Effects of autologous platelet-rich plasma injections on facial skin rejuvenation

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Abstract. Autologous serum platelet-rich plasma (PRP) has been used to rejuvenate wrinkled and aged skin for years; however, the molecular mechanism for the positive effects of PRP on the skin remains unclear. The present study aimed to clarify the potential molecular mechanisms for the role of PRP in wrinkled and aged skin rejuvenation, and provide evidence for future clinical applications. A total of 30 healthy females were recruited for PRP treatment and signed informed consent was obtained. A total of 3 autologous PRP injections were administered to each patient with 15-day intervals between injections. The effects of PRP injections were evaluated using the VISIA® Complexion Analysis System and skin computed tomography. A human organotypic skin model was established and treated with PBS or PRP before ultraviolet (UV)-B light (10 mJ/cm^2) irradiation. The distribution of the epidermal structure and dermal fibers were analyzed by hematoxylin and eosin and Masson's trichome staining. Expression of matrix metalloproteinase-1 (MMP-1), tyrosinase, fibrillin and tropoelastin was detected by reverse transcription-quantitative PCR, western blotting and immunofluorescence. The present results showed that PRP treatment improved skin quality in the participants. In addition, the VISIA® results showed that wrinkles, texture and pores were decreased in the PRP groups compared with the PBS treatment. The *in vitro* study demonstrated that PRP treatment ameliorated photoaging by inhibiting UV-B-induced MMP-1 and tyrosinase upregulation, and by inducing fibrillin and tropoelastin expression that was downregulated by UV-B. Collectively, it was demonstrated that PRP treatment ameliorated skin photoaging through regulation of MMP-1, tyrosinase, fibrillin and tropoelastin expression.

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Introduction

Both endogenous and exogenous factors have been demonstrated to cause skin aging. The endogenous factors are largely influenced by a variety of intrinsic genetic and epigenetic alterations, while the exogenous factors are characterized by 'photoaging' of the skin caused by ultraviolet (UV) rays in sunlight (1). Photoaging mainly impacts the conversion of synthetic collagen fibers to synthetic elastic fibers, as well as inflammatory infiltration of the dermis (2). UV-irradiation of skin induces elevated matrix metalloproteinase (MMP) expression, leading to degradation of fibrous connective tissue and reduction of collagen synthesis, and these biological processes are important mechanisms of skin photoaging (3-5). In addition, tyrosinase is a key enzyme that initiates melanogenesis and tyrosinase activity strongly correlates with melanin production (6). All of these effects result in collagen reduction and a decreased rate of epidermal turnover during aging (7).

Platelet-rich plasma (PRP) is a highly concentrated platelet plasma obtained from whole blood. In total, >1100 different proteins have been found in PRP, including immune system messengers, various enzymes and growth factors (8,9). These proteins have been demonstrated to participate in biological processes, such as cellular proliferation and differentiation, matrix remodeling and angiogenesis (8,9). PRP proteins enhance wound healing and tissue regeneration. Among all the proteins in PRP, growth factors are the most important components (10-12). Platelet-derived growth factor, transformation growth factor, insulin-like growth factor, epidermal growth factor, fibroblast growth factor and vascular endothelial growth factor have well established roles in angiogenesis, cell migration, cell proliferation and collagen deposition (8,13-15).

When PRP is implanted into damaged skin tissue, a variety of high-concentration growth factors are activated and a series of skin cell reactions occur. Cellulose, fibronectin and vitronectin from PRP aggregate with the growth factors released by platelets and function locally (8). These proteins can also act as a scaffold for nascent cells and tissues to promote the repair of damaged/aging skin. At the molecular level, PRP injection induces DNA synthesis and promotes the corresponding gene expression (16,17).

The aim of the present study was to evaluate the effects of PRP injections on prevention of UV-B-induced photoaging through

clinical practice and the use of an *in vitro* model. Furthermore, the aim of the present study was to elucidate the molecular mechanisms underlying PRP injections to facilitate the future clinical application of PRP injections as an anti-aging therapy.

Materials and methods

Clinical study design. The present study was conducted at The Inner Mongolia International Mongolian Hospital (Inner Mongolia, China) between July 20 and September 20, 2018. In total, 30 females between the ages of 30 and 50 years were recruited. Informed written consent was obtained from all participants before treatment. The individual patient also provided written informed consent for the publication of the facial images. The present study was approved by The Ethical Committee of Inner Mongolia International Mongolian Hospital. Exclusion criteria included unwilling patients and patients with abnormal renal function, coagulopathy, acquired immune deficiency syndrome, hepatitis B or other infectious diseases.

PRP was injected on the right sides of the faces of the patients, and an equal volume of normal saline was injected on the left side as a negative control. In total, ~1 ml PRP was injected at multiple sites on the right side of each patient's face at a depth of 2.0 mm. Injections were administered 3 times at 15-day intervals. Images using the noninvasive VISIA® Complexion Analysis System (the VISIA® multi-point positioning system; Canfield Scientific) were taken and computer tomography (CT) detection of the injection sites was performed before each injection and 2 weeks after the last injection. The 6th generation VISIA® skin tester from Canfield Scientific was used to detect skin texture. With advanced optical imaging, RBX® and software technology, the VISIA® skin tester automatically performed the quantitative evaluation for skin thickness, pigmentation, pores, wrinkles, skin smoothness, porphyrin, UV spots and brown spots. Using the reflectivity of the facial skin, the shape trajectory of wrinkles or textures can be reconstructed using the software algorithms.

Preparation of PRP. The method used for PRP preparation was as previously described (18). Briefly, whole blood was drawn into an anticoagulant tube and then transferred to a new tube containing 3.2% (w/v) trisodium citrate (9:1 v/v mixture). The blood sample was centrifuged at 110 x g for 15 min at room temperature and the resulting middle yellow PRP layer was centrifuged for another 8 min at 1,400 x g at room temperature to concentrate the platelets. The final concentration of platelets in PRP was $1009.91\pm219.43\times10^9$/l.

Human organotypic skin explant culture. An *in vitro* culture model of organotypic human skin (hOSEC) was established, according to the method by Frade *et al* (19). Excess skin of the breast or abdomen, collected during orthopedic surgery or breast surgery, was trimmed to remove the lower adipose tissue and cut into 1x1 cm² pieces. The skin sample was then cultured in a 6-well plate containing metal mesh and DMEM with 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin and streptomycin for 7 days. These experiments were approved by The Ethical Committee of Inner Mongolia International Mongolian Hospital (reference no. B2018-013). The skin samples were collected at The Inner Mongolia International Mongolian Hospital on July 12, 2018. The patient

agreed to the use of her samples in scientific research and written informed consent was provided.

UVB-induced photoaging and PRP treatment. The hOSEC was irradiated with UVB light at a dosage of 10 mJ/cm² every other day for 3 days. Before UVB irradiation, the medium was removed and PBS or PRP solution was added to the explants. After UVB irradiation, the medium was changed back to complete medium containing 10% FBS and 1% penicillin and streptomycin, and the explants were cultured for another 7 days.

Hematoxylin and eosin (HE) staining and Masson's trichrome stain. The skin explants were fixed with 10% neutral buffered formalin at room temperature for 24 h and then embedded with paraffin and cut into 4 mm pieces. The sections were deparaffinized at 65˚C for 4 h with gradient ethanol and then stained with HE for 10 min or Masson's trichrome stain for 6 min at room temperature. The images were captured of the sections using a light microscope (magnification, x200; Olympus Corporation).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA samples were isolated from the cultured skin tissue using TRIzol® reagent (Thermo Fisher Scientific, Inc.). Briefly, ~50 mg tissue was lysed in 1 ml TRIzol®, and then chloroform was added to the homogenates and the samples were centrifuged at 14,000 x g for 10 min at 4˚C to obtain the RNA fractions (supernatants). Isopropanol was added to the pellet RNA and then the samples were washed with 75% ethanol. The cDNA was synthesized from the RNA sample using HiScript® III RT SuperMix for qPCR with +gDNA wiper (Vazyme) according to manufacturer's protocol. The qPCR was performed using a CFX96 system (Bio-Rad Laboratories, Inc.) with ChamQ Universal SYBR® qPCR Master Mix (Vazyme). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 15 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for 30 sec; 95˚C for 15 sec, 60˚C for 60 sec and 95˚C for 15 sec. PCR primers were as follows: GAPDH: Sense 5'-TCAACAGCG ACACCCACTCC-3', anti-sense 5'-TGAGGTCCACCACCC TGTTG3'; tropoelastin: Sense 5'-GCTGACGCTGCTGCA GCCTA-3', anti-sense 5'-CAGCAAAAGCTCCACCTACA-3'; fibrillin-1: Sense 5'-TGACTGGCCCACACGTGCATAG-3', anti-sense 5'-TGACATTGACCCCTTGTTGACAGGA-3'; MMP-1: Sense 5'-GGGAGATCATCGGGACAACTC-3', anti-sense 5'-GGGCCTGGTTGAAAAGCA-3'; p53: Sense 5'-ATCGTGGAGGCATGAGCAGA-3', anti-sense 5'-TCTGGA GTTTCTGCTGCTGCTA-3'; and Tyrosinase: Sense 5'-CTC CGCTGGCCATTTCCCTA-3', anti-sense 5'-GGTGCTTCA TGGGCAAAATC-3'. GAPDH was used as a housekeeping gene for normalization of gene expression. The $2^{-\Delta\Delta Cq}$ method was used to quantify the relative gene expression (20).

Immunofluorescence. The skin graft was frozen in liquid nitrogen and placed in a constant temperature freezer. A small amount of optimal cutting temperature compound was added for cryosectioning (thickness, 4 μm). The sections were dried with a cold air blower and blocked with 5% BSA for 1 h at room temperature. Then, the frozen sections were incubated with specific antibodies against MMP-1 (1:800, R&D Systems, Inc.; cat. no. MAB901), tyrosinase (1:800, Abcam; cat. no. ab738), tropoelastin (1:800, Abcam; cat. no. ab21600) and fibrillin (1:800,
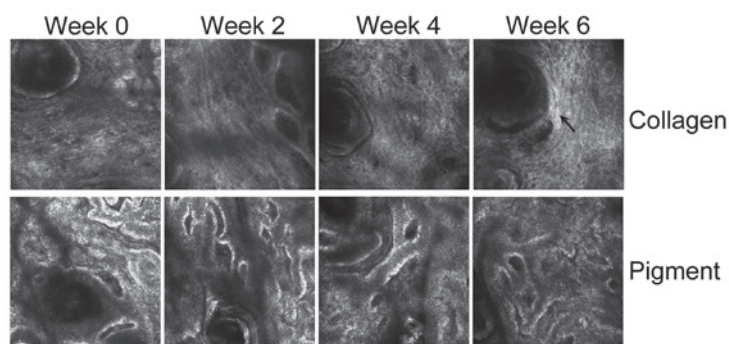
Figure 1. PRP treatments improve human skin conditions. Skin computed tomography examination (magnification, x200) of collagen (upper panel) and pigment (lower panel) around the injection sites before PRP treatment (week 0) and after PRP treatment (at 2, 4 and 6 weeks). Black arrow in the upper panel at week 6 indicates the collagen fibers. PRP, platelet-rich plasma.

Abcam; cat. no. ab53076) at room temperature for 1 h. After washing, the frozen sections were labeled with Alexa Fluor 488 goat anti-rabbit IgG (1:500, Invitrogen; Thermo Fisher Scientific, Inc.; cat. no. A11008) at room temperature for 1 h. Finally, the samples were imaged and analyzed with a confocal laser scanning microscope (Carl Zeiss AG) with x200 magnification.

Western blotting. The skin explants were irradiated 3 times with UVB light at a dosage of 10 mJ/cm^2 every other day. Then the skin explants were washed twice with cold PBS and ground in RIPA lysis buffer (containing protease inhibitor cocktail; Beyotime Institute of Biotechnology). The whole cell lysates were incubated at 4°C for 30 min, followed by centrifugation (12,000 x g for 15 min at 4°C). Protein samples were quantified using Bicinchoninic Acid protein assay method and 30 μg protein for each sample was separated by SDS-PAGE (4-18% gradient gel), transferred to a PVDF membrane and blocked with 5% milk at room temperature for 30 min. The membranes were then probed with specific antibodies against MMP-1 (1:1,000, R&D Systems, Inc.; cat. no. MAB901), tyrosinase (1:1,000; Abcam; cat. no. ab738), tropoelastin (1:1,000; Chemicon International; Thermo Fisher Scientific, Inc.; cat. no. MAB2503) and β-actin (1:2,000, Sigma-Aldrich; Merck KGaA; cat. no. A5441) at 4°C overnight. The next day, the membranes were incubated with goat horseradish peroxidase-conjugated anti-mouse IgG secondary antibody (1:10,000; Thermo Fisher Scientific, Inc.; cat. no. G-21040) at room temperature for 1 h. The protein bands were visualized with SuperSignal™ West Pico PLUS Chemiluminescent Substrate kit (Thermo Fisher Scientific, Inc.; cat. no. 34580) and analyzed using ImageJ (version 1.52a; National Institutes of Health).

Statistical analysis. Numerical data (n=3) are presented as the mean ± SD and were compared using unpaired Student's t-tests (GraphPad Prism version 6.0; GraphPad Software, Inc.). One-way ANOVA followed by Fisher's Least Significance Differences test were used for multiple comparisons. P<0.05 was considered to indicate a statistically significant difference.

Results

Changes in skin biophysical parameters and skin appearance upon PRP injections. All 30 females (median age, 43 years) completed 3 PRP injections within 1 month and no adverse
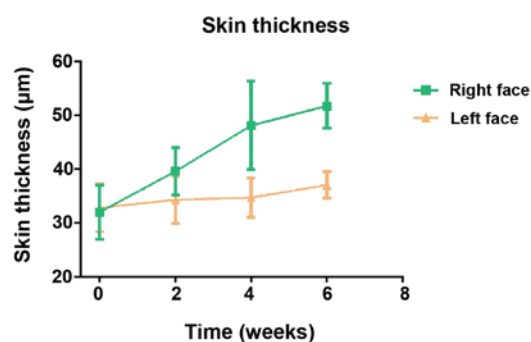

Figure 2. Measuring the skin thickness of the right-side face (PRP injected) and the left-side face (without PRP treatment) from week 0 to week 6. PRP, platelet-rich plasma.

effects were observed throughout the treatment. Data were collected before each injection (0, 2 and 4 weeks) and 2 weeks after the last injection (week 6). After 3 PRP injections, skin CT examination around the injection sites showed that the translucency of the pigment ring decreased, indicating a decrease in pigmentation. In addition, collagen was increased and denser than that before treatment (Fig. 1). Baseline (week 0) skin pores, texture, wrinkles and spots, which were assessed using the VISIA® multi-point positioning system, were compared with the follow-up measurements and are presented in Table I. Skin pore values continuously decreased with the progress of PRP treatment. At week 0, the value was 1094.26±351.42, but with continued PRP treatments, the value significantly decreased to 907.21±362.89 (P=0.045) at the final measurement. Wrinkle values measured 2 weeks after the last treatment (20.72±6.07) at week 6 were also significantly lower than that of week 0 (30.17±9.17; P<0.001). Similarly, the skin texture at week 4 (507.23±247.02; P=0.03) and week 6 (496.52±265.47; P=0.02) was significantly lower than that at week 0 (673.45±317.23). Although the spot values also decreased, the difference between week 0 and either of the 3 PRP treatments was not significant.

In order to further verify the role of PRP in improving human skin conditions, PRP injections were only performed on one side of the patient's face and the other side of the face was left untreated. With 3 rounds of PRP injections, the skin thickness markedly increased on the right-side face (with PRP treatment), but the left-side face (without PRP treatment) showed minimal changes (Fig. 2). Similarly, with 3 rounds of

Table I. Skin biophysical parameter changes upon platelet rich plasma injection.

Parameter	Week 0 Mean ± SD	Week 2 Mean ± SD	Week 2 P-value[a]	Week 4 Mean ± SD	Week 4 P-value[a]	Week 6 Mean ± SD	Week 6 P-value[a]
Texture	673.45±317.23	610.68±346.57	0.41	507.23±247.02	0.03	496.52±265.47	0.02
Wrinkles	30.17±9.17	27.19±10.01	0.18	24.66±8.34	0.01	20.76±6.07	<0.001
Spots	223.65±71.93	214.73±52.67	0.53	203.18±42.32	0.15	197.24±49.57	0.07
Pores	1,094.26±351.42	1,021.93±379.44	0.46	958.23±401.87	0.16	907.21±362.89	0.045

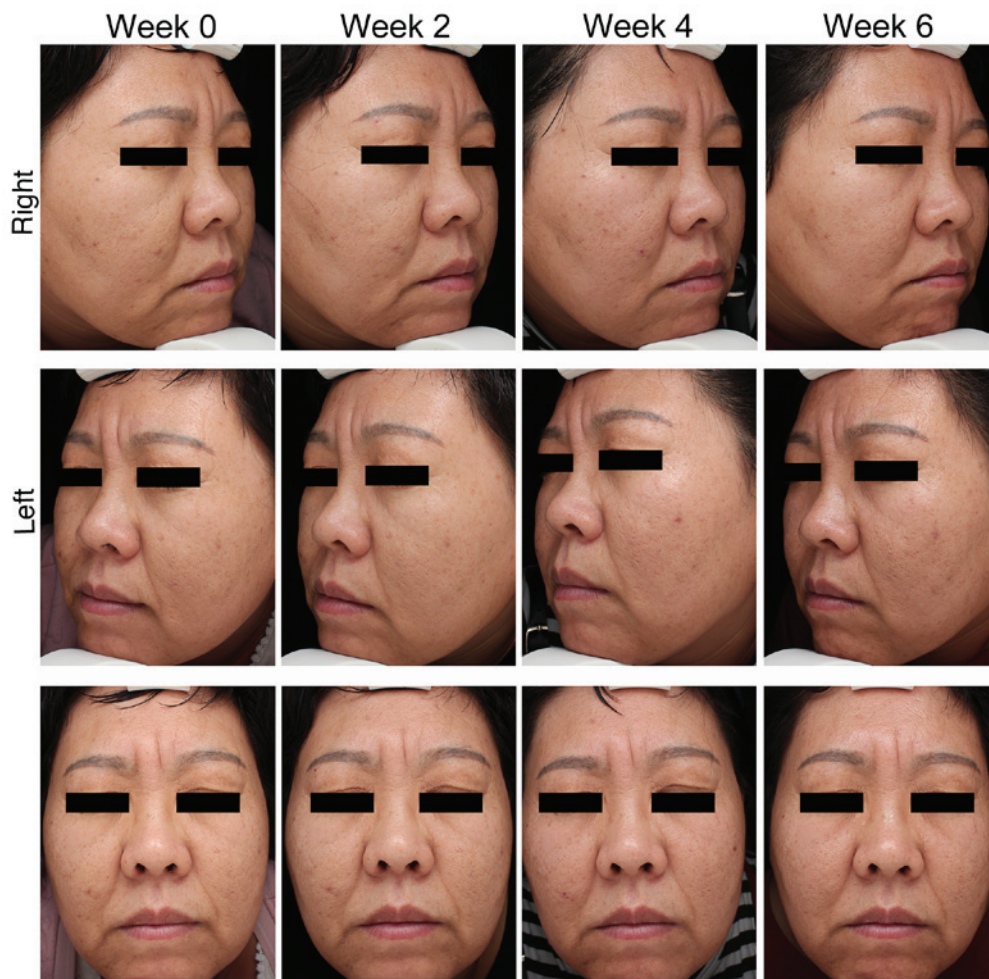[a]P-values vs. corresponding item on week 0.

Figure 3. Images show the skin conditions of the right face (PRP injected) and the left face (without PRP treatment) from week 0 to week 6. PRP, platelet-rich plasma.

PRP treatments, the right-side face showed better skin texture, less wrinkles and relatively smooth and firm skin, whereas the left-side face showed little changes (Figs. 3 and S1). Therefore, it was demonstrated that PRP injections effectively improved human skin conditions.

PRP protects skin against photoaging caused by UV light. A hOSEC was established using the method described previously (19). To observe the distribution of epidermal structures and dermal fibers, HE staining and Masson's trichrome staining were used to detect collagen in the human skin grafts. It was observed that the collagen fibers of skin grafts without UVB treatment were arranged neatly and densely, and the collagen staining was deep. After UVB irradiation, the collagen fibers were denatured, broken and arranged disorderly, and the collagen staining was light and showed markedly reduced collagen content. However, with PRP treatment, collagen fibers of the skin grafts were not altered after UVB irradiation
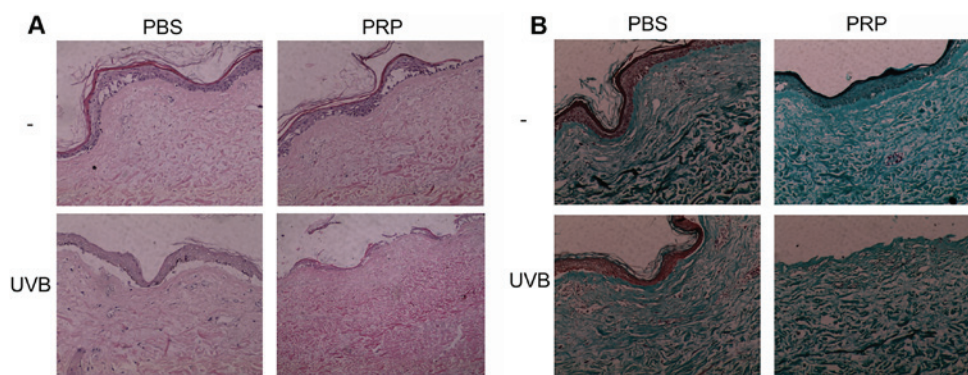
Figure 4. PRP protects against skin photoaging caused by UVB. Distribution of epidermal structures and dermal fibers in human skin grafts after treatment with UVB and/or PRP were determined by (A) hematoxylin and eosin staining and (B) Masson's trichrome staining (magnification, x200). PRP, platelet-rich plasma; UV, ultraviolet.
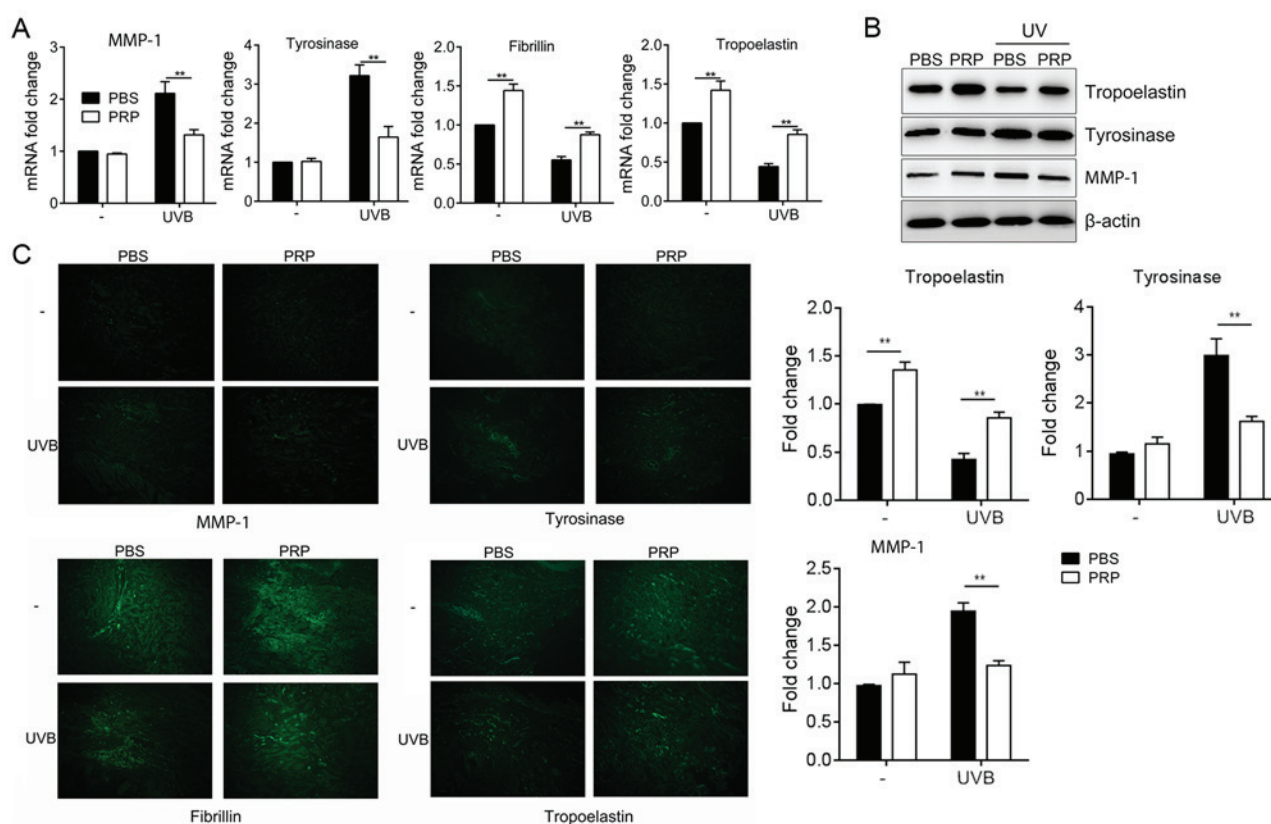

Figure 5. PRP inhibits UVB-induced MMP-1 and tyrosinase upregulation. (A) MMP-1, tyrosinase, fibrillin and tropoelastin mRNA levels in human skin grafts after UVB and/or PRP treatments were determined by reverse transcription-PCR using GAPDH as an internal control. Data are presented as the mean ± SD of three independent experiments. (B) MMP-1, tyrosinase, tropoelastin and β-actin protein levels in human skin grafts after UVB and/or PRP treatments were assessed by immunoblotting. Bar plots show the quantified fold changes of the western blotting bands. **P<0.01. (C) Distribution of MMP-1, tyrosinase, fibrillin and tropoelastin in human skin grafts after UVB and/or PRP were detected by immunofluorescence staining (magnification, x200). PRP, platelet-rich plasma; MMP-1, matrix metalloproteinase-1; UV, ultraviolet.

(Fig. 4). These results suggested that PRP can protect collagen fibers, delay collagen fiber changes, reduce elastic fiber chain scission and resist skin photoaging caused by UV rays.

PRP inhibits UVB-induced MMP-1 and tyrosinase upregulation to protect skin against photoaging. In order to explore the potential molecular mechanisms that mediate protection of PRP against photoaging, gene expression changes of MMP-1,

tyrosinase, fibrillin and tropoelastin were measured after treatment with UVB and/or PRP. It was identified that PRP significantly inhibited UVB induced-MMP-1 and tyrosinase upregulation, but significantly restored the expression of fibrillin and tropoelastin, which were downregulated by UVB treatment (Fig. 5). These observations were also made at both mRNA and protein levels (Fig. 5A-C). These results suggested that PRP may protect human skin against photoaging by

restoring the gene expressions of MMP-1, tyrosinase, fibrillin and tropoelastin.

Discussion

PRP has been widely applied for tissue repair in the fields of plastic surgery, oral and maxillofacial surgery, orthopedics and neurosurgery (8,12,15,18). As part of the diverse functional factors contained in PRP, autologous PRP has the best ratio of growth factors. The growth factor content in PRP is consistent with that in the patient's body, and compensates for the deficiencies of poor activity and low repair capacity of a single growth factor (8). In addition, there are no immunological problems and no risk of spreading diseases in allogeneic transplantation (21). Furthermore, PRP forms a gel, which protects platelets from damage and loss during injection, and allows platelets to secrete growth factors for an extended period to maintain a high concentration of growth factors (22,23). The benefits of PRP treatment in skin anti-aging repair come not only from the variety of high-concentration growth factors, but also from its gelatin state, which has plasticity and good support for skin wrinkles, cavities and skin relaxation (22,23). In addition, PRP also contains a large number of cell adhesion proteins, such as cellulose, fibronectin and vitronectin, that may keep skin smooth and tight (24). Physiologically, the growth factors in PRP have important roles in reducing the rate of aging by restoring the declining DNA synthesis that occurs with aging, resisting cell death and enhancing gene expression for tissue repair (25,26). A positive correlation between PRP and skin anti-aging has also been reported in both pre-clinical and clinical practice (25-28).

Consistent with previous studies (17,26), the present clinical study showed that PRP treatment improved skin conditions, including increased skin thickness, enhanced collagen content and reduced pigmentation. In addition, parameters assessed using the VISIA system, such as wrinkles, texture and pores were all decreased compared with pretreatment. Further evidence from the hOSEC experiments provided insight for the potential molecular mechanisms that explain how PRP treatments protect skin from photoaging.

UV is the primary external stress that causes oxidative stress in the skin. This reaction is initiated by reactive oxygen species and eventually results in premature skin aging by inhibiting transforming growth factor-β (TGF-β) activity, inducing MMP expression and activating the mTOR signaling pathway, culminating in the inhibition of autophagy (29,30). TGF-β in PRP may compensate for the localized reduction of TGF-β during photoaging. Furthermore, PRP is also reported to be involved in autophagy (31). MMP-1 and tyrosinase function in the degradation of fibrous connective tissue and the reduction of collagen synthesis, and are also important molecules in promoting skin photoaging (32-34). The present study demonstrated that PRP could inhibit UV-induced MMP-1 and tyrosinase upregulation to protect skin from photoaging. PRP also induced the expression of fibrillin and tropoelastin, and these factors have been reported to improve skin elasticity.

Therefore, PRP treatment directly implants a variety of active growth factors into aging skin. These factors change gene expression in skin cells, promote skin cell proliferation and differentiation, and rearrange the structure of skin tissues.

PRP injections are effective in improving skin conditions and protecting skin from photoaging, and thus have broad applications in anti-aging skin repair.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

TL and RD conceived and designed the study and wrote the manuscript. RD performed the experiments, analyzed the data and recorded the clinical characteristics of the patients. Both authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by The Ethical Committee of Inner Mongolia International Mongolian Hospital. Informed written consent was obtained from all participants before treatment. The skin sample experiments were approved by The Ethical Committee of Inner Mongolia International Mongolian Hospital (reference no. B2018-013). The patient agreed to the use of her samples in scientific research and written informed consent was obtained.

Patient consent for publication

The patient provided written informed consent for the publication of the facial images.

Competing interests

The authors declare that they have no competing interests.

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