The Therapeutic Effects of Baicalin on Vitiligo Mice

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Abstract

Vitiligo is a common disease of skin. Its pathogenesis is complex, resulting in the incapacity to find a targeted cure. Baicalin, which is isolated from Scutellariae radix, has been known to exhibit a number of pharmacological effects on autoimmune diseases. In this study, we explored the effects of baicalin on the recovery of vitiligo stimulated by monophenylketone in mice. We observed that Baicalin slowed down the progression of depigmentation, decreased the incidence of depigmentation, and reduced the area of depigmentation. Moreover, reflectance confocal microscopy (RCM) shown that Baicalin increased the epidermal melanocytes in depigmented skin. Baicalin decreased CD8+ T cell infiltration in mice skin, and decreased the expression of CXCL10 and CXCR3. Baicalin significantly decreased the levels of serum cytokine (IL-6, TNF-α, IFN-γ, and IL-13). Collectively, these data suggest that Baicalin play an important role in the occurrence and development of vitiligo.

Key words: Baicalin, Monobenzone, vitiligo
Introduction

Vitiligo is a skin disorder that does not cause fatality but results in significant psychosocial consequences. It is characterized by the progressive depigmentation of skin sections. The cause of this disease remains to be determined; however, various factors have been suggested to be involved in its pathogenesis. Recent work has suggested that a specific cytotoxic T-cell (CD8+T cell) reaction is responsible for the destruction of melanocytes (1). Moreover, melanocyte-specific cytotoxic CD8+T cells have been detected in the peripheral blood and skin lesions (2-3), and the quantity of melanocyte-specific cytotoxic CD8+T cells correlates with disease severity (4-6). CXCL10 could combine with its specific receptor CXCR3 to activate T cells and exhibit the function for mediating immune responses. CXCL10 and CXCR3 were over-expressed in various autoimmune diseases, and they involved in T cells homing into the diseased tissues to aggravate the degree of tissue damage (7-8). Recent observations support that cytokines acted as a vital factor in the nosogenesis of vitiligo. Compared to healthy control skin, the expression of TNF-α, IFN-γ, and IL-10 significantly higher than vitiligo-affected and perilesional skin.(9). These studies confirmed the participation of CD8+T cells and cytokines in the pathological mechanisms of vitiligo. Baicalin is a main active ingredient derived from the radices of Scutellaria baicalensis Georgi, which is a commonly used herbal medicine. It is a traditional Chinese herbal medicine with several pharmacological effects, such as anti-oxidation, anti-tumor, and anti-inflammation, and so on (10-12). In the present study, we exploited our animal model for of vitiligo to evaluate the potential use of Baicalins cytotherapy to induce recovery of from vitiligo lesions and inhibit the occurrence and development of vitiligo.

Materials and methods

Mice. All animal procedures were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. Female C57BL/6 mic (4-5 weeks old, 10-15 g) were obtained from SLRC Laboratory Animal (Shanghai, China). The mice were kept at room temperature (20-25 °C) and humidity (40-50%) in microisolator cages with sufficient supply of food and water.

Vitiligo model and Baicalin transplantation. Female SPF C57BL/6 mice at 4 weeks of age, were divided into four groups randomly, group I - III contained 15 mice in each group and 50 mg of oil-in-water (O/W) emulsion base were used respectively every day (1). In group II - III, mice were treated with 40% monobenzone cream (4-benzyloxyphenol, Sigma) cream were prepared by the Hangzhou Third people's Hospital Medical Centre pharmacy for use in animal experiments, shaved 2×2 cm on the abdomen.
spatulas were used to massage the creams completely. The mice were intraperitoneally injected with 2 mg of baicalin (Sigma-Aldrich) in 100 μL of 5% NaHCO3 aqueous solution every day in group III.

**Depigmentation evaluation.** In treatment groups, the depigmentation degree was objectively quantified by observers. We examined each exposed location and estimated the depigmentation degree as a percentage of the anatomic site. Points were awarded as follows: no evidence of depigmentation was defined as 0 point; >5% was defined as 1 point; >5–25% was defined as 2 points; >25–50% was defined as 3 points; >50–75% was defined as 4 points; and 75–100% was defined as 5 points. The sum of the scores was the depigmentation score. The average individual score of the mice in that group represents the vitiligo score of each experimental group.

**Reflectance confocal microscopy.** RCM (Vivascope 1500) was performed for medical imaging applications in vivo. Vivascope 1500 contained a diode laser at 830nm wavelength and power <35mW at the tissue level. It also has a×30 objective lens with 0.9 numerical aperture. To reduce motion artifacts, an adhesive ring allowed the xation of the optic to the tissue. The immersion medium between adhesive plastic window and skin was distilled water. and ultrasound gel was filled between plastic window and lens owing to their refractive indexes close to the epidermis (1.34). Compared with the surrounding skin structures (1.4), elainin has a high reflectance index (1.7) to produce a strong backscatter that visualize the melanocytes and pigmented keratinocytes clearly. It could further observe the cellular structures of the specimens in vivo owing to the images with a spatial resolution of 0.5–1.0mm in the lateral and 3–5 mm in the axial dimension. (13-14).

**Histological examination.** In order to detect the tissues from the perilesional skin of the vitiligo lesions and healthy skin by using microscopic. Tissue samples were taken out and formalin-fixed. Then embedded in paraffin, sectioned, and stained with hematoxylin and eosin were conducted according to standard methods.

**Immunofluorescence for confocal laser-scanning microscopy.** The perilesional skin of the vitiligo lesions and healthy skin were formalin-fixed and embedded in paraffin. Then the paraffin-embedded section (4-μm) were deparaffinized using xylene and ethanol, washed thrice for 2 min with distilled water and soaked for 5 min with PBS. Furthermore, antigen retrieval was performed in a microwave oven for 30 min and washed thrice for 2 min using distilled water, After that, we washed antigen retrieval with PBS twice for 2 min, and blocked for 30 min with serum. After washing with PBS, the sections were incubated in the rabbit anti-CD8 monoclonal antibody (ZSGB-BIO, China) overnight at 4°C. After that the sections were washed with PBS thrice for 5 min and incubated with fluorescein isothiocyanate-conjugated goat anti-mouse antibody (ZSGB-BIO, China) for 2 h and washed with PBS thrice for 5 min. At last, we mounted the sections under a no. 0 coverslip and using a glycerol-based mounting medium to cover
sections. Confocal laser scanning microscop were carried out to capture the images of immunofluorescence.

Cytokine Analysis. Enzyme-linked immunosorbent assays (ELISA) were performed to detected the cytokines levels in serum in recipient mice, including IL-6, TNF-α, IFN-γ, and IL-13 using commercial kits (MultiSciences Biotech Co, Ltd, China).

RNA isolation and real-time RT-PCR analysis.
SV total RNA purification kit (Promega, Shanghai, China) was carried out to extract total RNA from the perilesional skin of the vitiligo lesions from all groups. QuantiTect Reverse Transcription Kit (Qiagen, Germany) was used to reverse transcript reaction. Then Real time PCR was carried out using QuantiFast SYBR Green PCR Kit (Qiagen). β-actin was performed as endogenous control. The results were analyzed using the comparative Ct method, and expressed as percentage of control, with the control as 1.

Statistical analysis. All values are presented as means ± S.E.M. The differences between mean values of normally distributed data were estimated by one-way ANOVA (Dunnett's t-test) and two- tailed Student's t-test. *P<0.05 indicated significant differences were noted by ns.

Results

Intragastric administration of Baicalin promoted remission, and slowed down the occurrence and progression of vitiligo.

On the 15th day, in 11 of 15 mice in the model group, in the drug-exposed area we observed small white patches first and then gradually expanded, whereas no patches were discovered in group III. We found that distant white patches (depigmentation of non-exposed sites) were visible on the trunk, ears, and/or tail of the majority of mice on the 23rd day after monobenzone treatment (Fig. 1a-c). The incidence in the model group was 73.33%, much higher than that of group III, which was 26.67% (Fig. 1d). There was a remarkably difference between groups III and II. At non-exposed sites, we observed that the occurrence time and the incidence of depigmentation patches were delayed, and the depigmentation area score in group III was decreased significantly (Fig. 1e-f), indicating that Baicalin may prevent the occurrence and growth of vitiligo.
Figure 1. The occurrence and development of vitiligo were delayed by Baicalin in mice. (a-c) After grafting, representative images of the depigmentation at Days 40 were shown compared among the three groups. The C57BL/6 mice that treated with monobenzone cream developed depigmentation on the monobenzone exposed site (red circle) and nonexposed site (red arrow). (d) The depigmentation suppressed decreasingly in the mice occurred after treating with Baicalin. (e) Baicalin retarded the occurrence of vitiligo. (f) The depigmentation degree also alleviated due to Baicalin. (Color figure can be accessed in the online version.)

Intragastric administration of Baicalin promoted melanocyte formation in vitiligo lesions.

We further examined melanocyte content in mice using in vivo reflectance confocal microscopy (RCM).
The dermal papillary containing melanocytes and pigmented keratinocytes appeared as bright refractive granules under RCM in the untreated mice (Fig. 2a). The dermal papillary of the skin with depigmentation indicated lower number of bright granules in group II, suggesting the decrease of melanocytes and pigmented keratinocytes (Fig. 2b). In group III mice, the skin containing melanocytes and pigmented keratinocytes, exhibited bright refractive granules and dendrites under RCM (Fig. 2c).

**Figure 2.** (a) Respectively image of the healthy skin at the level of the dermoepidermal junctionin were captured by in vivo reflectance confocal microscopic (RCM) (group I ). (b) Bright refractive granules were observed in model group show as melanocytes and pigmented keratinocytes (red arrows). (c) In group III mice, the skin consisting of melanocytes and pigmented keratinocytes, exhibited as bright refractive granules and dendrites under RCM. (Color figure can be accessed in the online version.)

**Intragastric administration of Baicalin inhibited leukomonocytes and CD8⁺ T cells in vitiligo lesions.**

Histochemical assay suggested that the leukomonocyte infiltration was increased in the perilesional skin of distant depigmentation in group II (Fig. 3b). Only a small amount of leukomonocyte infiltration was observed in in group I and III (Fig. 3a, c).

The infiltrating lymphocytes included a substantial amount of CD8⁺ T cells in group II were measured by immunofluorescence staining with an anti-CD8 antibody(Fig. 3e). CD8⁺ T cells were significantly reduced in group III (Fig. 3f), and few CD8⁺ T cells were observed in healthy skin (group I) (Fig. 3d).
Figure 3. Hematoxylin and eosin staining were performed to detected the presence of leukomonocyte, and immunofluorescence staining for CD8\(^+\) T cells in depigmentation skin sections. (a) Few leukomonocytes were observed in the healthy skin. (group I) (b) The results demonstrated that the leukomonocyte levels were remarkably increased in the perilesional skin of the progressive vitiligo lesions. (c) Compared with group II, the leukomonocytes were significantly decreased in group III. (d) In the healthy skin, we found only few CD8\(^+\) T cells in group I. (e) In group II, the infiltrated lymphocytes contained a substantial amount of CD8\(^+\) T cells. (f) Compared with group II, the CD8\(^+\) T cells were significantly decreased in group III.

(Color figure can be accessed in the online version.)

**Baicalin Could Reduce Expression of Chemokine CXCL10 and Its Receptor CXCR3**

As demonstrated in Figure 4, high expression of CXCL10 and CXCR3 were obtained in the group II. Contrast to the model group (group II), obviously reduced levels of CXCL10 and CXCR3 was observed in the drug-treating group (group III). The level of CXCL10 and CXCR3 was lower in the mice of the control group (group I). Whereas Baicalin could inhibit the increase of CXCL10 and CXCR3.
Fig. 4. Real-time polymerase chain reaction were carried out to validate differentially expressed genes. The expression of CXCL10 and CXCR3 was lower in the mice of the control group (group I) and the drug-treating group (group III). High expression of CXCL10 and CXCR3 was observed in the group II. *P<0.05 vs. control group  # P<0.05 vs. model group
(Color figure can be accessed in the online version.)

**Intragastric administration of Baicalin inhibited inflammatory cytokines.**

Cytokines (IL-6, TNF-α, IFN-γ, and IL-13), analyzed from serum samples after the application of monobenzone cream, were significantly increased in monobenzone challenged animals when compared with those of the untreated group. This reached a maximum at the 25th day, when depigmentation on non-exposed sites became evident, and declined thereafter (Fig. 5). Baicalin treatment efficiently prevented the monobenzone-induced increase in IL-6, TNF-α, IFN-γ, and IL-13 when compared with cytokine levels in group II (Fig. 5).
Figure 5. Effects of Baicalin on concentrations of IL-6, TNF-α, IFN-γ, and IL-13 in serum of vitiligo mice. On days 15, 25, and 50, we collected the serum and the inflammatory cytokines IL-6 (a), TNF-α (b), IFN-γ (c), and IL-13 (d) were detected, which were significantly suppressed by Baicalin. # P<0.05 vs. control group * P<0.05 vs. model group (Color figure can be accessed in the online version.)

Discussion

Vitiligo is a common skin disorder characterized by progressive depigmentation of the skin due to selective loss of melanocytes, yet few successful therapies are currently available. Therefore, it is essential to find a safe and effective natural medicines.

Baicalin, a small-molecule monomer, which is derived from the dried root of Scutellaria, exhibits
anti-inflammatory, anti-oxidant, anti-apoptotic, and antibacterial properties. Baicalin or berberine loaded ultradeformable vesicles, which were able to incorporate high amounts of baicalin and berberine, and promote their skin permeation. It showed remarkable antioxidant and photoprotective capabilities, presumably correlated with the stimulation of melanin production and tyrosinase activity (15). Based on its anti-inflammatory activity, baicalin is widely used to treat various inflammatory diseases including atopic dermatitis and asthma (16). It was reported that baicalin could decrease expression of transforming growth factor-β1, IL-13 and vascular endothelial growth factor (17).

Besides, a study on a mouse model of vitiligo induced by monobenzone has reported previously (18). In this study, tyrosinase expression is dramatically decreased in the mice treated with monobenzone, which is similar to that in vitiligo patients' lesional skins. In healthy people who apply monobenzone to acquire a lightened skin tone, this process can induce depigmentation that is clinically and histologically indistinguishable from vitiligo (19). We demonstrated that depigmentation of exposed and found non-exposed sites on the trunk, ears, and/or tail of mice after treating with prolonged, indicating a systemic immune reaction to autologous melanocytes. Therefore, we used this model to detect Baicalin effect on the vitiligo. Baicalin could efficiently alleviate vitiligo severity in mice induced by monobenzone, through prolonging the time to depigmentation, reducing the ratio of the mice that developed depigmentation distantly, and alleviated the degree of depigmentation and repigmentation. Based on these results, Baicalin could be used as a prophylactic, mitigating, and therapeutic approach to vitiligo.

It has been reported that histological analysis of perilesional margins surrounding depigmented skin displays infiltration of activated T cells and other lymphocytes (20). Further, It had been demonstrated that the infiltration of active CD8+T cells takes place in the vitiligo perilesional margins (21). Furthermore, those CD8+T cells had significantly higher activation levels and higher cytotoxicity to autologous melanocytes than their counterparts from peripheral blood samples (21). These studies have suggested that cytotoxic CD8+T cells play a major role in the pathogenic mechanism of vitiligo. In this study, we found that monobenzone-induced depigmentation in mice also induced a loss of melanocytes in the lesional skin and CD8+T infiltration in the perilesional area, which was similar to lesions of human vitiligo. Baicalin showed significant effects on monobenzone-stimulated histopathological changes in skin by reducing inflammatory infiltration, especially CD8+T cell infiltration. The results reflect the idea that Baicalin could
attenuate vitiligo severity by inhibiting CD8+ T cell proliferation and inducing CD8+ T cell apoptosis. It indicates that Baicalin could be a potential candidate for interference in depigmentation.

C-X-C motif chemokine ligands CXCL9, CXCL10 and CXCL11, and its receptor CXCR3 are linked to the Th1 pattern. It has been suggested as one of the most relevant chemokine axes, which promote T cell migration in different autoimmune and inflammatory processes (22). It has been proved that CXCL9, CXCL10 and CXCR3 are elevated in vitiligo patients with an increased expression in active phase (23-24). The keratinocytes were reported to produce CXCL9 and CXCL10, which binds to its specific receptor CXCR3, could be able to attract CD8+ cells (25). In further, we explored the expression of CXCL10 and CXCR3. In consistent, highly increased expression of CXCL10 and CXCR3 was found in the mice treated with monobenzone. Contrast to the model group, Baicalin could obviously reduce the expression of CXCL10 and CXCR3.

More and more evidences shown that cytokines function as a vital factor in the pathogenesis of autoimmunity and play a role in depigmentation (26-27). The study by Swope et al. (28) indicated that cytokines including IL1-α, TNF-α, and IL-6 could inhibit melanocyte proliferation. The generation of proinflammatory cytokines including IL-1β, IL-6, IL-8, and TNF-α in patients with active vitiligo were remarkably enhanced compared with the control group (29). Increased concentrations of serum IL-13 are observed in vitiligo patients (30). Therefore, it is very vital for treatment of vitiligo to decrease inflammatory cytokines. In the present study, we revealed increased levels of serum IL-6, TNF-α, IFN-γ, and IL-13 in the model group, supporting the hypothesis that cytokines may involve in immunologic events in the pathogenesis of vitiligo. We also found that Baicalin could dramatically inhibit IL-6, TNF-α, IFN-γ, and IL-13. According to the results, Baicalin may be benefit for inhibiting cytokine secretion in vitiligo.

In summary, it is demonstrated that Baicalin has a potential therapeutic effect on depigmentation. Baicalin slowed down the appearance of depigmentation, lessened depigmentation incidence, and decreased the depigmentation area. Baicalin significantly receded histopathologic changes in the mice skins and inhibited the infiltration of CD8+ T cells and expression of chemokines CXCL10 and CXCR3. It also showed that Baicalin could significantly inhibit the level of inflammatory mediators. Our data indicated that Baicalin may be a potential agent for vitiligo treatment.
Acknowledgments

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Conflict of Interest

The authors declare no conflict of interest.
References


