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The comparison of the rejuvenation effects on the skin of Wistar rats between 10600 nm CO_2 fractional laser and retinoic acid

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Abstract. – OBJECTIVE: The fractional laser and topical retinoic acid treatment have been applied for skin rejuvenation; however, the possible molecular mechanism of promoting remodeling of dermis is not clearly. Here we aimed to compare the effects of 10600 nm CO_2 fractional laser and topical retinoic acid formulation on the skin collagen proliferation of Wistar rats, and to further explore the possible molecular mechanism of promoting remodeling of dermis.

MATERIALS AND METHODS: The hair on the back of Wistar rats was removed, and the back was divided equally into four regions with the cross-streaking method: A (the control group), B (the retinoic acid group), C (retinoic acid and fractional laser combination treatment group), and D (the fractional laser group). Specimens were collected at 3rd day and in 1-8 weeks after CO_2 fractional laser irradiation; then they were used for detection of the changes of dermis thickness and content of hydroxyproline in the four regions of the rats' back. Real-time PCR method was used to detect the dynamic changes of the expression level of type III procollagen mRNA and the expression levels of miR-29a, Akt and transforming growth factor-β (TGF-β) mRNA at 3rd week in the skin tissue of Wistar rats.

RESULTS: The thickness of dermis, content of hydroxyproline and expression level of type III procollagen mRNA in the treatment groups (B, C, and D) were found all significantly increased compared with those in the control group (A) ($p<0.05$); at 3rd week, up-regulation of Akt and TGF-β mRNA expression and down-regulation of miR-29a mRNA expression were observed in the treatment groups (B, C, and D). The difference in the combination treatment group (C) was the most significant ($p<0.05$).

CONCLUSIONS: These results demonstrate that retinoic acid formulation and CO_2 fractional laser both can promote collagen proliferation and reconstruction, with the skin rejuvenation efficacy in group C > group D > group B. miR-29a/Akt/TGF-β signal pathways may play a certain role in the promotion of collagen synthesis and proliferation.

Key Words:
CO_2, Fractional laser, Retinoic acid, miR-29a, Akt, Transforming growth factor-β.

Introduction

Skin photoaging not only influences the appearance, but also induce various kinds of skin diseases and even tumors[1,2]. Therefore, prevention and cure of photoaging have become a hotspot of research in dermatology. 0.05% of all-trans-retinoic acid is currently the only externally applied drug for photoaging approved by FDA; fractional laser is also a therapeutic method for photoaging, which is widely used in recent years. Prior studies[3-6] observed demonstrated that both retinoic acid and fractional laser have effects on skin rejuvenation, including recovering skin elasticity, decreasing wrinkles, and improving pigmentation. However, there have been few comparative studies on the skin rejuvenation efficacy and studies on the combination of the two. MiR-29a is a newly found MicroRNA closely correlated with tumors and various kinds of diseases, and it is involved in the biological processes in human body, including cell metabolism, differentiation, apoptosis and so on. Studies in recent years have found that miR-29a is closely correlated with fibroblasts, and it participates in regulation of fibrosis of organs like heart, lung, and liver. Maurer et al[7] demonstrated that in patients with systemic sclerosis and animal models of systemic sclerosis, expression of miR-29a significantly decreased, and inhibition of miR-29a or over-expression of miR-29a could up-regulate or down-regulate the levels of type I collagen and type III collagen mRNA and proteins. Studies[8] have found that miR-29a has negative regulatory effects on the PI3K/Akt signal pathway through inhibiting the post-transcrip-

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tional translation of Akt1, Akt2, and Akt3 in the pathway. Transforming growth factor β (TGF-β) is an important positive growth factor of dermis; it can stimulate transportation of amino acids and glucose in fibroblasts, promote glycolysis, induce synthesis of collagen proteins, fibronectin and extracellular matrix components, and regulate the growth and differentiation of fibroblasts. Therefore, it has an important modulation effect in wound healing and tissue fibrosis. Studies[9-15] demonstrated that PI3K/Akt signal pathway and TGF-β co-regulated the synthesis of collagen in fibroblasts through synergistic effects at multiple levels. It can be presumed that miR-29a is a switch at the upstream of PI3K/Akt signal pathway, and can inhibit the post-transcriptional translation of various genes of the signal pathway, and play the role of down-regulation. Akt and TGF-β signal pathways interact with each other in multiple links, and both participate in processes, including fibroblast proliferation, and synthesis and secretion of collagen. To explore therapeutic effects and possible mechanism of combination of fractional laser and retinoic acid, we investigated ed the rejuvenation effects on the skin of Wistar rats to provide theoretical basis for further studies on the molecular mechanism of skin rejuvenation effects of retinoic acid and fractional laser.

Materials and Methods

Laboratory Animals and Grouping

Forty-five clean female Wistar rats, with a body mass of 250-300 g provided by the Laboratory Animal Center of Shandong University (batch number: SCXK20140102). Laboratory animals were fed with routine diet and water in the same environment with natural illumination. 45 rats were randomized into 9 groups according to the time points of specimen collecting. The experiment was started after 1 week of acclimatization. The present study was approved by the Ethics Committee of the Shandong University.

Main Laboratory Apparatuses and Consumables

10600 nm CO_2 fractional laser was bought from Lumenis Inc. (San Jose, CA, USA); Real-time PCR instrument was LightCycler® 480 Real-time PCR System from Roche, (Basel, Switzerland); the 8-tube strips used for Real-time PCR were bought from Roche (Basel, Switzerland); NanoDrop 2000 trace nucleic acid quantitation instrument was bought from Thermo Fisher Scientific Inc (Waltham, MA, USA). RM2265 paraffin section microtome was bought from Leica (Wetzlar, Germany). 0.05% all-trans-retinoic acid cream was prepared by the preparation laboratory of Qilu Hospital of Shandong University (Jinan, Shandong, China). PrimeScript® RT reagent kit perfect Real-time for reverse transcription, PCR kit SYBR Premix Ex Taq (Perfect Real-time) for fluorescent quantitation and Trizol total RNA isolation kit were all bought from TaKaRa (Otsu, Shiga, Japan); hydroxyproline assay kit was bought from NanJing Jiancheng Biotechnology Company (Nanjing, Jiangsu, China). All the other reagents were bought from Shanghai Reagent Factory of China National Pharmaceutical Group (Shanghai, China).

Preparation Before the Experiment

The hair on the back of rats was removed with 8% sodium sulfide solution, with the depilation area of 10×6 cm^2. The area was divided equally into four regions through cross-streaking with saturated picric acid solution. The region near the head on the left side was taken as the normal control group (region A), the region near the tail on the left side was taken as the retinoic acid group (region B), the region near the tail on the right side was taken as the combination treatment group (region C), and the region near the head on the right side was taken as the fractional laser group (region D). The experiment started from 24 h after depilation.

Externally Applied Drug and Laser Radiation Method

From the first day of the experiment, region B and region C were applied with 0.05% retinoic acid cream for continuous 3 weeks. Region C and region D were irradiated with 10600 nm CO_2 fractional laser once on day 1, with parameters of the laser setting as:micro pulse energy, 15 mJ; density, 5%; frequency, 300 Hz; with a figure of square, 10 mm × 10 mm.

Histopathological Observation

3 days, 1 week, 2 weeks, 3 weeks, 4 weeks, 5 weeks, 6 weeks, 7 weeks, and 8 weeks after irradiation, the Wistar rats were sacrificed in batch. The skin specimens were collected from the regions, embedded and fixed with 10% paraffin to prepare to paraffin blocks, made into a series of continuous section slides with a thickness of 5 μm, and stained with HE method. The specimens

stained were observed under a microscope, and the thickness of dermis was measured with a micrometer.

Elisa Test

The skin was stripped from the back regions of the rats sacrificed at different time points, adipose tissue was removed as far as possible, 100 mg skin was weighed accurately from each region, and prepared to 2% skin homogenate with physiological saline, and the content of hydroxyproline in the specimens was determined with hydroxyproline kits, with the operating procedures done strictly according to the instruction manual of the kit.

Real-Time PCR Detection

The back specimens of rats sacrificed at different time points were immediately put into liquid nitrogen and ground into powder, to which Trizol reagent was added to extract total RNA, and the concentration and purity of RNA were determined with the nucleic acid quantitation instrument. Total RNA was reverse transcripted into cDNA according to the procedures in the instruction manual of reverse transcription kit, and the expression levels of type III precollagen, miR-29a, Akt, and TGF-β mRNA were detected with Real-time PCR method. Primer sequences were shown in Table I, and reaction conditions were as follows: 1- heat activation: 95°C 5 min, 1 Cycle; 2- amplification: 95°C, 10 s; 60°C, 20 s (to collect fluorescence signal), 40 Cycles; 3- melting curve analysis: 95°C, 15 s; 65°C, 15 s; fluorescence signals were continuously monitored during the process of the temperature rising to 95°C. Each sample was tested in triplicate. $^{\Delta}$Ct value of a corresponding index in a sample was obtained through minus the Ct value of GAPDH from the detection index Ct (cycle threshold) value of the same sample obtained with ABI7000 software. $^{\Delta}$Ct value of the control group was used for correction, and $2^{-\Delta\Delta CT}$ value was used to represent gene expression level.

Statistical Analysis

All data is analyzed with SPSS17.0 statistical software (SPSS Inc., Chicago, IL, USA), variable data of each group is expressed as mean±standard deviation. Differences between the mean values for individual groups were assessed using a one-way analysis of variance (ANOVA). Multiple comparison between the groups was performed using SNK method. $p<0.05$ is considered as having statistical significance.

Results

Histological Changes of Wistar Rats' Skin

At week 3, the thickness of dermis in the treatment groups (B, C, and D) significantly increased, the collagen fibers were of more compact alignment and trachychromatic, there was *de novo* collagen with sparse alignment, which was parallel to the epidermis, and *de novo* subsidiary glandular epithelium increased to different extents. The changes were most evident in the combination treatment group (C).

Determination of the Thickness of Dermis

On day 3 of the experiment, the thickness of dermis significantly increased in the treatment groups (B, C, and D), which was considered as caused by edema between collagen bundles; at week 1, the edema of dermis regressed, thus the thickness of dermis in each group returned to normal. At week 2, the thickness of dermis in the combination treatment group (C) increased the most significantly ($p<0.05$); thereafter, the thickness of dermis gradually increased in the treat-

Table I. Primer sequences of type III precollagen, Akt, and TGF-β.

Primer sequences		
Upstream	5'-TGGCACAGCAGTCCAACGTA -3'	Type III precollagen
Downstream	5'-AAGGACAGATCCTGAGTCACAGACA -3'	
Upstream	5'-TCCTGCACCTGGAGCTCTGTTA-3'	Akt
Downstream	5'-CTCAGGGCAGCAGGACATGTAG-3'	
Upstream	5'-GGTGGACCGCAACAACGTGAGCACTGAAGCGAAAGC-3'	TGF-β
Downstream	5'CGTGCGTGACATTAAAGAGTTGCCGATAGTGATGACCT-3'	
Upstream	5'-AAATGGTGAAGGTCGGTGTGAAC-3'	GAPDH
Downstream	5'-CAACAATCTCCACTTTGCCACTG-3'	

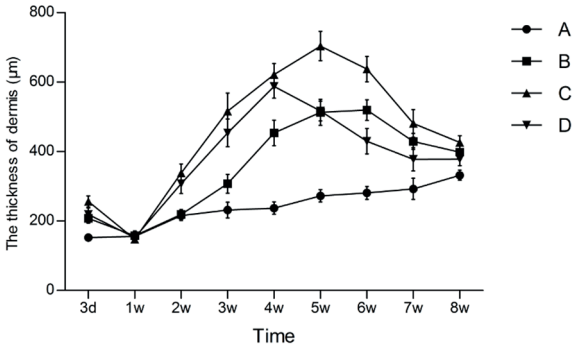

Figure 1. The effects of retinoic acid, fractional laser and the combination of the two on the thickness of dermis.

ment groups (B, C, and D), and it reached peak (519.45±29.63) at week 6 in the retinoic acid group (B), reached peak (703.71±42.35) at week 5 in the combination treatment group (C) and reached peak (587.71±34.37) at week 4 in the fractional laser group (D). Compared with the retinoic acid group (B), the thickness of dermis in the fractional laser group (D) increased more rapidly during the weeks 2-4 (p<0.05) (Figure 1).

Hydroxyproline Assay

On day 3, the contents of hydroxyproline in Wistar rats' skin in the treatment groups (B, C, and D) were lower than that in the normal group, and the decrease in the combination treatment group (C) was the most significant, which was considered due to the more obvious collagen edema caused by laser thermal injury and retinoic acid stimulation of the rats' skin in the combination treatment group (C). 1 week later, the contents of hydroxyproline gradually increased in the treatment groups (B, C, and D), with the slowest increase in the retinoic acid group (B), which reached peak at week 6; the contents of hydroxyproline in the combination treatment group (C) and the fractional laser group (D) reached peak at week 5. After week 5, the down regulation speed of the content of hydroxyproline in the combination treatment group (C) was significantly slower than that in the fractional laser group (D) (p<0. 05). During 1-4 weeks, compared with the retinoic acid group (B), the increase speed of the content of hydroxyproline in the fractional laser group (D) was significantly faster (p<0. 05). At week 8, the contents of hydroxyproline in the treatment groups (B, C, and D) significantly decreased compared with peak values. However, they were still higher than that in the normal control group (A) (Figure 2).

Expression of type III procollagen mRNA

At week 1, the expression of type III procollagen mRNA increased compared with before in the treatment groups (B, C, and D); at week 2, the expression of type III procollagen mRNA in the combination treatment group (C) and the fractional laser group (D) was significantly up-regulated (p<0.05); the increase in the combination treatment group (C) was the most significant, and it reached peak first at week 3; then, the expression in the fractional laser group (D) reached peak at week 4; the expression in the retinoic acid group (B) was slowly up-regulated and it reached peak at week 5 (Figure 3).

The Expression Levels of miR-29a, Akt, and TGF-β mRNA in Wistar Rats' Tissues at Week 3

The results of Real-time PCR assay demonstrated that at week 3, the expression levels of Akt and TGF-β mRNA in the Wistar rats' skin tissues in the treatment groups (B, C, and D) were higher than those in the normal control group (A), and the expression levels of miR-29a mRNA were significantly lower than that in the control group. The difference in the combination treatment group (C) was the most significant (Figures 4-6).

Discussion

Photoaging may lead to damages of cells and connective tissue[16] and decrease of collagen fiber in dermis. Therefore, no matter what method is used, the treatment objective of photoaging is to promote the emergence of new collagen fibers through certain stimulation to the dermis tissues

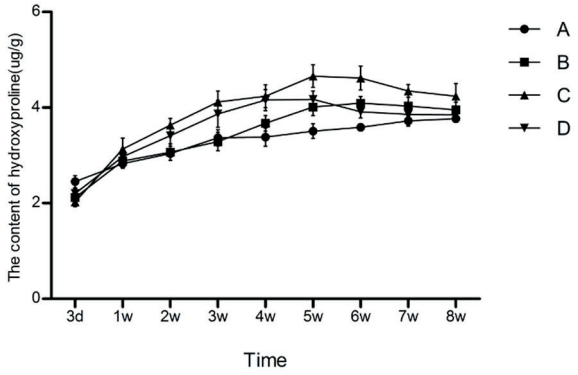

Figure 2. The effects of the retinoic acid, fractional laser and the combination of the two on the content of hydroxyproline.

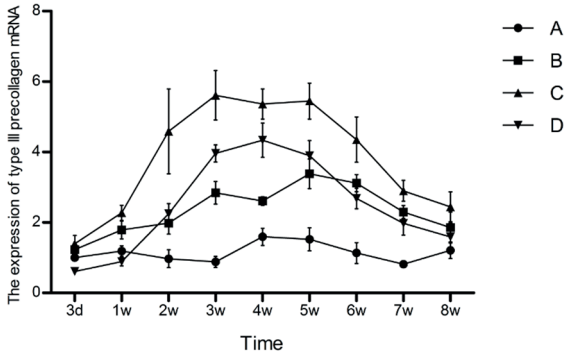

Figure 3. The effects of retinoic acid, fractional laser and the combination of the two on the expression of type III procollagen mRNA.

to recover the normal tissue structure of the skin, and increase the thickness of dermis. Many studies[4] in the past demonstrated that retinoic acid has the effects of prevention and cure on photoaging, and it can even histologically reverse the atrophy of epidermis and subcutaneous tissue to a certain extent and promote dermis reconstruction. Fractional laser, which comes into being in 2003, is a novel technique based on focal photothermal principle[17]. The minor light beam array emitted by the fractional laser only acts on a small part of skin, and forms columnar microscopic thermal zones (MTZs) in the skin[18]. There is a certain amount of residual normal tissues around each MTZ, and these normal tissues insert between thermal zones in micro-matrixes. Because the distance of the keratinocytes in adjacent normal tissues crawling to MTZ is shortened, the healing of the wound surface is faster than that caused by the traditional laser[19-22]. Especially for ablative fractional laser, because the waveband of ablative fractional laser is absorbed better by water, and the penetration is deeper, it has advantages in clinical improvement of photoaging[20,23]. In this study, the dermis thickness of rats' skin in the treatment groups increased significantly at week 2. According to the study

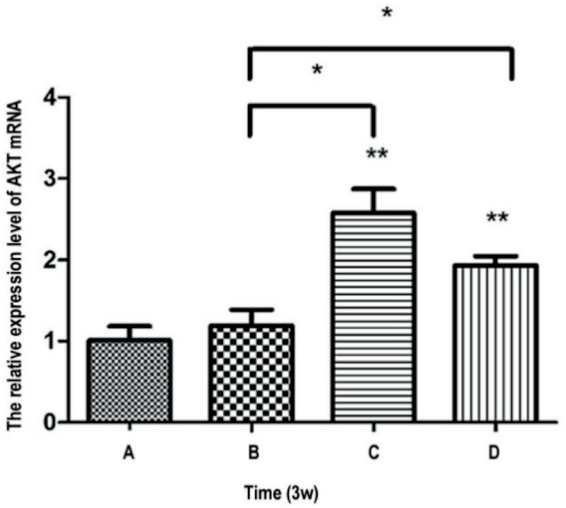

Figure 5. The expression levels of AKT mRNA in the skin tissues of Wistar rats at week 3. **, $p<0.05$, the treatment groups (B, C, D) *vs*. the normal control group (A); *, $p<0.05$, the combination treatment group (C) and the fractional laser group (D) *vs*. the retinoic acid group (B).

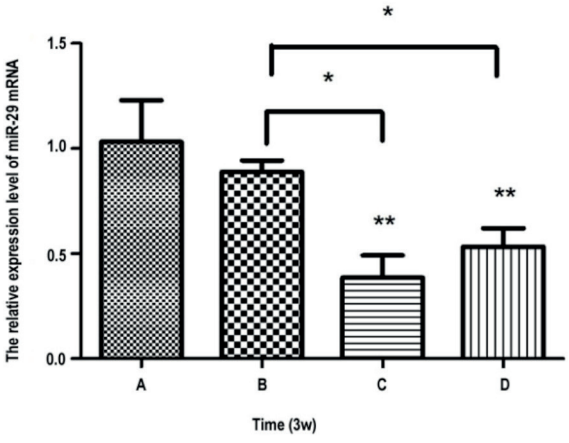

Figure 4. The expression levels of miR-29 mRNA in the skin tissues of Wistar rats at Week 3. **, $p<0.05$, the treatment groups (B, C, D) *vs*. the normal control group (A); *, $p<0.05$, the combination treatment group (C) and the fractional laser group (D) *vs*. the retinoic acid group (B).

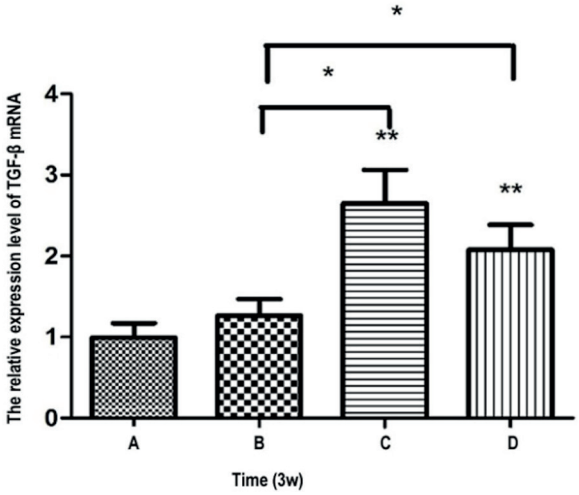

Figure 6. The expression levels of TGF-β mRNA in the skin tissues of Wistar rats at week 3. **, $p<0.05$, the treatment groups (B, C, D) *vs*. the normal control group (A); *, $p<0.05$, the combination treatment group (C) and the fractional laser group (D) *vs*. the retinoic acid group (B).

of Dahiya et al[24], because collagen fiber is an essential component of skin dermis, the increase of dermis thickness is mainly through deposition and rearrangement of collagen fibers. Collagen protein is the tissue basis for intensity and elasticity of skin; hydroxyproline is a distinctive aminoacid of collagen protein, and 7.46 times of the content of hydroxyproline is usually used to represent the quantity of collagen protein[25]. Collagen in dermis is mainly composed of type I collagen (80%) and a small quantity of type III collagen (10%). Procollagen from fibroblasts are the precursor molecules of mature collagen, and their levels may reflect the biosynthesis activity of collagen. In studies, it has been found that the levels of type I and III procollagen decrease in photoaging skin[26]. The results of this work suggest that retinoic acid and fractional laser can both promote the transcription of type III procollagen mRNA in the skin, thus to further promote synthesis of *de novo* collagen. At week 8, the expression levels of type III procollagen mRNA in groups B, C, and D were still higher than that in the normal control group, suggesting that fractional laser and retinoic acid were both safe and effective and had persistent effects in the treatment of photoaging skin. As an important cytokine regulating the transformation and proliferation of fibroblasts, TGF-β can promote proliferation of dermis collagen and improve photoaging. According to prior researches, it is considered that miR-29a, Akt, and TGF-β can interact and together form a regulation network to maintain the activity of fibroblasts, and may become new targets in the prevention and cure processes of photoaging diseases. The results of this report showed that at week 3, the expression levels of Akt and TGF-β mRNA in the treatment groups were up-regulated, and the expression level of miR-29a mRNA was down-regulated. The findings suggest that the application of retinoic acid and fractional laser can decrease the expression of miR-29a, thus the inhibition of downstream AKT activation is lost, AKT activation increases, the expression of TGF-β is induced, the proliferation of fibroblasts is stimulated in turn, and the synthesis of collagen protein in the skin is promoted. Therefore, miR-29a/Akt/TGF-β signal pathways are possibly involved in the mechanisms of skin rejuvenation by retinoic acid and fractional laser.

Conclusions

Retinoic acid and CO_2 fractional laser can both promote proliferation and recombination of dermis collagen, and improve photoaging. According to the changes of the thickness of dermis, the content of hydroxyproline in the skin and the expression level of type III procollagen mRNA determined in this experiment, the comprehensive effect of the combination treatment group is the best, and the effect in the CO_2 fractional laser group is superior to that in the retinoic acid group. The changes of the above three indexes consistently reflect proliferation of dermis collagen protein; therefore, CO_2 fractional laser has better effects of skin rejuvenation than retinoic acid. This difference is possibly due to the fact that CO_2 fractional laser has longer wave length and stronger ability of penetration, thus the laser can reach the middle layer of dermis or even deeper, mechanical effect and photothermal effect can have more influence on fibroblasts and blood vessels of dermis, so as to promote recombination and proliferation of collagen protein, and activate self-repair process of tissues. However, although the advantages of CO_2 fractional laser include rapid onset of effects and high peak value, it has the disadvantage of a short effective time. In this experiment, the thickness of dermis, content of hydroxyproline in skin and expression level of type III procollagen mRNA, all decreased quickly after reaching the indexes peak value. The three indexes of the retinoic acid group were up-regulated relatively slow; although the peak value was lower than that of the CO_2 fractional laser group, there was the advantage of persistent efficacy. The combination of CO_2 fractional laser and retinoic acid combines the advantage of a rapid onset of effects and a short time to peak of the fractional laser, and those of retinoic acid including persistent and stable efficacy. Therefore, the skin rejuvenation effects will be enhanced greatly. The indexes tested in this experiment show that there are obvious advantages in treatment of photoaging in the combination treatment group. These results demonstrate that retinoic acid formulation and CO_2 fractional laser both can promote collagen proliferation and reconstruction, with the skin rejuvenation efficacy in group C > group D > group B. miR-29a/Akt/TGF-β signal pathways may play a certain role in the promotion of collagen synthesis and proliferation.

Conflict of interest

The authors declare no conflicts of interest.

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