkle effect (Rt) of formulation with Pal-KVK

Acquisitions of the real 3D microtopography of the crow's feet area were taken with the DermaTOP (EOTECH-France), a three-dimensional measurement device. Figure 5 show an example of DermaTOP artificially colour-coded microrelief record from the crow's feet area from one volunteer before treatment (day 0) and after 7, 14,28 Days treatment with formulation with 100 ppm Pal-KVK. As a result of the smoothing and anti-wrinkle effect, we observed a significant efficacy of sample B (100ppm Pal-KVK) after a 28-days formulation treatment. As the Figure 5 shows, sample B treatment induced a significant decrease of crow feet wrinkles in DermaTOP analysis pictures.

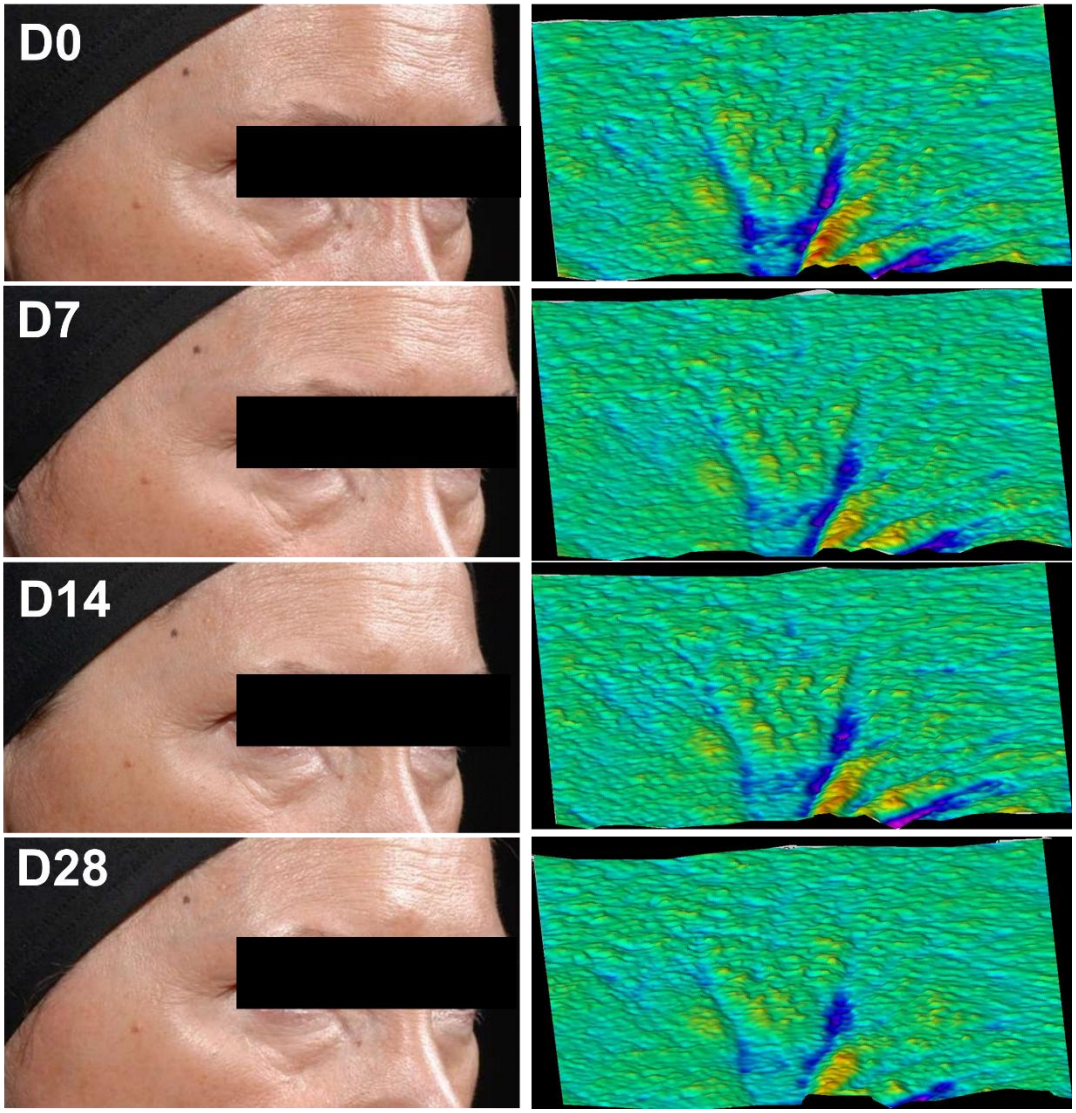

Figure 5. Example of DermaTOP artificially colour-coded microrelief record from the crow's feet area from one volunteer before treatment (day 0) and after 7, 14,28 Days treatment with formulation with 100 ppm Pal-KVK

As clinical in-vitro testing results, there were no noticeable variation in the cutaneous relief parameters on underneath eye wrinkles and on the lids sagging.

Subjective-self evaluation

After 28 days formulation with Pal-KVK and control sample treatment, subjective evaluation with self-assessment questionnaire and skin conductance response were analyzed. The products satisfied many of the subjects for their organoleptic characteristics and their efficacy especially on wrinkles.

The self-assessment showed that after 28 days of application, up to 78% subjects satisfied with the Pal-KVK formulation for immediate moisturized and soft skin, up to 76% subjects report reduction of under-eye-wrinkle, 75% subjects report crow's feet reduction, with 72% subjects reporting puffiness reduction, lines around the eyes and dark circles were also reduced for 70% subjects.

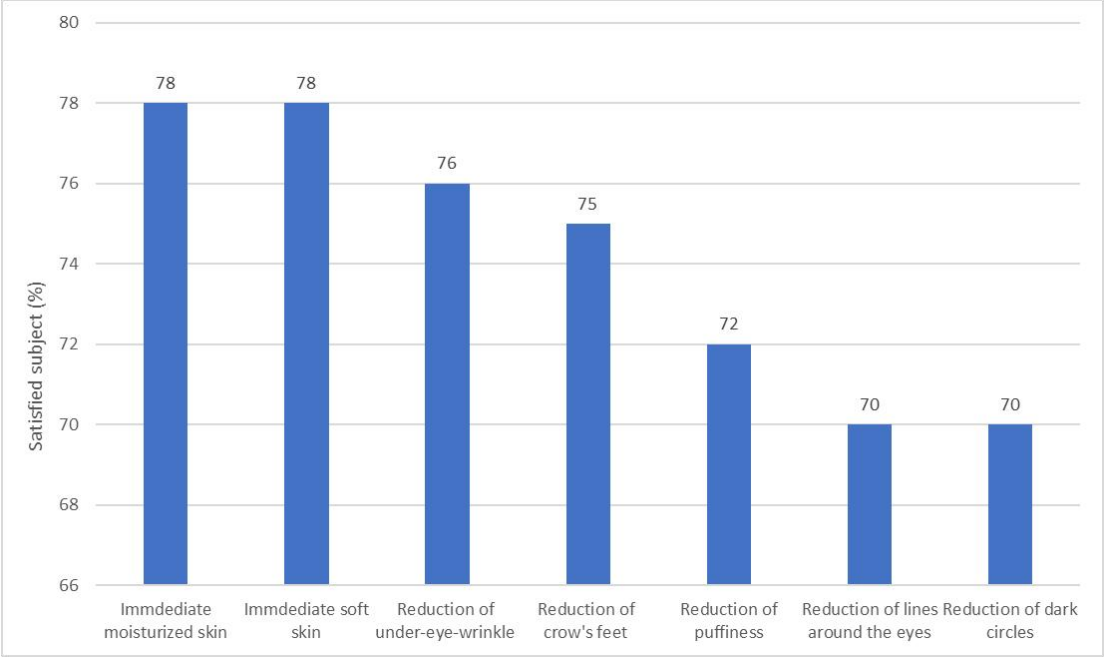

Figure 6 Self-assessment response for Subjects

Discussion.

As Pal-KVK induced a time-dependent release of active TGF-β during in-vitro LAP-TGF-β activation testing. The palm-KVK (10 µM) peptide shows stronger inhibited up-regulation of MMP-1, MMP-3, and pro-inflammatory cytokine expression (TNF-α and IL-8) than TGF-β at 12.5 ng/ml.

Under these study conditions, after 7, 14 and 28 days of use on wrinkles of crow's feet, the different studied parameters evidenced the following effects: a smoothing and anti-wrinkle effect on crow's feet, in both comparison with the initial state and with the placebo at each kinetics time.

Acquisitions of the real 3D microtopography of the crow's feet area were taken with the DermaTOP (EOTECH - France). As a result of the smoothing and anti-wrinkle

effect, we observed a significant efficacy of sample B (100ppm Pal-KVK) after a 28-days formulation treatment.

The self-assessment showed that after 28 days of application, more than 70% subjects reporting the reduction of puffiness, lines around the eyes and dark circles

The Pal-KVK peptide showed a high TGF-β potential function relate with time, and inhibit pro-inflammatory factors (TNF-α, MMP-1, MMP-3, and IL-8) in human normal keratinocytes. In clinical testing, the cream containing the peptide showed a significant smoothing and anti-wrinkle efficacy on crow's feet and underneath eye after 28 days twice-daily application. And in Subjective-self-evaluation, the Sample B with Pal-KVK satisfied most of the subjects for their organoleptic characteristics and their efficacy especially on wrinkles.

Conclusion.

As the peptide Pal-KVK proved to be a strong activator of LAP-TGF-β relate with time, the active ingredient would provoke the same cellular effects as those mediated by TGF-β directly. And the Pal-KVK showed inhibition of the pro-inflammatory factors (TNF-α, MMP-1, MMP-3, and IL-8) and good clinical smooth and anti-wrinkles efficacy. So, the Pal-KVK is a good anti-aging and skin rejuvenation ingredient in cosmetic filed, which will bring a lastingly younger and beautiful facial present to consumer.

Acknowledgments.

NONE.

Conflict of Interest Statement.

NONE.

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