



Anti-wrinkle Effect of a Palmitoyl Oligopeptide Complex on Human Keratinocytes and Fibroblasts Through TGF- β 1 Pathway

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Abstract: Many researchers revealed that dermal fibroblast which mainly synthesis the collagen and elastin in dermis were affects by skin ageing, such as the proliferation rate and gene expression level. Oligopeptides were verified by many reports on anti-aging focus on collagen synthesis, but few literatures revealed the molecular mechanism of oligopeptides on skin aging. The potential anti-ageing effect and the mechanism of PTC, a new palmitoyl oligopeptide complex, were evaluated. MTT assay was adopted to detect the anti-aging effect of PTC in both human dermal fibroblast HDF-a and keratinocyte NHEK. The mechanism of PTC was detected further by full transcriptome analysis using RNA-sequencing method, and ELISA analysis of key proteins. Skin wrinkles were measured by VC98 *in vivo*. PTC showed positive effect in cell proliferation and the biosynthesis of collagens, intergrins not only on fibroblasts, but also on keratinocytes. Moreover, many pathways were confirmed in the anti-aging effect of PTC, such as TGF- β 1 pathway and ECM-receptor interaction. A cream contained 5.0% PTC showed significant improvement of skin wrinkles and roughness. So that, PTC has a potentially beneficial effect on the cell proliferation, the biosynthesis of ECM related proteins and the expression of many genes which are involved in cell cycle and secretory features to slow down skin aging. The molecular pathway maybe targets the TGF- β 1 in both epidermis and dermis.

Keywords: Anti-wrinkle, A Palmitoyl Oligopeptide Complex, Transcriptome, TGF- β 1

1. Introduction

Skin is a multifunctional organ but, alongside every other organ system, is subject to both intrinsic (chronological) and extrinsic (environmental) aging. Skin function is mediated primarily by the structure of the epidermal and dermal layers. The epidermis as well as the dermis is becoming thinner and the dermal epidermal junction flattens in skin aging [1]. Many researchers revealed that dermal fibroblast which mainly synthesis the collagen and elastin contribute to maintaining the skin's elasticity and inhibit wrinkling of skin *in vitro* and *in vivo* [2-4], and many ingredient such as plants and peptides were improved have functions on maintaining skin elasticity, strengthening joints and retaining moisture [5, 6], especially the collagen and elastin. Due to the large size

of the collagen molecule unable to absorb, oligopeptides were been widely used recently, especially the palmitoyl oligopeptides, including tripeptide to six peptide.

Oligopeptides were verified by many reports on anti-aging focus on collagen synthesis [5-7]. During aging, the dermis undergoes progressive fragmentation that impairs fibroblast attachment with consequent reduction of size. In aged skin, reduced size of fibroblasts is accompanied by their decreased production of key extracellular matrix (ECM) components, such as type I collagen, fibronectin, connective tissue growth factor (CTGF) and transform growth factor (TGF- β) [8, 9]. Interestingly, many of the dermal ECM-related genes that are downregulated in aged human skin are regulated by the

TGF- β pathway [9]. And many other pathways are also involved in skin aging, such as IGF-FOXO pathway, MAPK pathway, rapamycin (mTOR) pathway [10-12]. There are few literatures revealing the molecular mechanism of oligopeptides on skin aging.

We combined two high throughputs screening methods focus on cell proliferation and type collagen I synthesis which is the common factors related to aging, to validate lots of active ingredients from different plants and peptides, and finally got a palmitoyl oligopeptide complex PTC, which consists of hydrolyzed collagen and oligopeptide. Then the anti-aging effect of PTC was identified in both fibroblasts and keratinocytes, and finally verified in human skin. The mechanism of PTC against skin aging was confirmed by transcriptome (RNA-seq) in human fibroblasts, and ELISA analysis confirmed the expressions of key proteins involved in TGF- β 1 pathways.

2. Materials and Methods

2.1. PTC Preparation

The palmitoyl oligopeptides (ZPC peptide, China) were mixed with different proportions and dissolved in 1, 2-hexanediol (CAS No. 6920-22-5), followed by hydrolyzed collagen added into the liquid. Finally, PTC solution was obtained through filtration.

2.2. Cell Proliferation Experiments

Human dermal fibroblast from adult (HDF-a) and normal human epidermal keratinocytes (NHEK) were used to detect the proliferation effect according to literatures [13, 14]. Briefly, Cells were seeded in the 96-well plate at a density of 5000 cells/well. When cultured overnight in FM or DMEM media with 10% FBS, the cells were continually incubated with various concentrations of PTC (0.004-1%) in the medium without FBS for 24 to 72 hrs respectively. Cells treated with vehicle served as a control. The cell proliferation rate was estimated by MTT (Sigma, USA) assay at 492 nm after incubation using the Multiscan FC microplate reader (Thermo Fisher Scientific, USA). From the values obtained, the percentage proliferation was calculated as in

$$\text{Viability (\%)} = \text{OD}_{492_{\text{treatment}}} / \text{OD}_{492_{\text{control}}} \times 100\% \quad (1)$$

2.3. Transcriptome Sequencing

Twenty-four hours after the PTC treated (0.5% and 1% concentration) in FM medium without FBS, the purified total RNA of the human fibroblast was obtained with Trizol (Thermo Fisher Scientific). The RNA (400 ng) was converted to the cDNA library by a TruSeq Stranded LT mRNA kit (Illumina, San Diego, CA, USA) [15-17]. Specific adapters for Illumina universal and qPCR were used to quantify the cDNA libraries. One unique identified sequence was added for sample separation after sequencing each library, thus allowing sequencing of all samples during the same run and minimizing technical variations. Libraries were sequenced on a HiSeq

2500 platform (Illumina) in the High Output mode.

2.4. Detection of Collagens

The synthesis of type I and III collagen were detected on HDF-a cells by immunofluorescent assay [18, 19]. HDF-a cells were treated with PTC (0.004-1%) similar with proliferation assay. For visualization of type I and III collagens, PTC treated HDF-a cells were firstly fixed with cold methanol (CAS No. 67-56-1; Sinopharm, China) at 4°C for 10 min, and then 0.5% triton X-100 (Sigma, USA) was incubated with cells for 15 min. After washing, cells were blocked with 3% BSA for 1.5 hrs at room temperature. Finally cells were stained with anti-collagen I and III antibody (Abcam, CA) at 4°C overnight and anti-rabbit IgG Alexa Fluor (R) 488 antibody (CST, USA) was used to detect the fluorescence. The nuclei were stained with DAPI (Abcam, CA) and the images were captured using fluorescence microscope (Leica, GER).

2.5. ELISA Detection

The DEJ composition proteins such as laminin 5, integrin β 1, fibronectin and four type collagens were detected on NHEK and HDF-a cells by ELISA assay [19-21]. After incubation period, the supernatant and cells were collected and estimated using commercially available ELISA kits (Elabscience, China) according to its manual. The percentage rate of growth was calculated as in

$$\text{Growth rate (\%)} = \text{OD}_{450_{\text{treatment}}} / \text{OD}_{450_{\text{control}}} \times 100\% \quad (2)$$

2.6. Skin Wrinkles Evaluation

The *in vivo* study was performed as a double blinded, randomized and placebo controlled study to follow the internationally recognized ethical principles. A clinical study of 30 subjects (the average age was 41.5) was performed to evaluate the effects of PTC on facial wrinkles and roughness [22, 23]. During 8 weeks treatment, participants applied 2 mg/cm² test formula (contained 5% PTC) and vehicle around the left and right eye every morning and evening after cleansing. Photo were taken and analyzed by visioscan VC98 (CK company, GER) using SELS multi parameter analysis at the following time points: baseline (T0), after 2, 4, 6 and 8 weeks of use (Tw). The reduction of skin wrinkle and roughness was calculated as in

$$\text{Reduction (\%)} = (\text{SELS}_{\text{Tw}} / \text{SELS}_{\text{T0}})_{\text{test formula}} / (\text{SELS}_{\text{Tw}} / \text{SELS}_{\text{T0}})_{\text{vehicle}} \times 100\% \quad (3)$$

2.7. Statistical Analysis

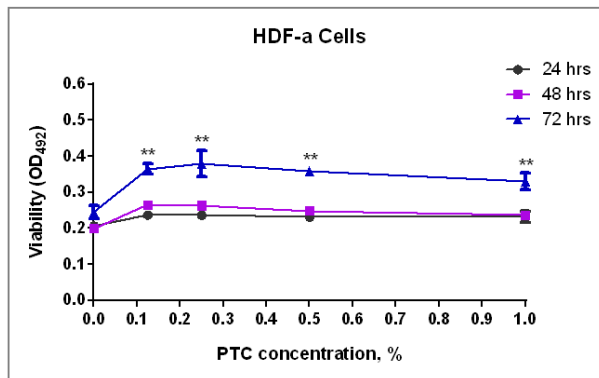
All experiments were repeated at least three times with different batches of cells. All data are presented as mean \pm S.D. with GraphPad Prism. Data were evaluated statistically using Student's t-test, and the statistical significance was set at $p < 0.05$. For transcriptome analysis, statistical significance was set at $p < 0.05$ and fold change ≥ 1.5 .

3. Results and Discussion

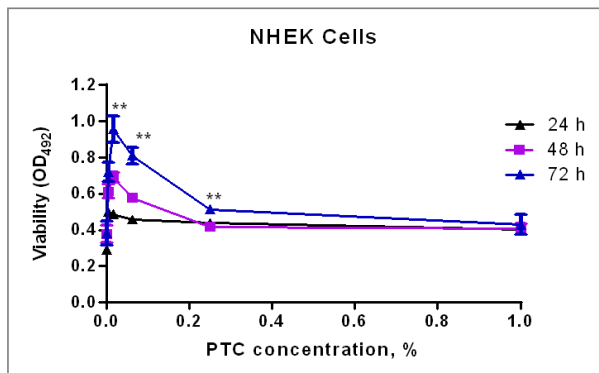
3.1. PTC Improves the Proliferation of HDF-a Fibroblasts and NHEK Keratinocytes

Cellular senescence plays a vital role in regulating cellular aging both in vitro and in vivo. The aging cells are identified with a distinct phenotype, which includes flat morphology, bulged cell size, slowed proliferation rate and changes in protein and gene level [24, 25].

MTT results showed that the highest cell viability rate were 155.3% after 0.25% of PTC treatment for 72 hrs on HDF-a cells (Figure 1a), and for 24 and 48 hrs PTC at 0.125% to 0.5% concentrations still significantly improved the cell viability. On NHEK cells, the cell viability was maximum induced to 239.4% after 0.016% of PTC treatment for 72 hrs (Figure 1b). It verified the proliferation efficacy of PTC in epidermal keratinocytes and dermal fibroblasts.



(a)



(b)

Figure 1. Proliferation effect of PTC on and fibroblast (a) and keratinocyte (b) cells.

3.2. PTC Affected Lots of Genes Involved in Cell Senescence

The gene expression changes with age in various human tissues including skin [26]. Previous studies reported that during replicative senescence, dermal fibroblasts presented a deregulation of genes involved in various skin cellular processes such as inflammatory response, cell cycle regulation and signal transduction [27-29].

Focusing on the key genes related to cell cycle, secretory features and macromolecular damage [30-32], we identified the anti-aging mechanism regulated by PTC. Total 935 genes were changed after 0.5% PTC treatment for 24 hrs, 439 upregulated and 496 downregulated. First significant variations in cell-cycle arrest were identified in table 1, such as cyclins, cyclin dependent kinases (CDKs) and CDK inhibitors (CDKN1A and CDKN2A). The proteins encoded by these genes are classified as the key protein required for cell cycle progression in G0/G1 to S phase.

Table 1. Modulated genes in cell-cycle arrest after PTC treatment.

Gene symbol	Description	Fold change
CCNA2	Cyclin A2	1.24
CCNB1	Cyclin B1	1.55
CCND1	Cyclin D1	1.68
CDK2	Cyclin dependent kinase 2	1.21
CDK4	Cyclin dependent kinase 4	1.51
CDKN1A	Cyclin-dependent kinase inhibitor 1A (p21, Cip1)	0.66
CDKN2A	Cyclin-dependent kinase inhibitor 2A	0.89
RB1	Retinoblastoma 1	0.82
TP53	Tumor protein p53	0.63

Furthermore, PTC can improve the expression of key senescence-associated insoluble factors in figure 2, such as collagens (COL5A3, COL6A1, COL7A1), elastin (ELN), mucoopolysaccharide (BGN, CD44), laminins (LAMA1, LAMC5), integrins (ITGA2, ITGB1), epiregulin (EREG), intercellular adhesion molecule 5 (ICAM5), fibrillin 1 (FBN1), fibronectin (FN), procollagen-lysine (PLOD1) and tenascin C (TNC) to strengthen cell function and adhesion [33-36]. All the proteins encoded by these genes are downregulated in senescent cells to strengthen skin aging.

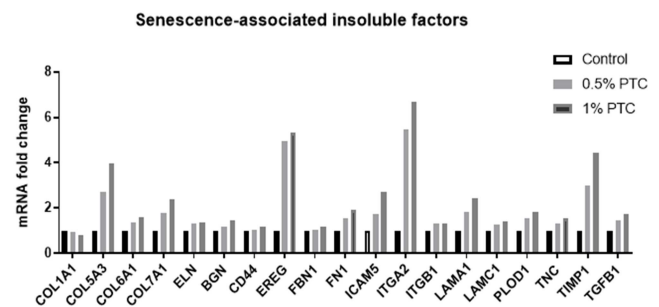


Figure 2. The mRNA expression changes of secretory features in fibroblast treated with PTC.

3.3. PTC Induced the Synthesis of ECM Proteins

Dermal fibroblasts are thought to be responsible for synthesising the three major groups of dermal ECM proteins including fibrillar collagens, elastic fibres and proteoglycans [2, 9]. According to the gene expression results, the content of ECM proteins was analyzed further.

As shown in figure 3, PTC treated cells synthesized more amount of type I collagen and type III collagen than untreated control cells after 48 hrs.

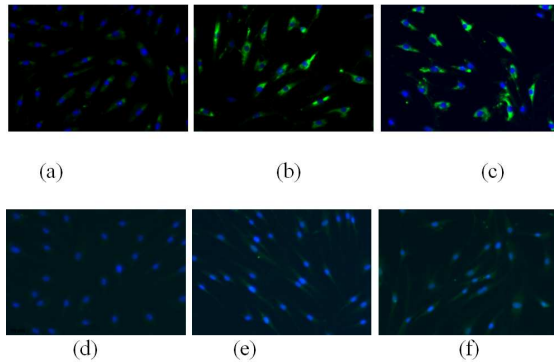


Figure 3. PTC increases the expression of collagens on HDF-a (100X). (a-c): type I collagen, control w/o FBS, PTC 0.25%, PTC 0.5%; (d-f): type III collagen, control w/o FBS, PTC 0.25%, PTC 0.5%.

We also tested the extracellular content of other ECM proteins for 48 hrs on HDF-a and NHEK cells treated with PTC (0.25% to 1%). As listed in table 2, when cells were treated with 0.25% PTC for 48 hrs, the extracellular content

of type I collagen was increased from 225.39 to 327.39 ng/ml and type III collagen increased from 0.66 to 1.31 ng/ml with a maximum growth rate of 145.2 and 198.5% respectively. The content of fibronectin was also increased from 400.31 to 740.20 ng/ml. Focusing on the ECM proteins secreted by keratinocytes, the extracellular amount of type IV and type VII collagen was regulated from 0.27 to 1.06 ng/ml and from 3.70 to 9.81 ng/ml by 1% PTC treated for 48 hrs, and the maximum rate of growth of integrin $\beta 1$ was moderately upregulated to 176.6% compared to untreated cells, while laminin 5 was not changed with data not shown.

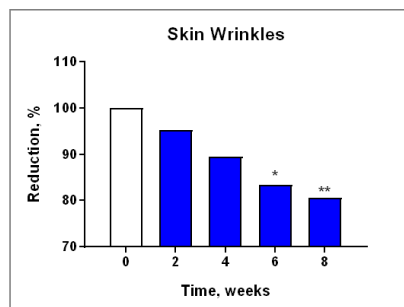
For the TGF- $\beta 1$, a cytokine that contributes primarily to the biosynthesis of collagen associated with cutaneous aging [37], was upregulated in both HDF-a cells and NHEK cells after PTC treatment for 48 hrs. The growth rate reached to 331.4% and 175.6% respectively. These results confirmed the fold change of gene expression which related to the pathway and cell functions in skin aging, from dermis to epidermis, especially the dermal-epidermal junction.

Table 2. The expression of ECM proteins after PTC treatment.

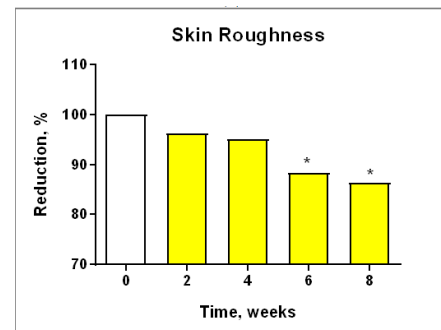
Protein name, ng/ml	Cells	Control	PTC 0.25%	PTC 0.5%	PTC 1.0%
Type I collagen	HDF-a	225.39	327.39	304.19	297.06
Type III collagen	HDF-a	0.66	1.31	1.22	1.21
Fibronectin	HDF-a	400.31	740.20	668.82	725.57
Type IV collagen	NHEK	0.27	0.54	0.62	1.06
Type VII collagen	NHEK	3.70	6.26	6.51	9.81
Integrin $\beta 1$	NHEK	8.86	8.97	9.70	15.65
TGF- $\beta 1$	HDF-a	0.035	0.136	0.152	0.116
TGF- $\beta 1$	NHEK	0.307	0.403	0.497	0.539

3.4. PTC Reduced the Wrinkles and Roughness in Human Skin

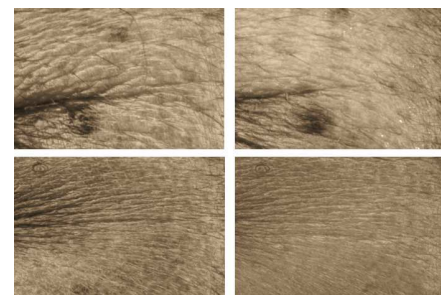
As a person ages, proliferation of cells in the basal layer reduces. The epidermis then becomes thinner, the contact surface area between dermis and epidermis decreases, and dermal epidermal junction flattening, resulting in skin wrinkles and roughness [38]. Furthermore, the clinical improvement of PTC (left side) was compared to the placebo (right side) on human skin around eyes (Figure 4). When applied with formulas contained 5% PTC for 8 weeks, the surface evaluation of wrinkles (SEw) and surface evaluation of roughness (SEsm) was reduced from 100% to 80.6% and 86.3% respectively. Macrophotography of the left side of the face applied of PTC for 6 weeks also showed a reduction in length and width of wrinkles compared to placebo.



(a)



(b)



(c)

Figure 4. PTC improved the skin wrinkles and roughness. (a-b) The reduction of skin wrinkles and roughness. (c) PTC before and after use of two subjects (above: Subject #10, 47 old-years; below: Subject #15, 32 old-years).

4. Conclusions

Cellular senescence is a cell state implicated in various physiological processes and a wide spectrum of age-related diseases. Dermis thickness decreases with age; thinning is accompanied by a decrease in both vascularity and cellularity. There is a decrease in the number of fibroblasts, the amount of glycosaminoglycans, collagens and elastin, as well as a loss of molecular integrity and tight junction [39]. PTC, a palmitoyl oligopeptide complex, can stimulate the proliferation and strengthen the synthesis of ECM proteins in dermal fibroblast, to against cell senescence.

In senescent cells, the CDK2 inhibitor CDKN1A, 1C and CDK4/6 inhibitor CDKN2A were accumulated to result in activation of retinoblastoma (RB) and p53 family proteins, inhibition of E2F transactivation, and consequent cell-cycle arrest [32]. PTC prevented the cell-cycle arrest by upregulating the mRNA expression of CCNA2, CCNB1, CCND1, CDK2, CDK4, and downregulating the mRNA expression of CDKN1A, 1C, 2A, RB family members and p53 family members, to promote cell proliferation.

For the other certain physiological processes related to cellular senescence, secretory features, PTC not only affected the mRNA level of key genes, such as COL5A3, COL6A1, COL7A1, ELN, BGN, CD44, LAMA1, LAMC5, ITGA2, ITGB1, EREG, ICAM5, FBN1, FN, TNC and TIMP1, but also regulated the protein synthesis, including four type collagens, fibronectin, integrin β 1 as well.

Transforming growth factor- β 1 (TGF β 1) acted as an important modulator of cell growth, inflammation, collagen synthesis and apoptosis. Many of the dermal ECM-related genes that are downregulated in aged human skin are regulated by the TGF- β pathway [40-42]. Focusing on the TGF- β 1 pathway, PTC not only enhanced the TGF- β 1 expression to promote the synthesis of collagens, elastin, fibronectin, and integrins in fibroblasts, but also promoted the synthesis of TGF- β 1 in keratinocytes to act as an inducer of cell renewal. The molecular pathway maybe targets the TGF- β 1 in both epidermis and dermis.

Further, PTC certified its anti-aging effect on the clinical study targeting facial wrinkles and roughness for 8 weeks. The surface evaluation of wrinkles (SEw) and surface evaluation of roughness (SEsm) was significantly reduced at week 6 compared to placebo. All data suggest PTC had a 3D theory against skin aging: from epidermal keratinocyte to dermal fibroblast, especially containing the structural proteins of DEJ.

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