Original Article



# Experimental study on stromal vascular fraction mediated inhibition of skin pigmentation in guinea pigs

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**Background:** Pigment disorder dermatoses are common diseases with complex mechanisms. There are various methods for the clinical treatment of pigmentation diseases, but these have a poor curative effect and many adverse reactions. Currently, looking for safe and effective whitening agents is a popular research topic. Stromal vascular fractions (SVFs) are a compound cell component of adipose-derived stem cells (ADSCs) that can promote tissue regeneration, healing, and vascularization. The purpose of this experiment was to investigate the inhibitory effect of SVFs on pigmentation in guinea pigs.

**Methods:** After guinea pig subcutaneous fat was digested and centrifuged, SVFs were isolated and quantified. SVF was injected into the pigmentation area of the prepared guinea pig pigmentation model. The amount of inducible nitric oxide synthase (iNOS) was determined using immunohistochemical analysis, histopathological staining, and the Fontana-Masson (F-M) method for measuring melanin formation.

**Results:** The skin of the guinea pigs obtained stable and homogenous coloration following three treatments with narrow-band ultraviolet B (NB-UVB). Hematoxylin-eosin (HE) staining revealed that compared to the control group, the cuticle, granular layer, and spinous layer were thicker and the number of epidermal melanocytes and melanin granules increased. While the quantity of pigment granules in the treated group dramatically decreased, it did not significantly change in the blank control group. F-M staining revealed that melanin granules greatly expanded following ultraviolet irradiation and were continuously distributed in basal cells and spinous layers. The entire epidermis was evenly covered in melanin granules. The level of melanin dramatically decreased following therapy. According to immunohistochemical labeling, epidermal cells' cytoplasm and membranes are where iNOS is primarily found. In the epidermis of the irradiated group, iNOS expression was much higher than in the control group, and following treatment, it decreased in the experimental group.

**Conclusions:** SVFs have a reliable treatment effect on ultraviolet B (UVB)-induced pigmentation in guinea pig skin. SVFs can significantly inhibit pigmentation, effectively shorten the fading time of pigmentation, and play a role in skin whitening, providing a new breakthrough for the treatment of pigmentation diseases.

**Keywords:** Stromal vascular fraction (SVF); pigmentation; narrow-band ultraviolet B (NB-UVB)

Submitted Oct 17, 2022. Accepted for publication Nov 23, 2022.
doi: 10.21037/atm-22-5433
**View this article at:** https://dx.doi.org/10.21037/atm-22-5433

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## Introduction

The regenerative cell cocktail obtained from adipose tissue by the enzymatic method, including stem cells, is called a stromal vascular fraction (SVF) (1). In freshly isolated SVFs, the percentage of adipose-derived stem cells (ADSCs) is approximately 70% to 90%, and the rest of the cells are endothelial cells and peridermal cells (2). SVFs can be used to treat aging, refractory wounds, and deep burns (3-6). Other researchers showed that SVFs and ADSCs also play important roles in promoting wound healing (7,8). Our preliminary experiment shows that SVFs have a significant inhibitory effect on melanogenesis in B16 cells induced by external factors (9). Narrow-band ultraviolet B (NB-UVB) irradiation is widely used worldwide to induce pigmentation in guinea pigs (10). In this study, a 308 nm excimer laser was used to irradiate the skin on the backs of brown-yellow guinea pigs to induce a pigmentation animal model. Melanogenesis is depended on keratinocyte-generated nitric oxide (NO), while the synthesis of NO is regulated by NOS activation [endothelial nitric oxide synthase (eNOS) and neuronal nitric oxide synthase (nNOS)] or synthesis [inducible nitric oxide synthase (iNOS)]. Therefore, the expression of iNOS is used as one of the indicators to evaluate the skin pigmentation in this experiment. After preparing and extracting SVFs, a local injection was performed in the pigmentation area to analyze and evaluate the therapeutic effect of SVFs on the pigmentation of guinea pigs. We present the following article in accordance with the ARRIVE reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5433/rc).

### Highlight box

**Key findings**

- SVFs have a reliable treatment effect on ultraviolet B (UVB)-induced pigmentation in guinea pig skin.

**What is known and what is new?**

- SVFs can be used to treat aging, refractory wounds, and deep burns.
- SVFs can significantly inhibit pigmentation, effectively shorten the fading time of pigmentation, and play a role in skin whitening.

**What is the implication, and what should change now?**

- It provides a new breakthrough for the treatment of pigmentation disease. Next, the relationship between SVF dose and efficacy should be further investigated. Further experimental studies are needed to clarify the specific mechanism of action.

## Methods

The experimental animals were provided by the Experimental Animal Center of Southern Medical University, and they were normal healthy male guinea pigs. Collagenase I was purchased from Shanghai Qiaoyuan Biological Pharmaceutical Co., Ltd. iNOS antibodies were purchased from Santa Cruz, and a streptavidin peroxidase (SP) immunohistochemistry kit was purchased from Fuzhou Maixin Biotechnology Co., Ltd. A 308 nm ultraviolet radiation (UV) therapeutic instrument was purchased from Chongqing Dema Photoelectric Technology Co., Ltd. Animal experiments were performed under a project license (No. SUMC2017-177) granted by the Animal Research Committee of Shantou University Medical College, in compliance with Chinese national guidelines for the care and use of animals. A protocol was prepared before the study without registration.

### *Experimental design*

Ten guinea pigs with large back areas, aged 2–3 months, and weighing 329–410 g, were selected. Two areas were selected on the back of each animal, one of which was depilated and irradiated with ultraviolet light. Hair was retained in the other area as a normal control. After the pigmentation model was completed, the pigmented area of the back was divided into 2 parts that were, on average, the same size. One part was injected with normal saline for the control group, and the other part was injected with SVFs for the experimental group. The normal control group, saline injection group, and SVFs group were evaluated using the naked eye and histopathological staining. Melanin synthesis was evaluated using the Fontana-Masson (F-M) method. Immunohistochemical analysis was performed to measure the content of iNOS (*Figure 1*). The content of iNOS and melanin synthesis were the main indexes of this study, and the secondary indexes were naked-eye observation and hematoxylin-eosin (HE) staining results.

### *Inclusion and exclusion criteria*

Ten healthy adult guinea pigs were randomly selected. The guinea pigs that completed the model of skin pigmentation entered the next experiment. If the model failed, such as if the guinea pig developed severe burns, an infection, or unstable pigmentation on the skin, or the animals died, those animals were withdrawn from the experiment.

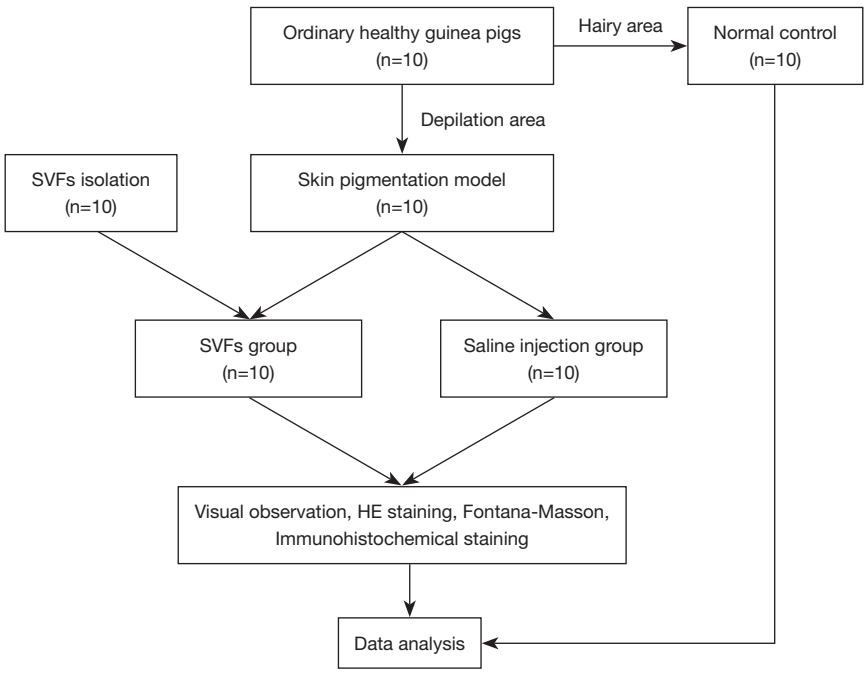


**Figure 1** Experimental design. SVFs, stromal vascular fractions; HE, hematoxylin-eosin.

### *Study methods*

#### A guinea pig pigmentation model was established by irradiating the backs of guinea pigs with a 308 nm ultraviolet therapy apparatus

The treatment head of the ultraviolet instrument with a wavelength of 308 nm was placed in direct contact with the exposed skin on the backs of the guinea pigs. The irradiation energy was 600 mJ/cm$^2$ each time, the stimulation was performed once a week for a total of 3 times, and the total irradiation energy was 1,800 mJ/cm$^2$. Skin redness was observed on the day of irradiation, obvious erythema was observed the next day, skin desquamation was observed 3–5 days later, pigmentation was observed 5–7 days later, and pigmentation reached its peak 3 weeks later, with uniform and stable pigmentation. The research team monitored the animals twice a day. Health was monitored through overall assessments of body weight (twice a week), food and water intake, animal activity, and wheezing and fur status. The score criteria of skin pigmentation are as follows: normal skin color is 0, light brown is 1, medium brown is 2, dark brown is 3.

#### SVF isolation

The guinea pigs were anesthetized using an intraperitoneal injection of 3% pentobarbital sodium with a dose of 40 mg/kg. The inguinal fat of the guinea pigs was removed with a routine aseptic operation. We washed the subcutaneous fat of the guinea pigs with green streptomycin in phosphate-buffered saline (PBS) 3 times and trimmed the tissue with sterilized scissors. We then added PBS to remove the upper oil fraction at 37 ℃ and added 0.25% type I collagen enzyme on a 250 rpm thermostatic table for 20–30 min to digest the tissue. The SVF of the hybrid cell group was observed by microscope and counted by counter. After SVF extraction, a cell suspension was made with 1 mL of normal saline, which was injected into the pigmentation area. One milliliter of normal saline was injected into the skin in the saline group.

#### Evaluating melanin synthesis using the F-M method

Guinea pig skin tissue was fixed with a 10% neutral formaldehyde solution and then paraffin-embedded and sectioned. Paraffin sections were dewaxed with water, cleaned with distilled water, treated with a silver ammonia solution in the dark, and incubated in the light at room temperature for 12–18 h. The excess silver particles were washed away. The slices were fixed in a 5% sodium thiosulfate solution for 5 min, washed with water for 5 min, re-dyed with hematoxylin solution for 1 min, differentiated with 1% hydrochloric acid ethanol for 10–15 s, and

 

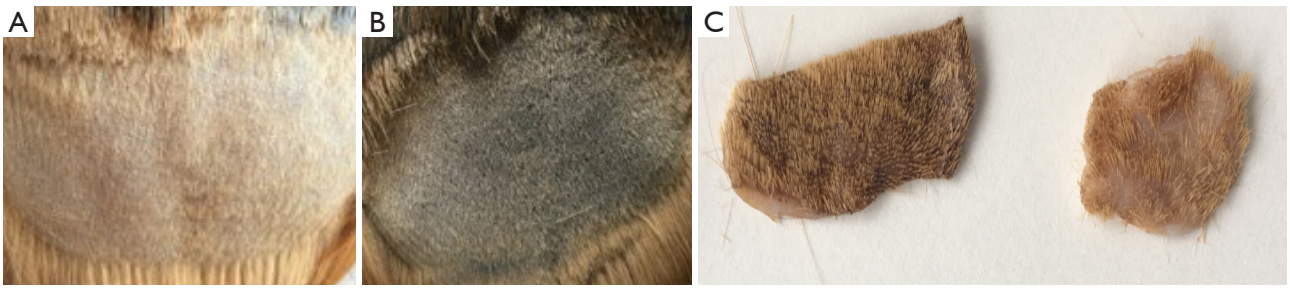


**Figure 2** Animal photos. (A) Normal skin. (B) Pigmentation after UVB308 irradiation. (C) Pigmented skin vs. normal skin.

returned to blue with ammonia for 10 s. Eosin solution was added and incubated for 30–60 s, conventional dehydration was performed, xylene was used to induce transparency, neutral gum sealing was performed, and inverted microscopy was used for observation. The evaluation criteria were divided into 5 grades: grade 1 was melanin particles occasionally seen in basal cells and spinous layer, grade 2 was discontinuous distribution of melanin, mainly concentrated in basal layer, and grade 3 was continuous banded melanin granules, mainly concentrated in basal cells and spinous layer. Grade 4 is a continuous banded distribution of melanin particles concentrated in the basal cells and spinous layer, and more melanin caps can be seen in keratinocytes; grade 5 melanin particles are densely distributed in the epidermis and more melanin caps.

### iNOS levels in the guinea pig skin were detected using immunohistochemistry

The procedure was carried out in strict accordance with the kit instructions, and diaminobenzidine (DAB) was used for color rendering. Immunohistochemical staining was performed on all samples using the SP method, and antigen repair was performed in a microwave oven before staining. PBS was used as a negative control. The scoring criteria of iNOS staining were as follows: no staining of cells was 0, dark yellow was 1, light brown was 2, dark brown was 3.

### *Statistical analysis*

SPSS 22.0 (IBM Corp, Armonk, NY, USA) was used for statistical analysis. The data are expressed as the mean ± standard deviation ($\bar{x}$ ± s). A $t$ test was used for pairwise comparisons, and the rank-sum test was used for comparisons of grade data between 2 groups. A value of $P<0.05$ was considered statistically significant.

## Results

After NB-UVB irradiation 3 times, stable and homogeneous pigmentation formed in the skin of the guinea pigs (*Figure 2*). After 4 weeks of treatment, SVFs obviously improved pigmentation in the inflamed skin of guinea pigs. The control group did not exhibit significant changes (*Figure 3*).

HE staining showed that epidermal melanocytes and melanin granules increased, and the cuticle, granular layer, and spinous layer were thicker compared with those in the control group. Inflammatory cells infiltrated occasionally into the dermis. After treatment, the number of pigment granules was significantly reduced, and a small number of melanin granules were observed in the basal layer and the spine layer (*Figure 4*). The pigment granules in the blank control group did not change significantly.

Melanin synthesis was evaluated. F-M staining showed that there were occasional melanin granules in basal cells and the spinous layers of the normal brown skin of the guinea pigs. After ultraviolet irradiation, the number of melanin granules increased significantly, and a large number of melanin granules was continuously distributed in basal cells and the spinous layers. Melanin granules were densely distributed in the entire epidermis. After treatment, melanin was significantly reduced. After 4 weeks of follow-up, there was no significant recurrence of melanin (*Figure 5*).

Immunohistochemical staining showed that iNOS was mainly located in the cytoplasm and membrane of epidermal cells. Positive cells were found in all epidermal layers and hair follicles except the stratum corneum. The expression of iNOS in the epidermis in the irradiated group was significantly higher than that in the control group, and the expression of iNOS decreased in the experimental

 

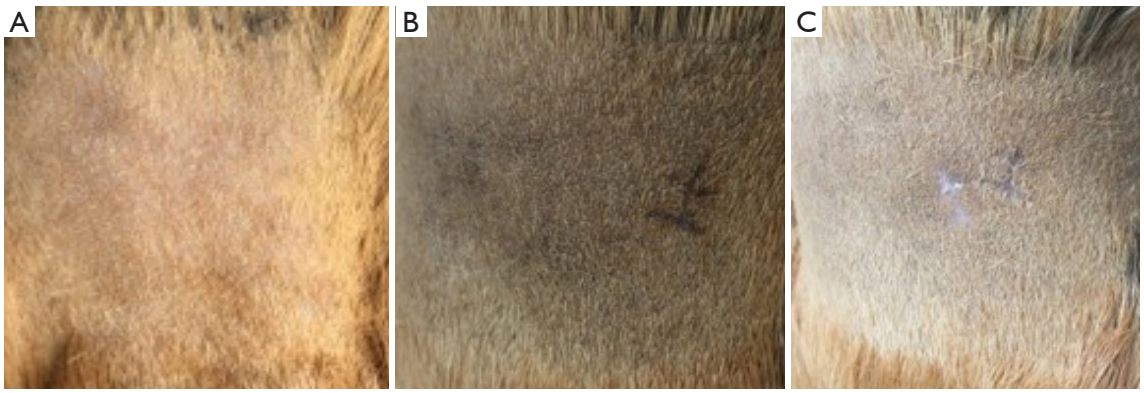


**Figure 3** Animal photos. (A) Normal skin. (B) Skin pigmentation after ultraviolet radiation. (C) SVFs were administered by an intradermal injection for 1 month. SVFs, stromal vascular fractions.

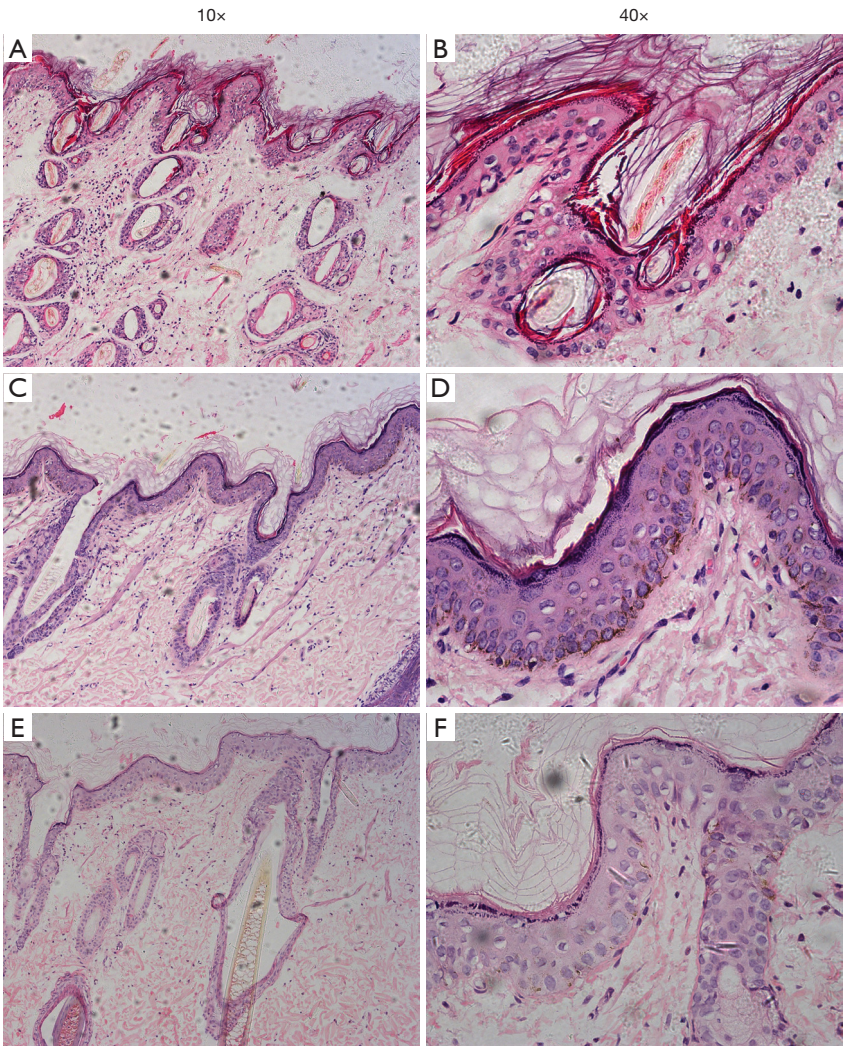


**Figure 4** HE staining of normal skin (A, 10×; B, 40×). HE staining of hyperpigmented skin (C, 10×; D, 40×). HE staining after SVF treatment (E, 10×; F, 40×). HE, hematoxylin-eosin; SVF, stromal vascular fraction.

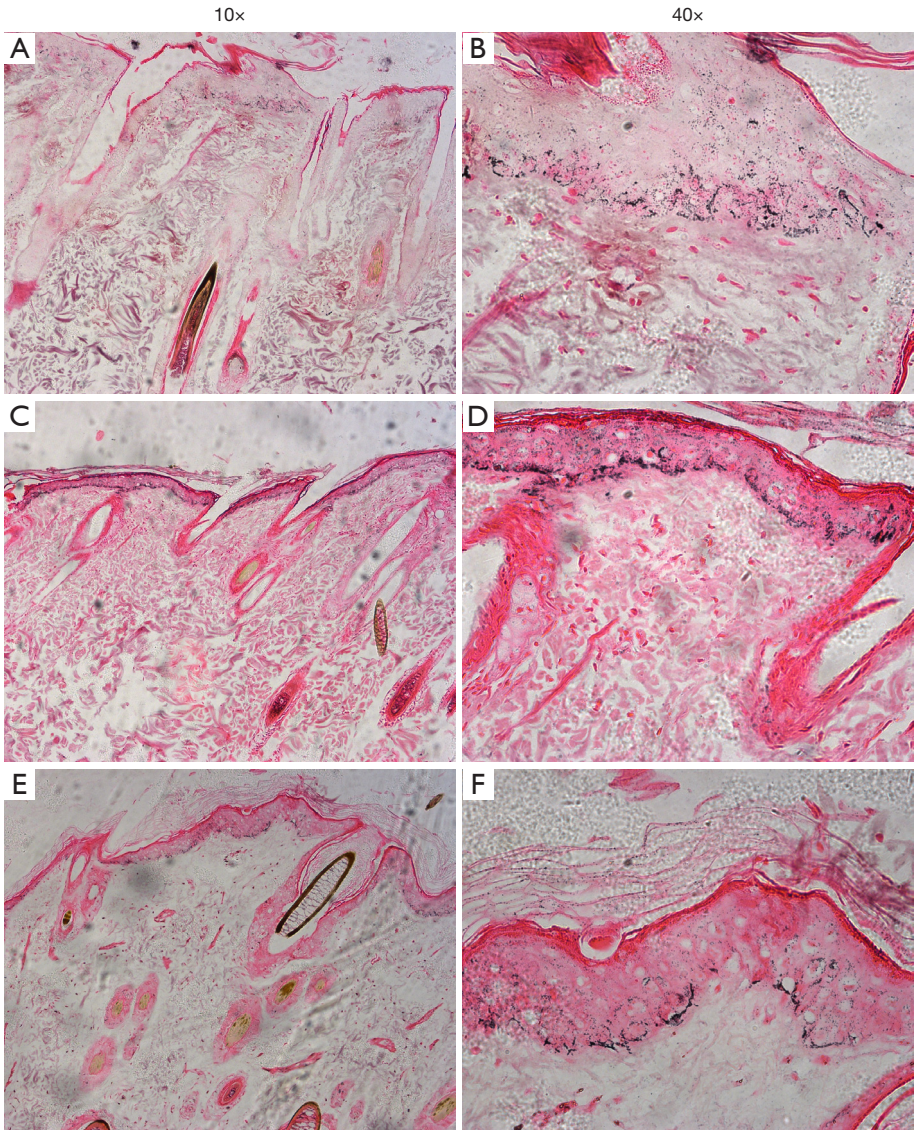


**Figure 5** Distribution of epidermal melanin granules in normal guinea pigs (Fontana-Masson; A, 10×; B, 40×). Distribution of melanin granules in guinea pig epidermis after pigmentation (Fontana-Masson; C, 10×; D, 40×). Distribution of epidermal melanin granules in guinea pigs after treatment (Fontana-Masson; E, 10×; F, 40×).

group after treatment (*Figure 6*).

Uniform and stable pigmentation were found on the back skin of guinea pigs after UVB irradiation (n=10). Photographs taken 4 weeks after treatment showed that SVFs significantly improved the pigmentation of guinea pigs (n=10), while there was no obvious change in the control group (n=10). The pigmentation scores of the 2 groups were statistically significant (P<0.05; *Table 1*).

F-M staining showed a significant increase in the number of melanin particles in the model group after UV irradiation (n=10) and a significant decrease in the number of melanin particles after treatment (n=10). The rank-sum test of melanin particle levels compared with those of the control group showed a significant difference (P<0.05; *Table 1*).

Immunohistochemical staining showed that the expression of iNOS in the epidermis increased in the UV irradiation group (n=10), and the expression of iNOS decreased in the experimental group after treatment (n=10). The levels of iNOS in the experimental group and the control group were significantly different (P<0.05; *Table 1*).

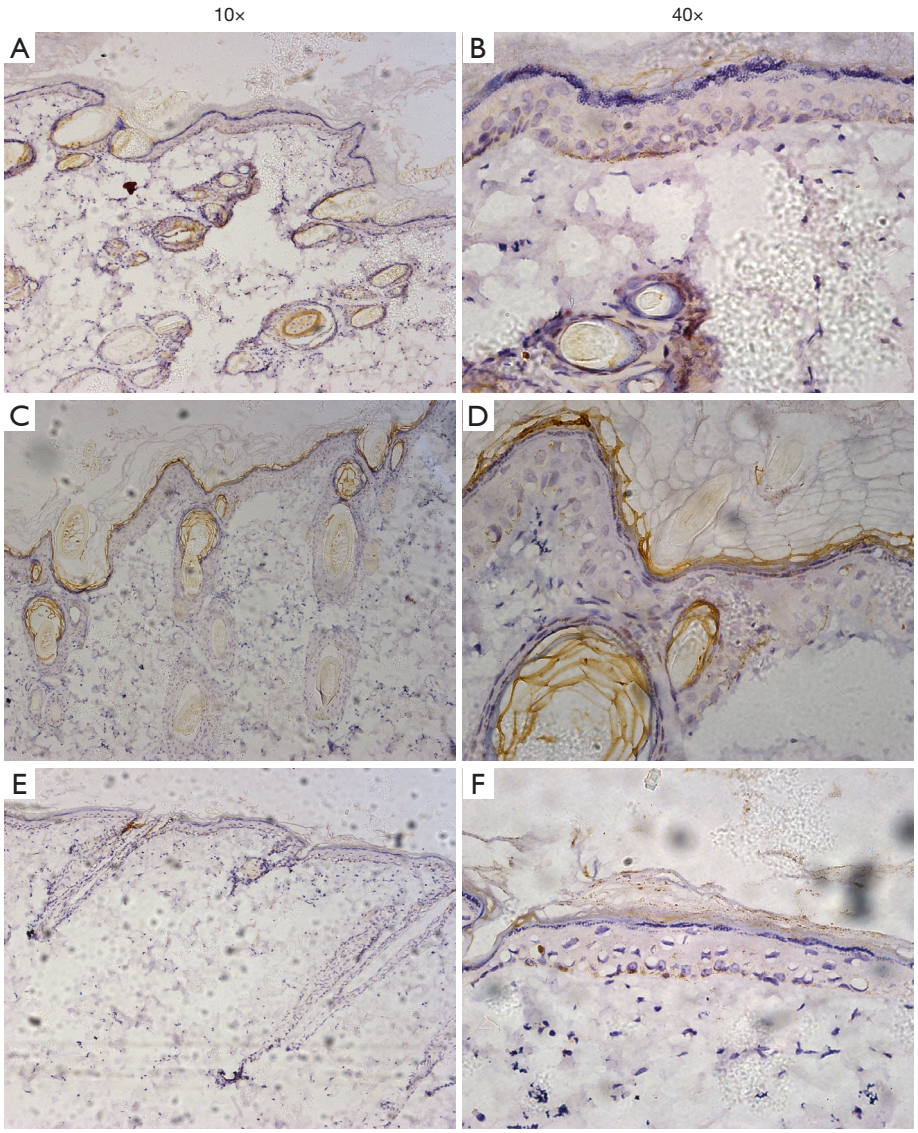


**Figure 6** Expression of iNOS in normal skin (SP method; A, 10×; B, 40×). Expression of iNOS in hyperpigmented skin (SP method; C, 10×; D, 40×). Expression of iNOS in the skin after treatment (SP method; E, 10×; F, 40×). The staining method is SP method of immunoenzyme labeling method. iNOS, inducible nitric oxide synthase; SP, streptavidin/peroxidase.

**Table 1** Pigmentation score, melanin granule content, and iNOS content in guinea pig skin (median)

| Group number | Pigmentation score | Melanin granule content | iNOS content |
|---|---|---|---|
| SVFs group (n=10) | 1.5 | 2.5 | 1.50 |
| Saline group (n=10) | 2.5 | 3.5 | 2.25 |

iNOS, inducible nitric oxide synthase; SVF, stromal vascular fraction.

## Discussion

Tyrosinase is a rate-limiting enzyme that determines the rate of melanin synthesis. This enzyme exists widely in animals and plants. The activity of tyrosinase is proportional to the amount of melanin. Studies on the function of tyrosinase have been carried out in many fields, such as medicine, cosmetics, chemistry, pharmacy, and agriculture (11,12). SVFs have a significant inhibitory effect on melanogenesis in

B16 cells induced by external factors, and the effect becomes more obvious with increasing doses (9).

After UVB irradiation, melanocytes and keratinocytes activate tyrosinase by releasing oxidative stress products or expressing various ligands, promoting melanin synthesis and melanosome transport and affecting pigment synthesis (13,14). UVB irradiation is the main stimulus leading to skin pigmentation. SVFs can achieve skin whitening by inhibiting pigmentation.

The reserves of SVFs in human adipose tissue are very large and easy to obtain, and there is no immune rejection of autologous applications. Moreover, freshly isolated SVFs from adipose tissue contain a sufficient amount of autologous ADSCs with biological activity (15). More importantly, the procedure can be completed in one session without an *in vitro* culture, is highly safe, and is easy to perform in clinical practice.

Melanin synthesis is not only influenced by tyrosinase but is also finely regulated by various other cytokines, including nitric oxide. Roméro-Graillet *et al.* (16) found that UVB could promote keratinocyte production of NO, thus regulating melanocytes. Nitric oxide exists for a very short time and is not easy to measure. Nitric oxide synthase is a key enzyme in the synthesis of nitric oxide. Many studies suggest that (17-19). UVB irradiation can incrementally regulate the expression of iNOS, and the increased expression of iNOS can result in the generation of a large amount of nitric oxide. The expression of iNOS decreased after the injection of SVF.

## Conclusions

Currently, tyrosinase inhibitors are the main effective ingredients in most cosmetics or whitening agents. Searching for tyrosinase inhibitors with lower toxicity and better whitening effects than that of current treatments is a developing trend in the treatment of pigmentation. By inhibiting tyrosinase activity, SVFs had a good effect on UV-induced pigmentation in guinea pig skin, and the therapeutic effect was reliable. This study may provide a breakthrough for the clinical treatment of pigmentation disorders. Further experimental studies are needed to clarify the specific mechanism of action.

## Acknowledgments

*Funding:* None.

## Footnote

*Reporting Checklist:* The authors have completed the ARRIVE reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5433/rc

*Data Sharing Statement:* Available at https://atm.amegroups.com/article/view/10.21037/atm-22-5433/dss

*Conflicts of Interest:* All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-5433/coif). The authors have no conflicts of interest to declare.

*Ethical Statement:* The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. Animal experiments were performed under a project license (No. SUMC2017-177) granted by the Animal Research Committee of Shantou University Medical College, in compliance with Chinese national guidelines for the care and use of animals.



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(English Language Editor: C. Mullens)

**Cite this article as:** Shen JP, Wu YX, Tang SJ, Peng LH. Experimental study on stromal vascular fraction mediated inhibition of skin pigmentation in guinea pigs. Ann Transl Med 2022;10(23):1268. doi: 10.21037/atm-22-5433

 