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Treatment with injectable platelet-rich fibrin in a rat model of melasma

Background: The management of melasma is challenging. Platelet-rich plasma (PRP) therapy has been shown to be beneficial, however, the use of anticoagulants for PRP is dangerous. *Objectives:* To evaluate the efficacy of recently developed blood-derived biomaterials (injectable platelet-rich fibrin [I-PRF]) in a rat model of melasma. *Materials & Methods:* Sprague Dawley (SD) rats were used to replicate an experimental animal model of melasma. SD rats exhibiting melasma were randomly divided into experimental and control groups. The experimental group was administered weekly intradermal injections of I-PRF, whereas the control group received an equivalent amount of saline. After four weeks, back skin was removed and evaluated based on (1) gross observation, (2) pathological examination and imaging analysis, and (3) biochemical detection. Data were analysed using SAS9.4 software. *Results:* I-PRF, a safe blood product without anticoagulants, inhibited melanin production in the epidermis and reduced oxidative stress damage in the cortex, improving melasma. *Conclusion:* I-PRF is a safe and cost-effective blood-derived biomaterial which is useful for the treatment of melasma.

Key words: I-PRF, melasma, photoaging, ultraviolet ray

Melasma is a common type of facial hypermelanosis that can substantially reduce quality of life and is often resistant to treatment [1]. Recent research has shown that melasma development is a complex process involving interactions between epidermal melanocytes, keratinocytes, dermal fibroblasts, mast cells, and vascular endothelial cells. Factors such as inflammation, reactive oxygen species, ultraviolet radiation, genetics, and hormones can exacerbate melasma [2]. Given its complex pathogenesis and various contributing factors, treating melasma can be challenging, and it often recurs. Emerging therapeutic approaches aim to counteract the pathogenesis and influential factors of melasma, with platelet-rich plasma (PRP) being one of the most promising treatments. PRP is an autologous blood product, and recent studies have demonstrated that it is effective in treating melasma [3-7].

However, this approach has some limitations. Firstly, the preparation of PRP requires the use of anticoagulants, which raises concerns among some individuals because of their known inhibitory effects on wound healing [8]. Secondly, the rapid release of growth factors in PRP results in a shorter duration of effect. Finally, PRP preparation is difficult and requires cumbersome instrumentation and materials [9].

The injectable platelet-rich fibrin (I-PRF) is a novel biomaterial derived from blood that does not require anticoagulants, and has been widely used to treat androgenetic alopecia, the rejuvenating area under the eye, temporary correction of facial skin folds, and treatment of hard-to-heal wounds and ulcers. Its unique property is that it remains in a fluid state for approximately 15 min, after which it forms a gel-like membrane. This drug can be injected within a 15-min timeframe. It has a three-dimensional fibrin network, which helps in the slow release of growth factors over a period of time; therefore, the effect lasts longer [10]. I-PRF has been utilized for the treatment of wounds and alopecia, owing to its rich content of various growth factors such as PDGF, TGF-B, VEGF, EGF, IGF-1, *etc*. One of the growth factors in I-PRF, transforming growth factor beta (TGF-β), plays a vital role in the treatment of melasma. In addition, I-PRF induces collagen synthesis, thereby improving skin quality and texture. Most importantly, I-PRF preparation is simple and requires minimal instrumentation and materials, making it cost-effective [11].

In this study, we investigated the efficacy of I-PRF in treating melasma in rats by conducting a randomised, controlled study in an animal model.

Materials and methods

Reagents, antibodies, and equipment

Malondialdehyde (MDA, A003-1-2) and superoxide dismutase (SOD, A001-3-2, EC No.1.15.1.1) assay kits were purchased from Nanjing Jiancheng Bioengineering Institute. An LD-2500 regular refrigerated centrifuge

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(Jiangsu Xinkang Medical Equipment Co., Ltd.) was used. Additionally, a TL 20 W/01 narrow-spectrum medium-wave 20 W 311-nm ultraviolet UVB phototherapy tube (East Valley Lighting Technology Co., Ltd.) was used. The CMIAS series 98A multifunctional true-colour pathological image analyser was produced by the Image Center of Beijing University of Astronautics. SOX10 (10422-1-Ap) and purchased from Proteintech Group.

Preparation of the melasma rat model

In this study, we utilized 34 female Sprague Dawley (SD) rats of clean grade, aged between 9 and 10 weeks and weighing between 260 and 290 g. The UVB-free group consisted of six rats while the UVB-applied group comprised 18 rats. Another 10 rats were used for blood collection for I-PRF preparation. The hair of all rats was removed from an area measuring 4 × 4 cm. Rats in the UVB-applied group were irradiated with UVB once daily. A distance of 20 cm was maintained between the irradiated skin of the rats and the lamp source to maintain the required radiation dose (300 mJ/cm^2). The rats were intramuscularly injected with 25 mg/kg progestin once daily. After four weeks, six rats were sacrificed and their tissues were subjected to immunohistochemical staining with SOX10 and ELISA for MDA and SOD. Data were collected and statistically analysed to determine whether the model was successfully established.

Separation of I-PRF

Ten rats were anaesthetised, and orbital blood was collected. The blood was quickly centrifuged in a refrigerated centrifuge at 400 g (700 rpm) at room temperature for three minutes. The upper yellow-orange coloured liquid contained I-PRF (*figure 1, 2*).

Figure 1. Direct products obtained after centrifugation for blood collection.

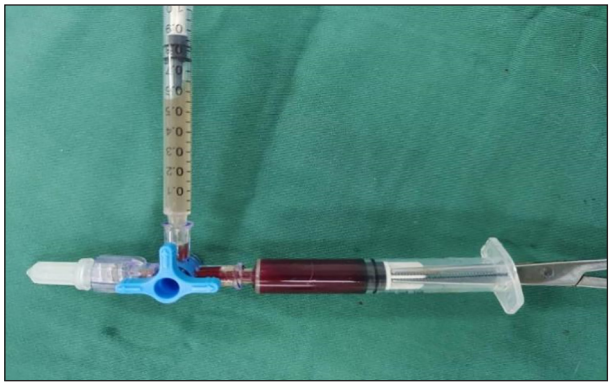

Figure 2. Separation of IPRF.

Intracutaneous injection of I-PRF

Twelve rats with melasma from the UVB-applied group were randomly divided into two groups. Group A, which was the treatment group, received weekly intracutaneous injection of I-PRF. The rats received an intradermal injection of I-PRF at 0.1 mL/cm^2 on the skin of the skin. The remaining six rats in group B were used as controls and received the same amount of physiological saline.

Observation

Thirty days later, the skin colour of the rats from treatment and control groups was visually evaluated using the Melasma Severity Scale (MSS). This scale uses a four-grade scoring system to evaluate the difference between the colour of the lesion and that of the normal skin around the lesion: 0=no difference; 1=mildly darker; 2=moderately darker; and 3=severely darker. Three blinded observers evaluated the rats in both the treatment and control groups in a mixed and blinded manner. The arithmetic average of the scores of the three observers was used to analyse the differences between the groups.

All animals were euthanised using the carbon dioxide ventilation method. A biopsy of the pigmented skin sites in each animal was performed for histological examination and biochemical index determination.

Half of the tissues were preserved in 10% formaldehyde, and 4-μm thick sections were acquired from paraffin-embedded tissues. These were then stained with haematoxylin and eosin, immunohistochemically stained with SOX10, and analysed using pathological imaging. Changes in the number and area of melanin-positive cells labelled with SOX10 were observed and analysed.

The remaining tissues were homogenised, following the manufacturer's instructions, to evaluate MDA content and SOD activity.

Statistical analysis

All data collected were transferred to an electronic database and evaluated using the SAS9.4 software package. Statistical significance was set at $p<0.05$.

Results

The effect of UVB

Visual evaluation

After establishing the model, the skin of the UVB-applied group showed extensive erythema, pigmentation, and scales (*figure 3*). The MSS score in the UVB-applied group was significantly higher than that in the UVB-free group ($p<0.0001$) (*table 1*).

Histological evaluation

The average area, surface density, number of targets, and density of positive melanin granules were significantly higher in the UVB-applied group compared with the UVB-free group (*table 2*).

The average grayscale value in the UVB-applied group was significantly lower than that in the UVB-free group ($p<0.05$).

The integral and mean optical densities of the UVB-applied group were significantly higher than those of the UVB-free group ($p<0.05$) (*table 3*).

SOX10-labelled melanin-positive cells in the UVB-applied group increased significantly and were distributed in the basal cell mass of hair follicles, showing a strong positive reaction (*figure 4*).

Pathological analysis indicated that the degree of pigmentation in the UVB-applied group was higher than that in the UVB-free group.

Biochemical index

The MDA content in the UVB-applied group was significantly higher than that in the UVB-free group ($p<0.05$) (*table 4*). The SOD content in the UVB-applied group was significantly lower than that in the UVB-free group ($p<0.05$) (*table 4*). Altogether, these data indicated that the degree of oxidative stress was higher in the UVB-applied group than in the UVB-free group.

Table 1. Comparison of MSS score between UVB-free and UVB-applied groups.

Group	MSS value on 30th day
UVB-free group	0.0500±0.0837
UVB-applied group	2.5333±0.4885
t	–12.27
p	<0.0001

The effect of PRF

Visual evaluation

Four weeks after treatment, the skin of the rats in the treatment group was less pigmented and shiny than that of the control group (*figure 5*). The MSS score in the treatment group was significantly lower than that in the control group ($p<0.0001$) (*table 5*).

Histological evaluation

The average area, surface density, number of targets, and density of positive melanin granules were significantly lower in the treatment group than in the control group (*table 6*).

The average grey scale value was significantly higher in the treatment group than in the control group ($p<0.05$) (*table 7*). The integral and mean optical densities of the treatment groups were significantly lower than those of the control group ($p<0.05$) (*table 7*).

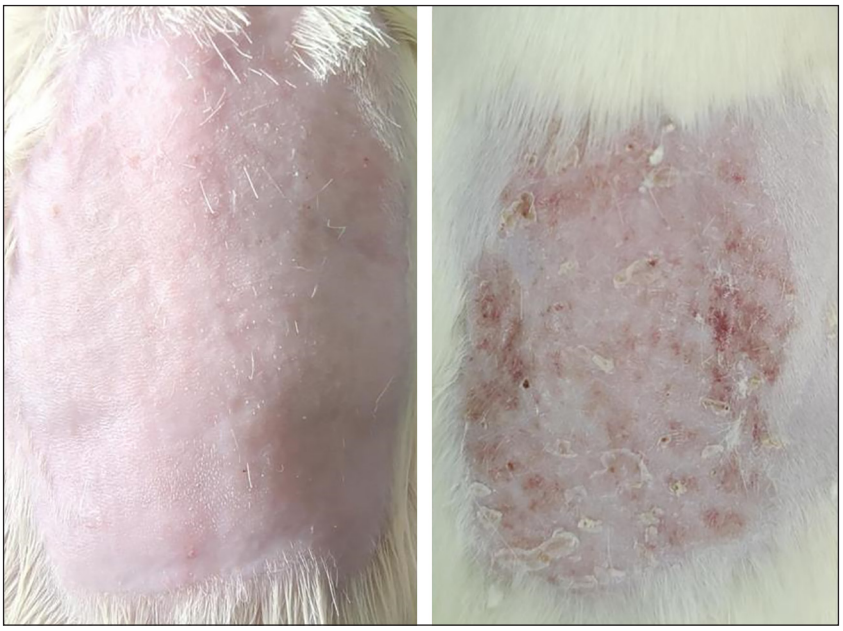

Figure 3. Appearance of back skin in the healthy group (L) and modelling group (R).

Table 2. Average area, surface density, number of targets, and density of positive melanin granules in the UVB-free and UVB-applied groups ($x\pm s$).

Group	n	Average area	Surface density	Number of targets	Density
UVB-free group	6	50.47±1.56	0.0072±0.0012	19.83±4.70	12.11±3.36
UVB-applied group	6	65.08±2.10	0.0213±0.0026	42.83±5.03	29.50±5.95
t		–5.56	–11.87	–8.17	–6.62
p		0.0003	<0.0001	<0.0001	0.0003

Table 3. Average grayscale value, integral optical density, and mean optical density in the UVB-free and UVB-applied groups ($x\pm s$).

Group	n	Average grayscale value	Mean optical density	integral optical density
UVB-free group	6	110.4±8.48	0.36±0.452	21.40±1.74
UVB-applied group	6	84.36±7.23	0.47±0.457	33.51±2.05
t		5.72	–4.22	–4.50
p		0.0002	0.0018	0.0012

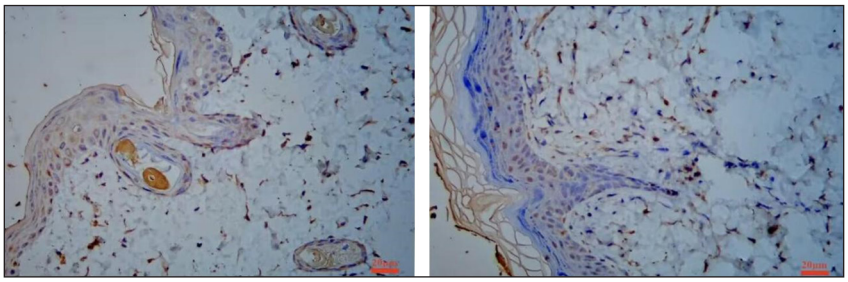

Figure 4. Immunostaining of the UVB-free group (L) and UVB-applied group (R).

Table 4. Comparison of MDA and SOD between UVB-applied and UVB-free groups ($x\pm s$, n=6, μmol, U/mg protein).

Group	MDA	SOD
UVB-free group	164.7±3.58	12.83±1.69
UVB-applied group	181.5±5.94	9.96±1.17
t	–5.94	3.41
p	0.0001	0.0067

The number of melanin-positive cells labelled with SOX10 was significantly reduced in the treatment group compared to that in the control group (*figure 6*). Pathological analysis indicated that the degree of pigmentation was lower in the treatment group than in the control group.

Biochemical index

The MDA content in the treatment group was significantly lower than that in the control group (p<0.05) (*table 8*). In contrast, SOD content in the treatment group was significantly higher than that in the control group (p<0.05) (*table 8*). Altogether, these data indicated that the degree of oxidative stress in the treatment group was significantly improved compared to that in the control group.

Discussion

Melasma, also known as pregnancy mask, is a benign, acquired pigmentation disorder that significantly affects quality of life. Managing melasma remains challenging [12]. Treating melasma is difficult because of the complexity of its pathogenesis, which involves multiple mechanisms. Topical hydroquinone, alone or in combination, is considered the gold standard for melasma treatment. Although topical hydroquinone is highly effective, long-term use can result in side effects, and recurrence is frequent after withdrawal. Therefore, novel therapeutic approaches are urgently needed. PRP, a recent approach for treating melasma, has provided significant therapeutic benefits in recent years [13, 14], which produces large quantities of growth factors when activated, which can improve pigmentation [15-17]. However, several problems are associated with the use of PRP for the treatment of melasma, including the use

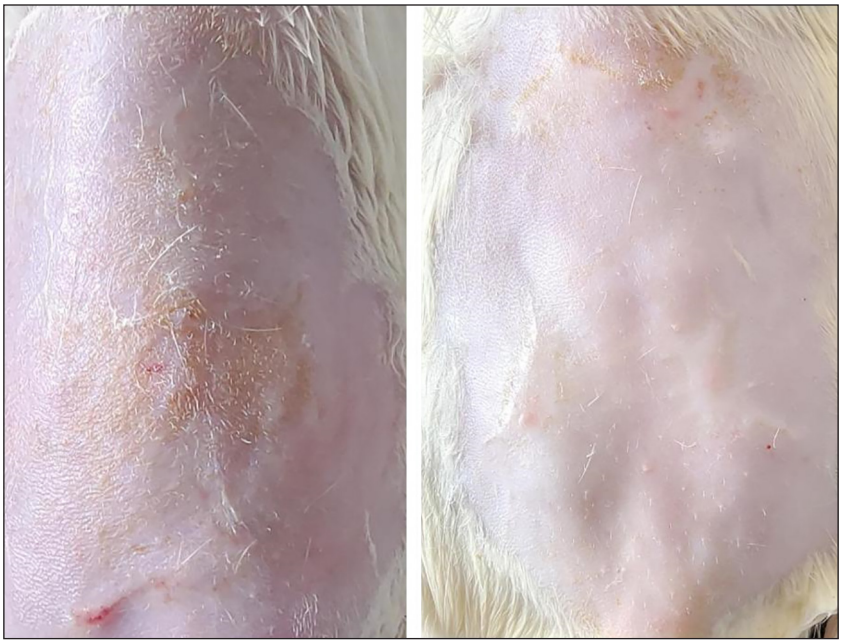

Figure 5. Skin of the control group (L) and treatment group (R).

Table 5. Comparison of MSS value between treatment and control groups.

Group	MSS value on the 60th day
Control group	0.8167±0.3817
Treatment group	2.0833±0.2639
t	–6.69
p	<0.0001

of anticoagulants, cumbersome preparation steps that may increase the risk of bacterial contamination, and a shorter maintenance period when growth factors are present at higher concentrations due to the fast rate of protein coagulation [18]. These factors contribute to its cost-ineffectiveness as a treatment option [19].

I-PRF is a recently developed blood-derived biomaterial that has several advantages and few weaknesses. It has a three-dimensional fibrin network that helps in the slow release of growth factors over time; therefore, the effect lasts longer [10]. I-PRF can also gradually release growth factors over a 14-day period. Therefore, I-PRF not only maintains the growth factor concentration at a higher level but also maintains it for a longer time [20].

The rich content of various growth factors, such as PDGF, TGF-B, VEGF, EGF and IGF-1, in IPRF has made it a widely used treatment for androgenetic alopecia and hard-to-heal wounds and ulcers. The distinctive

Table 6. Average area, surface density, number of targets, and density of positive melanin granules in the treatment group and control group (x±s).

Group	n	Average area	Surface density	Number of targets	Density
Control group	6	61.55±4.42	0.201±0.0014	40.84±5.36	28.38±4.70
Treatment group	6	53.32±5.69	0.114±0.0007	23.01±5.83	19.10±2.54
t		2.72	12.88	5.25	4.25
p		0.0302	<0.001	0.004	0.003

Table 7. Average grayscale value, integral optical density, and mean optical density in the treatment and control groups (x±s).

Group	n	Average grayscale value	Mean optical density	Integral optical density
Control group	6	88.47±5.62	0.42±0.025	31.41±2.73
Treatment group	6	102.37±6.81	0.35±0.21	24.61±2.56
t		–3.82	4.79	4.44
p		0.0036	0.0007	0.0013

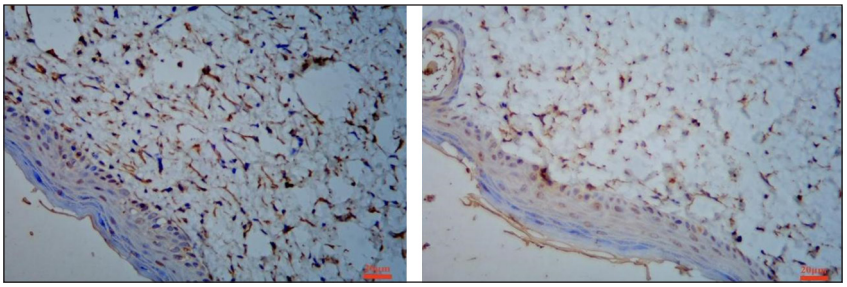

Figure 6. Immunostaining of control group (L) and treatment group (R).

Table 8. Comparison of MDA and SOD between treatment and control groups ($x \pm s$, n=6, μmol, U/mg protein).

Group	MDA	SOD
Treatment group	170.5±0.98	14.85±1.19
Control group	180.00±4.42	11.00±1.48
t	4.62	–4.96
p	0.0010**	0.0006**

three-dimensional fibrin network of I-PRF offers real advantages for the treatment of these diseases.

In particular, TGF-β not only inhibits melanogenesis by downregulating microphthalmia-associated transcription factor promoter and paired-box homeo-c gene (PAX 3), but also inhibits the production of tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2, reducing the production of melanin [21, 22]. At the same time, epidermal growth factor can inhibit the expression of prostaglandin-E2 and tyrosinase enzyme activity, thus lowering melanin production [23]. Recent studies have shown that oxidative stress plays an important role in melasma pathogenesis [24]. Moreover, I-PRF contains many leukocytes with anti-inflammatory and antibacterial properties [25]. Since I-PRF does not require anticoagulants or chemical activators, it is easier to prepare and safer to use for treatment than PRP. Finally, I-PRF preparation is simple and requires minimal instrumentation and materials, making it cost-effective [10, 26].

This study aimed to examine whether I-PRF is equally effective in treating melasma or not. The construction of the rat melasma model was a key part of this study, as it was the key to properly evaluating I-PRF treatment. Therefore, it was necessary to rigorously determine whether the melasma model was successfully established or not.

Chronic exposure to UV light and high hormone levels are significant pathogenic factors for melasma. A rat model of melasma was established using ultraviolet irradiation and intramuscular injection of progestin. The melasma model was evaluated using the MSS, skin histological evaluation, measurement of MDA content in skin homogenates, and evaluation of SOD activity:

(1) The MSS values in the UVB-applied group were higher than those in the UVB-free group [24, 27].

(2) Tissue immunostaining of UVB-applied rats showed a significant increase in SOX10-labelled melanin-positive cells, with clusters distributed in the basal cell mass of hair follicles, showing a strong positive reaction. Furthermore, the density of melanocytes and coloured spot areas differed significantly based on the statistical analysis. The skin of UVB-applied rats showed obvious hyperpigmentation compared with that of the UVB-free rats.

(3) MDA is a commonly used index to measure the degree of oxidative stress, which reflects the degree of membrane lipid peroxidation. The mechanism of action of SOD is primarily associated with the removal of superoxide anion free radicals, which have harmful effects on human health. Both can clearly reflect the degree of damage caused by oxidative stress in the skin and can be effective indicators for identifying the success of modelling and evaluating the efficacy of treatment. The elevated MDA and reduced SOD levels in the UVB-applied group indicated that the degree of oxidative stress in this group was elevated. Thus, we concluded that the development of the melasma rat model was successful [28, 29].

After the successful development of the model, six rats were assigned to the treatment group and six to the control group. The two groups were also evaluated using the MSS, skin histological evaluation, MDA content of the skin homogenate, and SOD activity measurement:

(1) The MSS values in the treatment group significantly improved compared to those in the control group. Hyperpigmentation was significantly reduced in the treatment group.

(2) In the treatment group, the number of melanin-positive cells labelled with SOX10 was significantly reduced compared to that in the control group. Weakly positive reactions were observed in the basal cell mass of the hair follicles. Pathological imaging analysis of melanocyte area density in the treatment group showed a significant improvement compared to that in the control group.

(3) Inflammation was effectively controlled in the treatment group. The oxidative index was significantly reduced and antioxidant enzyme activity was significantly enhanced, indicating that oxidative stress damage in the melasma architecture was effectively reversed.

The reasons for the effectiveness of I-PRF treatment may be as follows:

(1) I-PRF can not only maintain the growth factor concentration at a higher level but also maintain it for a longer time [20].
(2) TGF-β can inhibit the production of tyrosinase, tyrosinase-related protein-1 (TRP-1), and TRP-2, reducing the production of melanin.
(3) Epidermal growth factors can inhibit the expression of prostaglandin-E2 and tyrosinase, thereby lowering melanin production.
(4) I-PRF contains a large number of leukocytes, which may ameliorate skin damage caused by oxidative stress.
From this study, we can conclude that I-PRF has a remarkable effect on melasma treatment. However, the optimal dosing concentration and frequency require further control experiments.

Conclusion

Considering several previous studies, PRP provides significant therapeutic benefits for the treatment of melasma, however, the use of anticoagulants, cumbersome preparation steps, and high risk of bacterial contamination are issues of concern.
I-PRF is a recently developed blood-derived biomaterial that lacks these limitations. Therefore, in this study, we investigated whether I-PRF is useful for the treatment of melasma in rats or not by conducting a randomised, controlled study in an animal model. The results show that I-PRF is useful in the treatment of melasma, and is a safer and cost-effective blood-derived biomaterial.
Further randomised controlled studies comparing the efficacy of I-PRF and PRP are required. The optimal dosage and frequency of use also require further investigation. Additionally, certain scholars have posited that the construction of the melasma model neglects genetic susceptibility, and it is expected that future iterations of the model will be refined accordingly. ■

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