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Original Article

The therapeutic effect of sufficient oxygen-rich PRP injection in facial rejuvenation by multiple objective evaluations in 26 cases

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ABSTRACT

Background: Ozone can enhance the expression of some growth factors (GFs) in platelet rich plasma (PRP), recent study showed oxygen-rich PRP (ozonized PRP) have better therapeutic effects on bone and joint diseases. PRP injection has been widely used in the treatment of facial rejuvenation, but the efficacy of sufficient oxygen-rich PRP in facial rejuvenation has not been studied.

Objective: Firstly, we examined whether ozone treatment can increase the concentration of GFs of PRP in vitro. And then a variety of subjective and objective detection methods were used to evaluate the effect of sufficient(10–12 mL each time for the injection of face and neck) oxygen-rich (ozonized PRP) PRP injection in facial rejuvenation by follow-up for 6 months. At last, we investigated the satisfaction, side effects and pain score of the treatment through a questionnaire survey.

Methods: The concentration of main GFs in PRP treated with different dose of ozone in vitro was measured by ELISA. Clinical picture, the collagen thickness of dermis by reflectance confocal microscope(RCM), skin conditions (including spots, ultraviolet (UV) spots, brown spots, red area, pores, wrinkles, texture and porphyrin) by VISIA were collected before treatment and each month follow-up visit after treatment until 6-month follow-up period was finished. Patients' satisfaction, side effects and pain score were collected at the end of follow-up period.

Results: PRP treated by high-dose ozone (57 μg/mL, ozone/PRP volume ratio:1/1) in vitro showed a significant increase in endothelial growth factor (EGF) and transforming growth factor-β (TGF-β) compared to baseline($P < 0.05$). Collagen thickness of forehead, cheek and neck improved significantly compare to the baseline until to the 6 months after treatment. Spots, UV spots, brown spots, red area and texture improved significantly compare to the baseline($P < 0.05$). All of participants reported improvement and have a median pain score of 4.19. No serious adverse events were observed.

Conclusions: Ozone treatment can increase the concentration of GFs such as EGF and TGF-β in PRP in vitro. Sufficient oxygen-rich PRP injection may be an effective and promising method to treat facial rejuvenation.

1. Introduction

Skin aging is a natural process of temporal and physiological changes, which is characterized by a progressive loss of functionality and regenerative capacity [1]. As living standards have improved, people are becoming more conscious about their facial appearance. As research into the mechanisms of skin aging progressed, many effective interventions for preventing and reversing facial aging have been discovered. Numerous cosmetic techniques have been developed to meet growing demand. Platelet-rich plasma (PRP) has emerged as a therapeutic option for a multitude of skin conditions, and is gaining attention as a potential approach for facial rejuvenation [2]. Platelets not only to halt bleeding and clot blood but also to secrete numerous growth factors (GFs) and proteins upon activation, crucial for tissue repair and regeneration. PRP, characterized by a high concentration of platelets obtained

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through centrifugation of whole blood, facilitates cell migration, proliferation, and angiogenesis by releasing a multitude of GFs [3]. Its application in the realm of skin aesthetics, encompassing facial rejuvenation, hair restoration, and the treatment of acne scars [4–6].

Studies have shown that PRP releases different GFs including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), and endothelial growth factor (EGF), which can attach to cell surface receptors, activate cell signaling pathway, and result in gene expression and protein synthesis needed for tissue repair and regeneration [7–10]. These properties provide the foundation for the application of PRP in facial rejuvenation treatments. The role of PRP for the treatment of skin rejuvenation has been demonstrated in recent reports [2,4,11].

Ozone, a gas molecule composed of three oxygen atoms, serves as a powerful oxidizer. It is a broad-spectrum and efficient bactericidal gas, which can kill bacteria, fungi, viruses [12,13]. Furthermore, ozone's mechanisms of action also include immunoregulation, antioxidant defenses, and epigenetic alteration [14]. Based on the above multiple biological functions, ozone has been used to treat a variety of skin conditions, such as acne, psoriasis, systemic sclerosis, skin ulcer, scar and atopic dermatitis [15–17].

Recently, research on the combination of ozone and PRP is most prevalent in the fields of orthopedics and sports medicine, with promising outcomes [18–20]. Studies indicate that ozone can enhance the expression of some GFs in PRP which play a crucial role in skin tissue remodeling [21], but the role of ozone in enhancing the concentration of GFs in PRP in facial injections has not been studied yet.

Therefore, to figure out the ozone intervention effect on concentration of GFs in PRP, the aim of the present study was to assess whether different dose of ozone treatment of PRP increase the concentration of GFs. On this basis, we use subjective and objective methods to investigate the effectiveness of sufficient ozonized PRP (oxygen-rich) in facial rejuvenation.

2. Methods and materials

2.1. Platelet-rich plasma preparation

Prior to PRP collection, abnormal blood routine and coagulation function were assessed to exclude any anomalies. Female participants were requested to collect PRP outside of menstruation periods. PRP was obtained using the standard cell separation method in the department of Blood Transfusion, with the 15-anticoagulant sodium citrate solution. The ratio of whole blood to anticoagulant was 1:1. Blood was collected using the full-automatic apheresis system (COM. TEC, Fresenius KABI, Friedberg, GER). This system efficiently yielded PRP from the autologous blood of each participant, significantly reducing erythrocytes and leukocytes. It automatically adjusted centrifugation parameters based on the participant's blood cell parameters, operating at room temperature (25 °C). Depending on the machine parameters' settings, the platelet enrichment concentration typically increases to 4 times that of the original platelet count. Comparison of plasma components is shown in Table S1. The collected PRP was divided into three blood bags and then stored in the transfusion department according to the blood product storage requirements.

2.2. Detection of GFs' concentration in PRP treated by different dose of ozone

PRP was collected from 7 donors who did not take antiplatelet or anticoagulant drugs within 2 weeks before donation. The donors were 34–58 years old. Each sample were divided into 5 groups: control group, calcium-activated group (10%) [22], ozonized low-dose group (ozone/PRP: 1/3), ozonized medium-dose group (ozone/PRP: 1/2), and ozonized high-dose group (ozone/PRP: 1/1). The calcium-activated group was mixed with calcium gluconate according the volume ratio 9:1, stand for 13min, and then the supernatant was assayed by centrifugation. In the ozonized groups, PRP was mixed with 57 μg/mL ozone gas and rotated for 5 min, then the remaining gas was discharged after standing. The flow diagram of GFs measurement is shown in Fig. 1a. EGF, PDGF-bb and TGF-β was measured using an ELISA kit (Elabscience, Houston, USA), according to the manufacturer's instructions.

2.3. Participants

26 patients who had received sufficient amount of oxygen-rich PRP in outpatient, Department of Dermatology, the Xiangya Third Hospital of Central South University were enrolled from January 2021 to December 2021. Inclusion criteria include: (1) male and female, were adults aged 18–65 years; (2) patients who need facial rejuvenation are willing to participate in this study; (3) patients with normal blood routine and coagulation function. Exclusion criteria include: (1) pregnant woman or lactating women; (2) blood or platelet disorders; (3) collagen-related diseases; (4) relevant skin treatment in recent 3 months; (5) facial surgery or dermal fillers within one year; (6) active skin disease or infection in the treatment area; (7) history of skin tumor (8) infectious diseases such as HIV, syphilis, hepatitis B, hepatitis C or other inflammatory diseases; (9) serious mental disorders, psychological disorders, important organ diseases or other basic diseases; (10) skin allergies. This study was conducted in accordance with the World Medical Association code of Ethics (Declaration of Helsinki) and was approved by the Medical Ethics Committee of the Third Xiangya Hospital, Central South University (Rapid I 22160). Written informed consent was obtained from all patients. The flow diagram of the clinical study design is shown in Fig. 1b.

2.4. Treatment protocol

All of participants were fully informed and signed informed consent. The anesthetic lidocaine cream was coated on the treatment area for 40–60 min, then PRP injection was performed to the face and neck. A total volume of PRP was 12 mL, 8 mL PRP was injected into the face and 4 mL into the neck. Half volume of PRP was injected without any treatment, and half volume of PRP was activated by ozone (PRP/ozone:1/1, according to the ELISA result in vitro) before injection. Patients received injections at one-month intervals for a total of 3 injections. All of patients were asked to revisit clinic to evaluate the therapeutic effect at one-month intervals until 6 months after the last injection.

2.5. Assessment

Participants were required to take clinical photographs, record skin data before and after each treatment. Throughout the follow-up period, participants' adverse reactions were recorded. After treatment, patients rated their satisfaction with the treatment effects and reported pain severity during and after treatment. All 26 participants underwent three sessions of treatment at 1-month intervals. The same skin condition measurements were performed before each injection. After the completion of the three injection treatments, the participants' skin conditions were measured every month until 6 months after treatment.

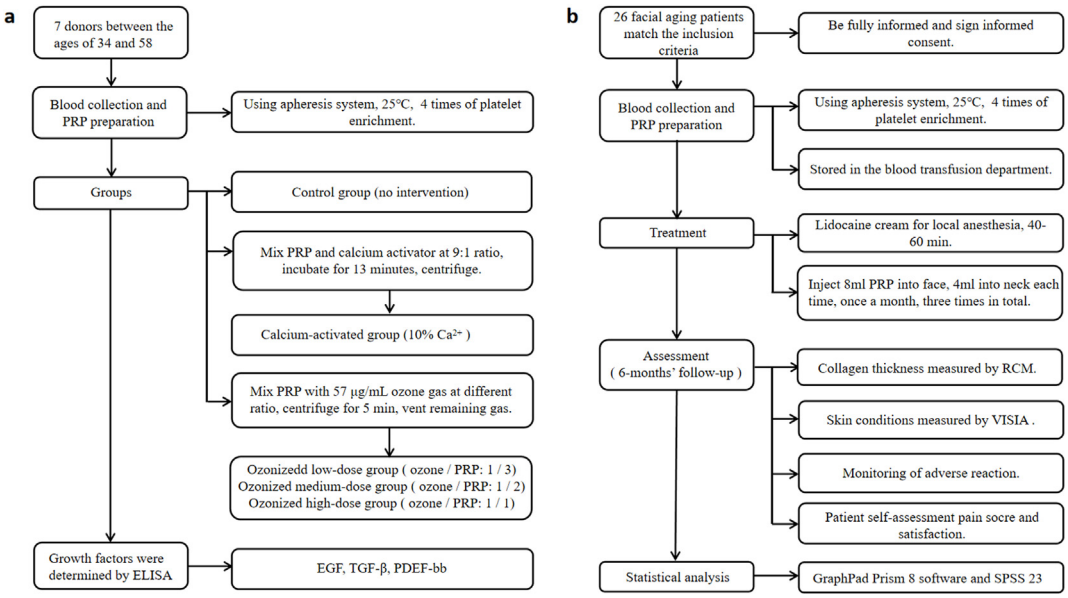

Fig. 1. (a) The flow diagram of growth factor measurement experiment. (b) The flow diagram of the clinical study design.

2.5.1. Objective evaluation

Collagen thickness of dermis was analyzed using a reflectance confocal microscope (RCM, VIVASCOPE, LUCID INC, Rochester, NY, USA). Before and after each treatment, the collagen thickness of the left and right foreheads, cheeks, and necks were measured. Take three different parts from each area for measurement, and take the average value of the results. Clinical photographs and images by VISIA Skin Analysis Imaging System (Canfield Scientific Co., Fairfield, NJ, USA) were taken at baseline and each month after the whole treatment. The evaluation by VISIA included spots, UV spots, brown spots, red area, wrinkles, texture, pore size and porphyrin. The mean values were then calculated from these readings.

2.5.2. Subjective evaluation

Patient satisfaction with the results was evaluated at the last follow-up. Treatment satisfaction was assessed on a 4-point scale as follows: 0, no improvement; 1, mild improvement; 2, moderate improvement; 3, significant improvement. Throughout the entire treatment, adverse reactions including edema, escharosis, hemorrhage, bruising and redness were recorded according to the standard of none, mild, moderate and severe.

Pain severity after injection in the participants was evaluated by a visual analog scale (VAS) based on a chart numbered from 0 (no symptoms) to 10 (maximum severity).

All assessments were performed at the baseline, and once a month after treatment. All measurements were carried out in the same area and location by the same physician assistant.

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism 8 software and SPSS 23. The results of GFs expression and the RCM measurements of collagen thickness were analyzed by one way ANOVA (multiple comparisons). The Paired *t*-test was used to analysis data of VISIA. The VAS score and subjective score results are expressed as the mean ± SD. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Concentrations of main GFs in PRP treated with different concentrations of ozone in vitro

The release of GFs from PRP was investigated by ELISA including EGF, PDGF-bb, and TGF-β (Fig. 2). Compared with untreated PRP, the high-dose ozonized group showed a significant increase in EGF compared to baseline ($P < 0.05$), and the differences in the low-dose and medium-dose groups were not significant ($P > 0.05$).

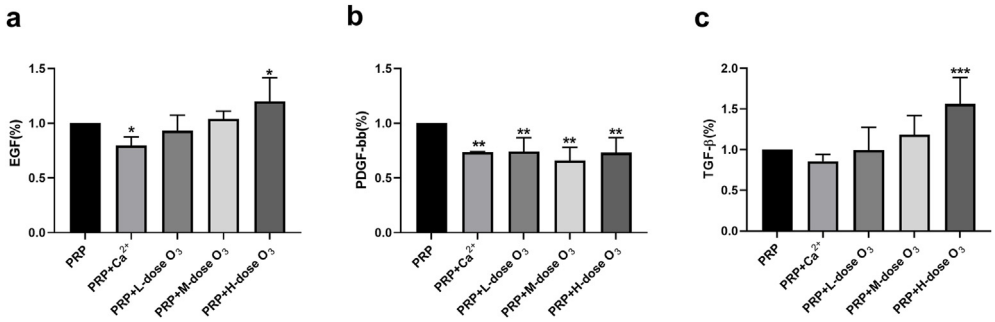

Fig. 2. Concentrations of growth factors in PRP treated with different concentrations of ozone. (a) epidermal growth factor (EGF), (b) Platelet-derived growth factor-bb (PDGF-bb), (c) Transforming growth factor-beta (TGF-β). *$P < 0.05$. PRP + L-dose ozone, the ratio of ozone and PRP is 1: 3. PRP + M-dose ozone, the ratio of ozone and PRP is 1: 2. PRP + H-dose ozone, the ratio of ozone and PRP is 1: 1.

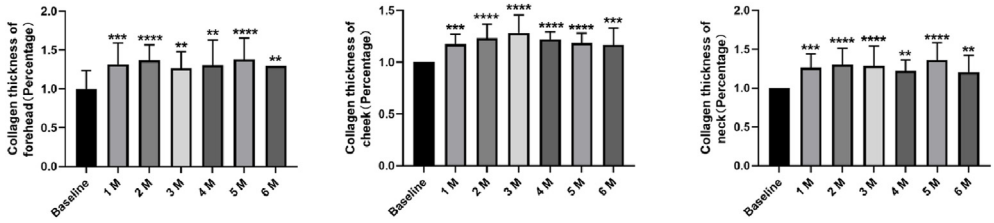

Fig. 3. Collagen thickness of forehead, cheek, neck of patients at baseline and months 1–6 after injection measured by RCM. *p < 0.05.

However, the unexpected is that the EGF release in the calcium-activated group was significantly reduced ($P < 0.05$). Similarly, TGF-β in high-dose ozonized group increased significantly ($P < 0.001$), while there was no significant change in the calcium-activated group, low-dose ozonized PRP group, and medium-dose ozonized PRP group ($P > 0.05$). Compared to the control group, the concentration of PDGF-bb in the calcium-activated group and ozonized PRP group was significantly reduced ($P < 0.01$), this is different from the trend of the other two GFs.

3.2. Collagen thickness of forehead, cheek and neck measured by RCM

The patients' forehead, cheek, and neck collagen thickness were continuously monitored with RCM before injection and at each follow-up visit until 6 months after injection. The results show that all the collagen thickness increased significantly in the first month after injection ($P < 0.01$) at the 3 monitored sites. The collagen thickness increase peaked during the follow-up period of 2–4 months after injection. It is amazing that the collagen thickening of the three sites reached even over 30% at the time point of maximum collagen thickening. The collagen thickness gradually decreased after 4-month follow-up visit, but the thickening of collagen was still significantly increased even at the end of the 6-month follow-up (forehead: 1.30 ± 0.21, face: 1.17 ± 0.16, neck: 1.20 ± 0.22, $P < 0.01$). It means that the percentage changes in collagen thickening 6 months after the injection were about 30% increasing in the forehead, 17% increasing in the cheek, and 20% increasing in the neck. (Fig. 3).

3.3. Skin conditions measured by VISIA

Skin conditions such as spots, UV spots, brown spots, red area, wrinkles, texture, pore size and porphyrin were measured by VISIA at baseline and 6-month follow-up visit after injection. Compared with baseline data obtained before the first treatment (week 0), we observed a significant improvement in spots (166.0 ± 38.95 vs. 146.6 ± 37.81, $P < 0.01$), UV spots (457.7 ± 116.8 vs. 377.2 ± 113.2, $P < 0.01$), brown spots (587.1 ± 53.50 vs. 553.5 ± 59.94, $P < 0.01$), red area (283.8 ± 80.22 vs. 236.0 ± 92.34, $P < 0.01$) and texture (363.7 ± 215.6 vs. 279.6 ± 169.5, $P < 0.05$). The wrinkles (31.85 ± 18.49 vs. 30.54 ± 16.74, $P > 0.05$), pore size (653.3 ± 375.1 vs. 549.6 ± 335.4, $P > 0.05$), and porphyrin (1457 ± 1067 vs. 1241 ± 1060, $P > 0.05$) were not significant improved, but it still showed good trends in improvement (Fig. 4).

3.4. Patients' satisfaction and pain scores investigated by subjective evaluation

At 6-month follow-up visit, patients had anonymously filled out a satisfaction questionnaire. By patients' treatment satisfaction, 12 (46.20%) reported significant improvement, 7 (26.90%) reported moderate improvement, 7 (26.90%) patients report mild improvement. The patients reported that the treatment could effectively improve facial rejuvenation, and the most important effects were the improvement of pigmentation and skin inflammation. Within 12 months after injection, 14 (53.85%) patients asked for the treatment again. After treatment, patients exhibited a more uniform and fair skin tone, with a noticeable reduction in wrinkles and scars (Fig. 5). Patients had a median pain score of 4.19 (4.19 ± 2.46) during the injection by the VAS scale (Fig. 5).

3.5. Adverse reaction

The results of adverse reaction were shown in Table 1. Most patients experience edema and bruising after injection and all adverse reactions were mild or moderate in severity. No serious adverse events were observed during the 6-month follow-up period.

4. Discussion

When PRP acts on damaged or aged skin tissues, platelet degranulation releases a variety of GFs, cytokines and other active

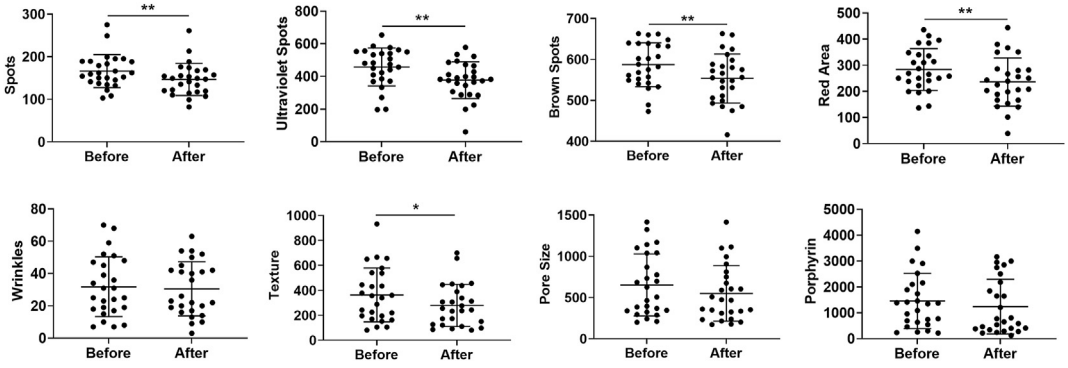

Fig. 4. The VISIA measurements at the baseline and 6-month follow-up visit after injection. Before, at the baseline. After, 6-month follow-up after injection.

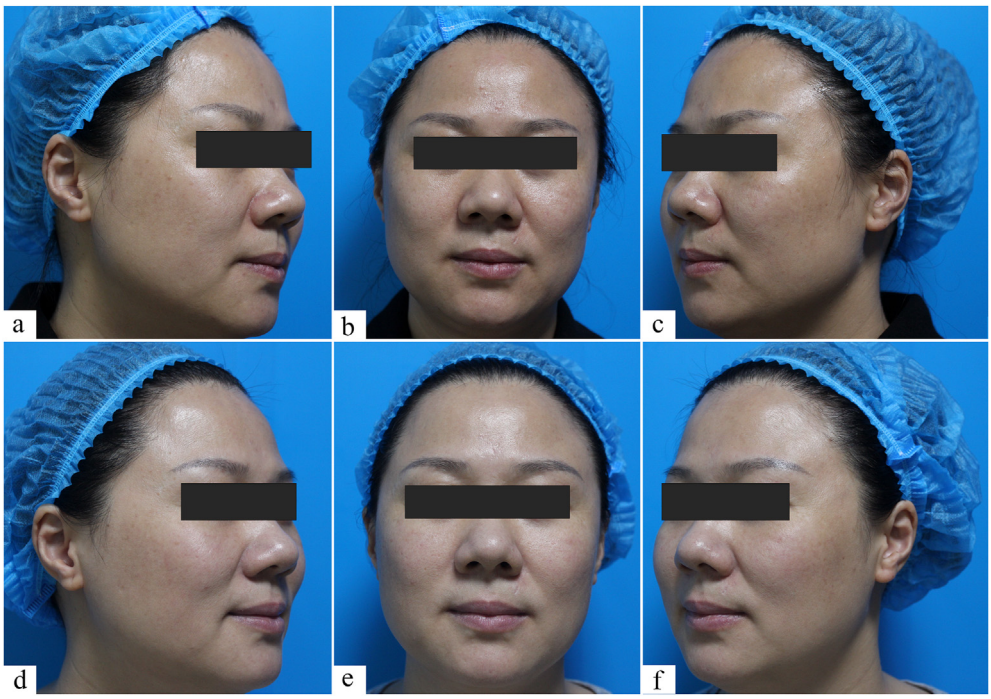

Fig. 5. (a) clinical photograph token before treatment, (b) clinical photograph token at 6-month follow-up after treatment.

Table 1
Adverse reactions in participants.

Severity of adverse reactions	Number (%)				
	Edema	Bruising	Hemorrhage	Redness	Escharosis
None	6 (23.08)	24 (92.31)	23 (88.46)	6 (23.08)	23 (88.46)
Mild	17 (65.38)	2 (7.69)	3 (11.54)	17 (65.38)	2 (7.69)
Moderate	3 (11.54)	0 (0)	0 (0)	3 (11.54)	1 (3.85)
Severe	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

substances to promote tissue repair and regeneration, including TGF-β, PDGF and EGF [23]. These GFs can stimulate collagen synthesis, inhibit extracellular matrix degradation, promote fibroblast proliferation and increase hyaluronic acid production [24]. Hence, PRP plays a vital role in promoting collagen regeneration, angiogenesis, and facial rejuvenation [23,25,26].

Whether to use an activator to induce platelet degranulation is a controversial issue, with conflicting results reported in several studies [4,27,28]. Thus, we measured the concentration of main GFs in PRP activated by different activators including calcium and ozone treatment by ELISA. In our study, we found that the addition of calcium activators resulted in decrease release of some GFs in PRP. This indicates the necessity for further research on activation methods and dosages. Our previous experiment showed that the release of some main GFs such as EGF and TGF-β by high dose of ozone treatment had a significant increase, while the PDGF-bb was significant decreased. These results suggest that the activation of ozone may have different effects on the concentration of different GFs. After ozone treatment, the concentration of some main GFs in PRP was increased, while the concentration of some main GFs decreased. The result indicated that maybe we can choose different treatments to balance the concentration of main GFs in clinic. Therefore, in the subsequent clinical treatment, we divided PRP into 2 halves, 1 half was injected without any activation, and the other half was injected after high dose ozone activation.

In current studies, PRP isolated kits and centrifuges are commonly used to collect PRP, with only 4–5 mL of PRP collected each time [29]. In our study, blood cell separator was utilized to obtain 35–60 mL of PRP at once. With the improvement of PRP collection technology, we can collect enough PRP for three injections at one time, and the concentration of PRP is more than four times, which can ensure that we have sufficient and high-quality PRP for clinical treatment.

All current studies on the application of PRP for facial rejuvenation have reported significant improvement. However, variety in evaluation criteria for medications, treatment protocols, and outcomes have created challenges in drawing definitive conclusions from clinical research [29]. In our study, we used RCM and VISIA system specially to assess treatment efficacy objectively during the follow-up period, aiming to address these challenges and provide one precise evaluation criteria. The results show that treatment significantly increases skin collagen thickness in forehead, cheek and neck. During the whole follow-up period, collagen thickness improved significantly compared with the baseline. It's worth noting that the increase in collagen thickness was not continuous, likely due to the natural aging process, where skin collagen thickness tends to decrease over time. Furthermore, we observed a significant improvement in patients' skin spots and red areas, suggesting that the combination therapy has an anti-inflammatory effect and reduces vascular lesions. Significant improvements were also observed in UV spots and brown spots, indicating that this

therapy can reduce skin pigmentation. The improvement of vascular lesions and pigmentation suggests that PRP injection may play a role in the treatment of melasma, and indeed, we also found that patients' melasma was obviously relieved by visual observation after PRP treatment in our study, this is consistent with the results of several recent clinical studies [30,31].

The activation of ozone and sufficient volume of PRP in our study prompt us to pay more attention to the safety and pain management. In our study, minor adverse events were noted, including edema, escharosis, hemorrhage, and redness, primarily limited to the injection site and usually resolved quickly. Most patients thought the injection pain is tolerable, but the pain score is still around 4 and there remains an urgent need to develop strategies that minimize patient discomfort during treatment.

5. Conclusion

In conclusion, our findings suggest that sufficient oxygen-rich PRP injection may be an effective and promising method to treat facial rejuvenation. High-dose ozone treatment enhances GFs concentration in PRP, aiding facial rejuvenation. Collagen thickness measurement and VISIA system are helpful for an objective assessment of the effects of this combined treatment.

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Data availability

Data will be made available on request.

Ethics statement

The patients in this manuscript have given written informed consent to publication of their case details.

Author contribution

JL and TZ was responsible for part of article writing. LZ, XT, JZ, and YF participated in the research and investigation process. RG, ZT and LG were responsible for part of study design. LJ and GZ performed the data curation and data analysis. JL obtained the funding. All authors participated in the data collection, critically revised the manuscript, and gave final approval to the vision submitted for publication.

Declaration of competing interest

The authors declare no conflict of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.reth.2024.05.013.

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