Abstract

Keishibukuryogankayokuinin (KBY) is a traditional Japanese herbal medicine widely used to treat skin pigmentation. The scientific basis for its use is, however, unclear, and studies evaluating its mechanism and effectiveness are sparse. In this study, we compared the tyrosinase inhibitory effects of KBY and Keishibukuryogan (KB, which has the same composition of KBY, except Coix Seed [CS]) and CS under exposure to UV radiation as well as under non-exposure conditions. Neonatal human epidermal melanocytes obtained from a darkly pigmented donor were used. These cells were cultured in a final concentration of 500 μg/ml or 1000 μg/ml, to which KBY, KB, and CS were added. After incubation for 72 h, cells were stained with Fontana-Masson stain and counted. Tyrosinase activity was measured by its dopa oxidase activity, and tyrosinase expression was estimated using real-time PCR. For UV radiation, cells were exposed to UVB radiation for 90 s per day for 3 days. Under non-exposure conditions, tyrosinase activity significantly increased with both KBY and KB but significantly decreased with CS, regardless of the concentration. In addition, tyrosinase expression significantly decreased but only with KBY at both concentrations. Under UV radiation exposure, tyrosinase activity significantly increased with KBY and KB at both concentrations while tyrosinase expression significantly decreased with KBY and KB; a significant increase was, however, observed with CS at both concentrations. These results suggest that taking KBY after sunburn is effective against skin pigmentation, and the combination of KB and CS is useful for skin depigmentation.

Keywords

Keishibukuryogankayokuinin, Coix Seed, UVB, Tyrosinase, Melanocytes
1. Introduction

Recently, the aging population in Japan has become a growing social problem [1] [2]. As life expectancy increases, it is important that we not only live longer, but also enjoy a high quality of life. Aging and the quest to “maintain a youthful appearance” have therefore attracted a heightened interest from not only the point of view of science, but also from society; the latter has been occurring throughout human history [3]. Among others, senescence of the skin affects appearance. In fact, spots and wrinkles due to photoaging, are major factors that indicate aging [4]. Rexbye et al. reported that a youthful facial appearance correlates with prolonged survival and that several environmental factors are associated with facial aging [5]. Furthermore, it has been known that skin conditions, such as dermatitis or skin rash, impact our psychological and physical functions in daily life [6] [7]. Lifestyles that prevent facial skin aging therefore lead to overall improvement in one’s health [8] [9]. Although skin aging is associated with one’s appearance, preventing and treating aging skin improve one’s mental health and the health of the body.

The color of the skin surface is not uniform. If the skin’s internal pigment such as melanin and hemoglobin is excessively secreted, it is perceived as spots, color irregularity or dullness, and spots especially are pigmentation caused by excessive secretion of melanin in the epidermis [10]. Although the mechanism of pigmentation is not completely clear, tyrosinase has been identified as a rate-controlling enzyme [11] [12]. In fact, many cosmetics and quasi-pharmaceutical products are sold as whitening agents for spots which inhibit the activity or reduce the expression of tyrosinase [11]. It is also noteworthy that contact dermatitis and various adverse effects have been reported [13] [14]. Pigmentation inhibitors that are confirmed to be efficacious by evidence-based medicine are expected [12].

Keishibukuryogankayokuuin (KBY), a traditional Japanese herbal medicine that is widely used in clinical settings, is recommended to remove spots from the skin [15]. Traditional Japanese herbal medicines have been established owing to over thousands of years of study on their use as therapeutics. These studies have led to the confirmation of beneficial, herbal combinations without harmful effects [16]; however, currently, there is a lack of scientific evidence to support their use, and only very little is known about the efficacy and mechanism of KBY. Therefore, in this study, we investigated the potential mechanism of KBY, as well as Keisibukuryogan (KB, which has the same composition of KBY, except Coix Seed [CS]) and CS, based on tyrosinase.

2. Materials and Methods

2.1. Traditional Japanese Herbal Medicine

The dried extract powder of KBY and KB used in the study were supplied by Tsumura & Co. (Tokyo, Japan). KBY is composed of six dried medicinal herbs—Coix Seed, Cinnamon Bark, Peony Root, Peach Kernel, Poria Sclerotium,
and Moutan Bark. KB is composed of five dried medicinal herbs—Cinnamon Bark, Peony Root, Peach Kernel, Poria Sclerotium, and Moutan Bark. CS was purchased from Tsumura & Co. and extracted with boiling water for 1 h. The extracts were evaporated and dried.

2.2. Cell Culture and UV Radiation

We obtained neonatal human epidermal melanocytes from a darkly pigmented donor (HEMn-DP; Gibco, USA) and cultured these cells in Medium 254 (Gibco) containing Cascade Biologics HMGS 100X (Gibco). The mixture was incubated at 37°C and 5% CO₂. After culture, KBY, KB, and CS were added in a final concentration of 500 μg/ml or 1000 μg/ml. After incubation for 72 h, the cells were used for the experiments. For UV radiation, cells were exposed to UVB radiation using a Handheld UV Lamp (Analytik Jena US LLC, USA) for 90 s per day for 3 days.

2.3. Cell Staining

We captured images after staining the cells with the Fontana-Masson Stain Kit (ScyTek Laboratories, Inc., USA) following the manufacturer’s protocols.

2.4. Cell Viability

After cell culture, cell viability was measured using Cell Counting Kit-8 (Dojindo Molecular Technologies, Inc., Japan) following the manufacturer’s protocols. After incubation at 37°C for 1 h, absorbance was measured at 405 nm.

2.5. Tyrosinase Activity

Tyrosinase activity was measured via its dopa oxidase activity with reference to the method described by Fujiwara et al. [17]. After cell culture, cells were recovered using Trypsin/EDTA (Cell Applications, Inc., USA). The supernatant was removed, and Triton™ X-100 (Sigma-Aldrich, USA) was added to elute intracellular substances from the melanocytes. N,N-Dimethylformamide (Sigma-Aldrich) adjusted to 4% with PBS pH 7.4 (Gibco) and 3,4-Dihydroxy-L-phenylalanine (Sigma-Aldrich) adjusted to 5 mM with PBS were added to the melanocyte solution. Absorbance at 0 min was measured at 405 nm, followed by incubation at 37°C for 30 min. Absorbance was again measured at 30 min.

2.6. Tyrosinase Expression

Tyrosinase expression was estimated with real-time PCR by referring to the method described by Masaki [11]. Cells were collected using RNeasy Mini kit (QIAGEN, Netherlands), and total RNA was extracted. Reverse transcription was carried out using 100 ng of RNA with the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, USA) to synthesize cDNA. The reaction conditions were: 25°C for 10 min, and 37°C for 120 min. qPCR was performed
using PikoReal 96 (Thermo Fisher Scientific, USA). We used TaqMan® Universal PCR Master Mix, No AmpErase®UNG (Applied Biosystems) and TaqMan® Gene Expression Assays as probes. We also used tyrosinase (TYR: Hs00165976_mL; Applied Biosystems) as the target gene and GAPDH (Hs99999905_mL; Applied Biosystems) as the standard internal gene. The reaction solution was 20 μL and reaction conditions were: 95°C for 10 min, 95°C for 15 s, and 60°C for 1 min \times 50.

2.7. Data Analysis

The experimental data are expressed as mean ± S.E. Paired t-test was used to detect any differences in each condition. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Cell Staining

Figure 1 represents the results of staining for the investigation of cell viability.

3.2. Cell Viability

Under non-irradiated conditions with a final concentration of 1000 μg/ml, we observed a significant decrease in cell viability with KBY (73.91 ± 0.76, p < 0.001) and KB (56.90 ± 0.73, p < 0.001) (Figure 2(a)). However, with 500 μg/ml as the final concentration, cell viability was observed to significantly increase with KBY (110.92 ± 0.71, p = 0.032) (Figure 2(a)). Under UV exposure with a final concentration of 1000 μg/ml, we observed a significant increase in cell viability with KBY (120.62 ± 1.48, p = 0.011) and CS (134.08 ± 2.84, p = 0.002) (Figure 2(b)). Similarly, with 500 μg/ml as the final concentration, cell viability significantly increased with KBY (144.34 ± 1.13, p < 0.001), KB (156.24 ± 1.64, p < 0.001), and CS (137.36 ± 2.29, p = 0.010) (Figure 2(b)).

3.3. Tyrosinase Activity

Under non-irradiated conditions, tyrosinase activity significantly increased with KBY (500 μg/ml: 142.30 ± 2.02; 1000 μg/ml: 151.18 ± 0.97) and KB (500 μg/ml: 181.79 ± 3.64; 1000 μg/ml: 188.21 ± 1.26), but significantly decreased with CS (500 μg/ml: 80.30 ± 0.54; 1000 μg/ml: 81.61 ± 0.52), regardless of the concentration (p < 0.001) (Figure 3(a)). In contrast, tyrosinase activity was significantly increased with KBY (500 μg/ml: 161.62 ± 2.44; 1000 μg/ml: 232.57 ± 4.71) and KB (500 μg/ml: 231.62 ± 3.57; 1000 μg/ml: 285.49 ± 6.87) at both concentrations (p < 0.001) when exposed to UV (Figure 3(b)). Furthermore, the rate of increase for KBY was significantly lower than KB under all conditions (p < 0.001).

3.4. Tyrosinase Expression

Under non-irradiated conditions, tyrosinase expression significantly decreased only with KBY at both concentrations (500 μg/ml: 84.04 ± 0.47, p < 0.001; 1000
Figure 1. Fontana-Masson staining of HEMn-DP with or without UV exposure. KBY, KB, and CS were added to make a final concentration of 500 μg/ml or 1000 μg/ml. (a) Under non-irradiated conditions, 500 μg/ml, (b) Under non-irradiated conditions, 1000 μg/ml, (c) Under UV exposure, 500 μg/ml, (d) Under UV exposure, 1000 μg/ml.

Figure 2. Effects of KBY/KB/CS on cell viability. (a) Under non-irradiated conditions, and (b) With UV exposure. Results are expressed as mean ± S.E. *p < 0.05, **p < 0.01, ***p < 0.001 vs. control.
Figure 3. Effects of KBY/KB/CS on tyrosinase activity. (a) Under non-irradiated conditions, and (b) With UV exposure. Results are expressed as mean ± S.E. ***p < 0.001 vs. control. ###p < 0.001 when KBY is compared to KB.

μg/ml: 84.41 ± 1.43, p = 0.020) (Figure 4(a)). Similarly, with UV exposure, this expression was significantly decreased with KBY (500 μg/ml: 63.75 ± 0.58; 1000 μg/ml: 53.44 ± 0.53) (p < 0.001) and KB (500 μg/ml: 65.53 ± 0.65; 1000 μg/ml: 61.99 ± 0.94) (p < 0.001) (Figure 4(b)). However, a significant increase was observed with CS at both concentrations (500 μg/ml: 140.11 ± 1.11; 1000 μg/ml: 122.35 ± 0.87) (p < 0.001) (Figure 4(b)).

4. Discussion

Melanin protects our skin from UV damage, but an increase in its production can cause abnormal pigmentation which can affect one’s psychosocial behavior and trigger issues with appearance [18]. Many studies have been conducted to develop whitening agents and many have reported that various naturally occurring compounds inhibit the activity or expression of tyrosinase [19] [20] [21]. For example, kojic acid, hydroquinone, and arbutin are direct inhibitors of tyrosinase activity [18] [21] [22]. Although the effectiveness of hydroquinone (as an example) has been demonstrated when used as a treatment, its use has been considered problematic due to cytotoxicity to melanocytes [18] [23]. An ideal whitening agent has no side effects and gives a potent and selective skin whitening effect to pigmented areas.

KBY, a traditional Japanese herbal medicine widely used in clinical settings, is recommended to remove spots from the skin [15]. However, these medicines presently lack scientific evidence and for KBY, there is very little known about its efficacy and mechanism. KBY is used to relieve the following symptoms: menstrual irregularity, automatic imbalance syndrome (which resembles climacteric disturbance in women), acne, spots, and roughness of the hands and feet. However, KB is not indicated for the relief of symptoms of the skin because it lacks CS, which has an effect on the skin. CS is a crude drug that is prepared by drying mature seeds, excluding the seed coat of *Coix lacryma-jobi* Linne var. *mayuen* Stapf [24]. Although its active ingredient and pharmacological effect remain unclear, it has been used to treat warts and skin-related issues [25] [26].
Figure 4. Effects of KBY/KB/CS on tyrosinase activity. (a) Under non-irradiated conditions, and (b) With UV exposure. Results are expressed as mean ± S.E. *p < 0.05, ***p < 0.001 vs. control.

Melanocytes respond to various factors such as the surrounding environment (e.g., UV), or neighboring cells in the skin [27] [28]. In the early stage of melanogenesis, L-tyrosine or L-3,4-dihydroxyphenylalanine (L-DOPA) is converted to the corresponding o-dopaquinone by enzymatic oxidation of tyrosinase [28] [29]. This tyrosinase catalyzes two rate-limiting steps during melanogenesis: 1) hydroxylation of tyrosine to L-DOPA, and 2) oxidation of L-DOPA to dopaquinone [30]. While tyrosinase regulates the rate-limiting steps in the early stage of melanogenesis, tyrosinase related protein (TRP)-1 and TRP-2 are involved in modifying eumelanin in the later steps [21]. Furthermore, microphthalmia-associated transcription factor (MITF) is the major transcription factor in the regulation of tyrosinase, TRP-1, and TRP-2 [21] [22]. Extensive studies on the whitening effects have been performed to elucidate the activity and expression of these enzymes, specifically tyrosinase [21]. We also investigated the effect of KBY-induced melanin inhibition, especially the effect on tyrosinase activity and expression, in HEMn-DP.

We measured cell viability to exclude the possibility that the tyrosinase inhibitory effects of KBY, KB, and CS might be caused by cell growth inhibition [28]. Under non-irradiated conditions and with a final concentration of 1000 μg/ml, a significant decrease in cell viability was observed with KBY and KB; however, with 500 μg/ml as the final concentration, a significant increase in cell viability was observed with KBY. These results and the result of Fontana-Masson staining suggest that KBY and KB might exert cytotoxicity (i.e., they may have an optimal concentration). However, as reported for other traditional Japanese herbal medicines, there are cases where they exert their effect only under certain conditions [31] [32]. In fact, under UV exposure and with a final concentration of 1000 μg/ml, cell viability was significantly increased with KBY and CS. In addition, with 500 μg/ml as the final concentration, cell viability was significantly increased with KBY, KB, and CS. As a result, no suppression of cell viability was observed under any condition, and the increased cell viability suggested that
these compounds protect cells from UV damage. Together, these results suggest that the tyrosinase inhibitory effects of KBY, KB, and CS on melanin production were not attributable to their cytotoxicity. Moreover, KBY could be a safe whitening agent without influencing melanocyte growth if consumed after sunburn.

For tyrosinase activity under non-irradiated conditions, a significant increase was observed with both KBY and KB; however, a significant decrease was observed with CS, regardless of the concentration. Under UV exposure, tyrosinase activity was significantly increased with KBY and KB at both concentrations, and the rate of increase in KBY was significantly lower than that of KB under any conditions. In brief, neither KBY nor KB inhibited tyrosinase activity, but CS had an inhibitory effect on this activity. From these results, we found that mixing CS with KB is important because the former plays a role to maintain the increase in tyrosinase activity to a minimum.

In contrast, when tyrosinase expression was considered under non-irradiated conditions, a significant decrease was only observed with KBY at both concentrations. Under UV exposure, tyrosinase expression significantly decreased with KBY and KB but was significantly increased with CS at both concentrations. These results suggest that KBY has a potent inhibitory effect on the expression of tyrosinase. Interestingly, for CS, the expression of tyrosinase increased under UV exposure. Although a further study is needed, with the inclusion of the tyrosinase activity results, we considered that the amount of CS mixed into KBY is a prescribed dose derived from years of experience, and this suppresses the tyrosinase activity promoting effect of KBY because of the compensatory tyrosinase activity inhibitory effect of CS. In addition, including this amount of CS does not hinder KBY’s inhibitory effect on the expression of tyrosinase.

5. Conclusion

These results suggest that taking KBY after sunburn is effective against skin pigmentation, and the combination of KB and CS is useful for skin depigmentation.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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