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ARTICLE

Whitening Effect of Watersoluble Royal Jelly from South Korea

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Abstract

Royal jelly has been widely used as a health supplement worldwide. However, royal jelly has been implicated in allergic reactions, and we developed a water-soluble royal jelly (WSRJ) without the allergy inducing protein. In this study, we aimed to identify the anti-melanogenic efficacy of WSRJ. B16F1 melanoma cells were first treated with 10 nM α-melanocyte stimulating hormone (α-MSH) and then with various doses of WSRJ. In addition, we investigated the mRNA and protein expression of melanogenesis-related genes such as tyrosinase, tyrosinase related protein-1 (TRP-1) and TRP-2 by reverse transcription-polymerase chain reaction and western blotting. WSRJ directly inhibited tyrosinase and cellular tyrosinase activity, which decreased melanin synthesis in α-MSH stimulated B16F1 melanoma cells a level comparable to that observed with arbutin. WSRJ decreased the mRNA and protein expressions of tyrosinase, TRP-1, and TRP-2, which was comparable to that observed with arbutin. WSRJ has strong anti-melanogenic activity, which invoice direct inhibition of tyrosinase enzyme activity and suppression of expression of melanogenesis related genes. Results from this study suggests that WSRJ is a potential candidate for the treatment of skin pigmentation.

Keywords: royal jelly, water-soluble royal jelly, α-MSH, tyrosinase, TRP-1, TRP-2

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Introduction

Melanogenesis is a biosynthetic pathway for melanin synthesis from melanocytes located in the lowest layer of epidermis in human skin (Yokota *et al*., 1998). Melanin is synthesized in melanosomes which are specialized pigment organelles in melanocytes. Melanin plays an important role in protecting the skin and hair from ultraviolet (UV) irradiation. However, excessive exposure to UV radiation causes hyperpigmentation disorders such as melasma, nevus, freckles and solar lentigenes (Urabe *et al*., 1998). When the skin is exposed to UV radiation, α-melanocyte stimulating hormone (α-MSH) is secreted, which binds to melanocortin 1 receptor (MC1R). MC1R is mainly regulated by melanogenic enzymes including tyrosinase, tyrosinase related protein (TRP)-1 and TRP-2 (Hearing, 1999). Tyrosinase is a rate-limiting enzyme involved in melanin production that catalyzes the hydroxylation of L-tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA) and the conversion of L-DOPA to dopaquinone (Urabe *et al*., 1998). Dopaquinone can be converted to a semi-stable dopachrome in the absence of sulfur-containing compounds. TRP-2 catalyzes the conversion of dopachrome to 5,6-dihydroxyindole-carboxylic acid and TRP-1 oxidizes 5,6-dihydroxyindole-carboxylic acid to indole-5,6-quinone carboxylic acid, ultimately leading to melanin synthesis (Hearing and Tsukamoto, 1991; Solano *et al*., 2006). Hence, down-regulation in these enzymes leads to a decrease of melanin synthesis.

Royal jelly (RJ) is secreted from the hypopharyngeal and mandibular glands of worker honeybees, and is well known to be essential for the growth of the queen honeybee (Jozef, 2001). RJ is widely marketed and used in various health promoting products including cosmetics, food supplements, beverages, and commercial medical products for all age groups (FAO, 2007). RJ is composed of water, protein, total sugars, lipids, vitamins, free amino acids, and 10-hydroxy-2-decenoic acid (10-HDA). RJ has been reported to confer a number of physiological and pharmacological benefits including anti-tumor (Tolanai and Morgan, 1962), anti-hypertensive (Tokunaga *et al*., 2004), anti-inflammatory (Kohno *et al*., 2004), wound

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healing (Park *et al*., 2012), and anti-photoaging (Park *et al*., 2011) properties in vivo and in vitro. We demonstrated in a previous study that RJ reduces melanin synthesis by down-regulating tyrosinase mRNA transcription (Han *et al*., 2011)

However, RJ has been linked to allergic contact dermatitis, acute asthma and anaphylaxis in countries with high consumption of RJ (Rosmilah *et al*., 2008). Several studies have reported that RJ-sensitive subjects possess immunoglobulin E (IgE) binding proteins between 47 and 55 kilo Daltons and these have been recognized as the major allergens of RJ (Rosmilah *et al*., 2008). In our preliminary data, we confirmed this effect in water-soluble RJ (WSRJ), wherein the allergens had been eliminated (Kim *et al*., 2013). We also reported that the only difference between the components of RJ and those of WSRJ is the presence of allergic proteins in the former.

To our knowledge, there are no reports on the inhibitory effects of WSRJ on melanogenesis regulated by melanogenic enzymes. Therefore, in the present study we investigated the potential anti-melanogenic effects of WSRJ. To determine the effects of WSRJ on melanogenesis, we evaluated tyrosinase activity and melanin levels in α-MSH-stimulated B16F1 melanoma cells. In addition, we explored the possible mechanisms of WSRJ by examining the regulation of mRNA and the levels of tyrosinase, TRP-1 and TRP-2 proteins.

Materials and Methods

Reagents

Dulbecco's Modified Eagle's Medium, fetal bovine serum and penicillin/streptomycin (100 IU/50 μg/mL) were obtained from Invitrogen (Grand Island, USA). Antibodies against tyrosinase, TRP-1, TRP-2 and β-actin were purchased from Abcam (Cambridge, USA). All other chemicals including 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, α-MSH, dimethyl sulfoxide (DMSO), mushroom tyrosinase and L-DOPA were purchased from Sigma-Aldrich (USA).

Preparation of WSRJ

Fresh royal jelly of *Apis mellifera* L. was collected from Inje County, Gangwon Province, Korea. Upon receipt, it was stored at -20°C until used. Lyophilized RJ was extracted twice with 70% ethanol. The supernatants were enriched and lyophilized for 48 h. Lyophilized RJ powder was dissolved in distilled water by stirring for 1 h and the pH was neutralized to 7.4 by sodium hydroxide. The extracts were re-lyophilized and kept at -20°C until use.

Cell culture and WSRJ treatment

B16F1 melanoma cells purchased from the Korean Cell Line Bank (Korea) were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% (v/v) fetal bovine serum and 1% penicillin/streptomycin at 37°C under 5% CO_2 in air atmosphere. B16F1 melanoma cells were seeded at a density of 5×10^3 cells/well in 6-well plates. After 24 h, the cells were cultured in fresh media supplemented with 10 nM α-MSH for 48 h, and then treated with various doses of WSRJ. After 24 h, the cells were harvested and used for various assays.

Cell viability assay

After incubation, the cultured medium was replaced with 50 μL MTT solution (1 mg/mL in PBS) in each well. After incubation at 37°C for 4 h, the solution was carefully removed, and 100 μL DMSO was added. The absorbance of each well was measured at 570 nm using a microplate reader (Bio-Tek Instruments, USA).

Melanin content assay

After cell cultivation, the cells were washed with PBS and harvested by trypsinization. The cell pellets were homogenized in lysis buffer containing 50 mM sodium phosphate, 1% Triton X-100 and 2 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation at 14,000 rpm for 15 min, the melanin pellets were dissolved in 200 μL 1M NaOH containing 10% DMSO at 80°C for 1 h. Absorbance was measured at 405 nm using a microplate reader. The melanin content was determined using an authentic standard of synthetic melanin. Protein content was determined using a Bradford assay with bovine serum albumin (BSA) as the protein standard.

Mushroom tyrosinase activity assay

An *in vitro* mushroom tyrosinase inhibition assay was performed as described previously by Lee *et al*. (2010) with slight modifications. We added 120 μL of 8.3 mM L-DOPA in 80 mM phosphate buffer (pH 6.8) to each well of a 96-well plate and 40 μL of the same buffer or WSRJ at various doses, followed by 40 μL of 125 U mushroom tyrosinase in 80 mM phosphate buffer. After 30 min incubation at 37°C, absorbance was measured at 490 nm using a microplate reader.

Tyrosinase activity assay

A tyrosinase activity assay was performed according to

the method described previously with slight modifications (Lee *et al*., 2010). B16F1 cells were lysed in 20 mM sodium phosphate (pH 6.8), 1% Triton X-100 and 1 mM PMSF, and centrifuged at 14,000 rpm for 15 min. The protein content of each supernatant was determined using the Bradford assay with BSA as the protein standard. Tyrosinase activity was determined in a reaction mixture (1 mL) containing 50 mM phosphate buffer (pH 6.8), 2.5 mM L-DOPA and 300 μg supernatant protein. After incubating at 37°C for 15 min, absorbance was measured at 475 nm using a microplate reader.

Western blot for tyrosinase, TRP-1 and TRP-2

Cells were lysed with ice cold lysis buffer containing protease inhibitors and centrifuged at 14,000 rpm for 10 min. The protein content of each supernatant was determined using a Bradford assay with BSA as the protein standard. Samples (20 μg) were separated by polyacrylamide gel (10%) electrophoresis, and then transferred to polyvinylidene membranes (0.45 μm, Millipore, USA). Membranes were blocked with 5% non-fat dry milk in Tris buffered saline (TBS) containing 0.1% Tween 20 (TBST) for 1 h. After blocking, membranes were incubated with tyrosinase, TRP-1, TRP-2, and β-actin (Abcam) in TBST overnight at 4ºC. After washing in TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies at a dilution of 1:5000 (GE Healthcare Life Science, UK) for 1 h at room temperature. After washing with TBST, proteins were visualized using an enhanced chemiluminescence detection system. Densitometric analysis was performed using Quantity One (Bio-Rad, Hercules, USA) to scan the signals.

Reverse transcription-polymerase chain reaction (RT-PCR) for tyrosinase, TRP-1 and TRP-2

Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. Reverse transcription of total RNA (1 μg) was performed using a QuantiTect Reverse Transcription Kit (Qiagen, Germany). The reaction was terminated by heating at 95ºC for 5 min. cDNA was amplified using a PCR Premix Kit (i-Taq, iNtRON Biotechnology, Korea) for denaturation at 94ºC for 5 min; 94ºC for 30 s, 5ºC for 30 s and 1 min at 72ºC for 30 cycles, followed by 10 min at 72ºC for elongation using PCR Thermal Cycler Dice (TaKaRa, Japan). The primer sequences were as follows: mouse tyrosinase, 5'-GGCCAGCTTTCAGGCAGAGGT-3'(forward) and 5'-TGGTGCTTCATGGGCAAAATC-3' (reverse); mouse TRP-1, 5'-GCTGCAGGAGCCTTCTTTCTC-3'(forward) and 5'-AAGACGCTGCACTGCTGGTCT-3'(reverse); mouse TRP-2, 5'-GGATGACCGTGAGCAATGGCC-3'(forward) and 5'-CGGTTGTGACCAATGGGTGCC-3'(reverse), and mouse glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control, 5'-ACCACAGTCCATGCCATCAC-3'(forward) and 5'-TCCACCACCCTGTTGCTGTA-3'(reverse). The PCR products were analyzed by 1.5% agarose gel electrophoresis with ethidium bromide. The signal intensity of each band was quantified and normalized to the GAPDH signal. Densitometric analysis was performed using Quantity One (Bio-Rad, Hercules, USA) to scan the signals.

Statistical analysis

All data are expressed as the mean±standard error of the mean (SEM). Statistical differences among groups were calculated by analysis of variance followed by Duncan's multiple range test (SPSS Version 18.0, USA). Differences with a P value less than 0.05 were considered significant.

Results

Effects of WSRJ on cell viability

The optimal dose from the cell viability assay using MTT in B16F1 melanoma cells are shown in Figure 1. The cell viability was 109±5.3% at 1 μg/mL, 114±2.0% at 5 μg/mL, 110±4.2% at 10 μg/mL, 116±2.1% at 50 μg/mL and 109±8.9% at 100 μg/mL during a 24 h treatment. WSRJ clearly was showed the non-cytotoxic to B16F1 melanoma cells.

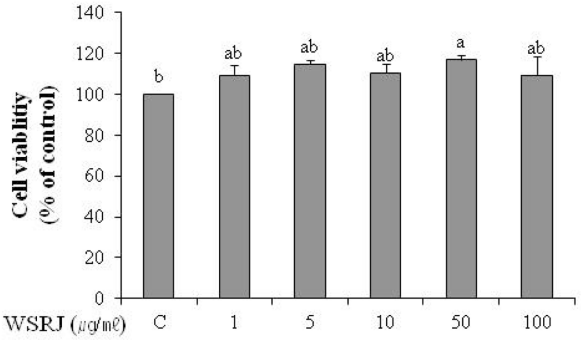

Fig. 1. Cell viability after WSRJ in B16F1 cells. B16F1 cells were treated with 10 nM α-MSH for 48 h and then further 24 h with WSRJ at 1-100 mg/mL. Cell viability was determined by measuring the absorbance at 570 nm using a microplate reader. Data are presented as mean±SEM of five independent experiments. Different letters indicate a significant difference with $p<0.05$.

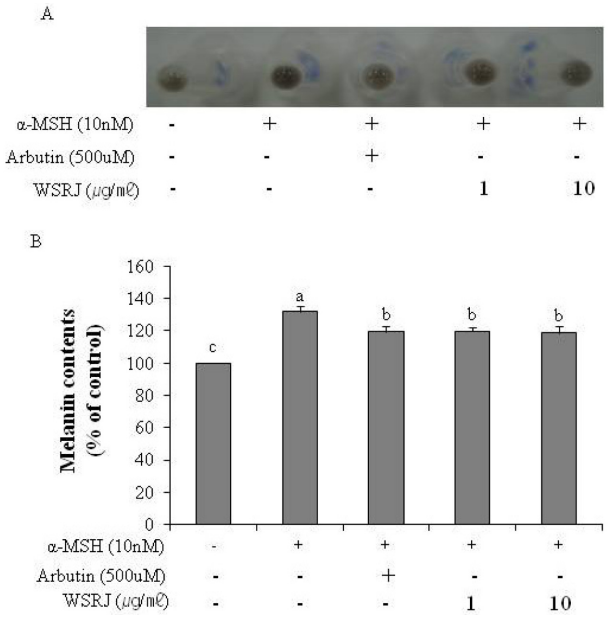

Fig. 2. Inhibitory effect on melanogenesis in B16F1 cells. B16F1 melanoma cells were stimulated with 10 nM α-MSH for 48 h and the medium was replaced with fresh medium with or without various concentrations of WSRJ and the cells were incubated for 24 h. (A) Photograph of cell pellets. (B) Synthesized melanin contents. Data are presented as mean±SEM of five independent experiments. Different letters indicate a significant difference with $p<0.05$.

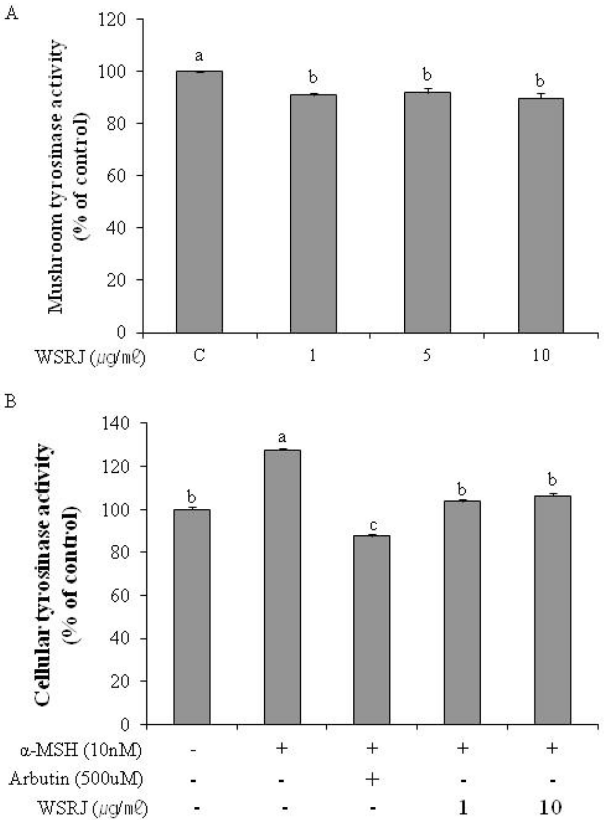

Fig. 3. Inhibitory effect of WSRJ on tyrosinase activity in B16 F1 cells. (A) Tyrosinase activity was determined using mushroom tyrosinase and by measuring the absorbance of dopaquinone that is produced by an oxidative reaction with mushroom tyrosinase as the enzyme and L-DOPA as the enzyme substrate. (B) Cellular tyrosinase activity was measured using B16F1 melanoma cell lysates. Data are presented as mean±SEM of five independent experiments. Different letters indicate a significant difference with $p<0.05$.

Suppression of melanin synthesis by WSRJ

Arbutin is an effective and well known anti-melanogenesis agent, and was used as a positive control. WSRJ significantly ($p<0.05$) suppressed α-MSH stimulated melanin synthesis compared to that in α-MSH only treated B16F1 melanoma cells, while arbutin also significantly reduced ($p<0.05$) melanin synthesis (Fig. 2).

Inhibition of tyrosinase activity by WSRJ

We further evaluated the direct inhibitory action of WSRJ on tyrosinase activity by using mushroom tyrosinase, and measured the absorbance of dopaquinone which is produced by an oxidative reaction with mushroom tyrosinase as the enzyme and L-DOPA as the enzyme substrate. Fig. 3A shows the inhibition of DOPA oxidase activity of mushroom tyrosinase by WSRJ in B16F melanoma cells. At a dose of 10 μg/mL, WSRJ reduced mushroom tyrosinase activity by 11%. We then examined the inhibitory effect of WSRJ on cellular tyrosinase activity in α-MSH stimulated B16F1 melanoma cells. WSRJ inhibited cellular tyrosinase activity in the crude lysates compared to α-MSH only treated B16F1 melanoma cells (Fig. 3B). As expected, the inhibitory effect of the positive control arbutin on tyrosinase activity was observed. These results indicate that WSRJ suppresses melanin synthesis by directly inhibiting tyrosinase and cellular tyrosinase activity in α-MSH stimulated B16F1 melanoma cells.

Suppression of tyrosinase, TRP-1 and TRP-2 protein expression by WSRJ

The melanogenic enzymes such as tyrosinase, TRP-1 and TRP-2 synthesized the melanin. To investigate whether WSRJ can influence melanogenic protein expression, Western blotting analysis was carried out using the lysate of B16F1 melanoma cells treated with WSRJ and stimulated by either α-MSH. When cells were stimulated by α-MSH, a significant increase of tyrosinase protein was observed, and TRP-1, 2 expression also increased. As shown in Fig. 4, WSRJ dramatically inhibited tyrosinase, TRP-1

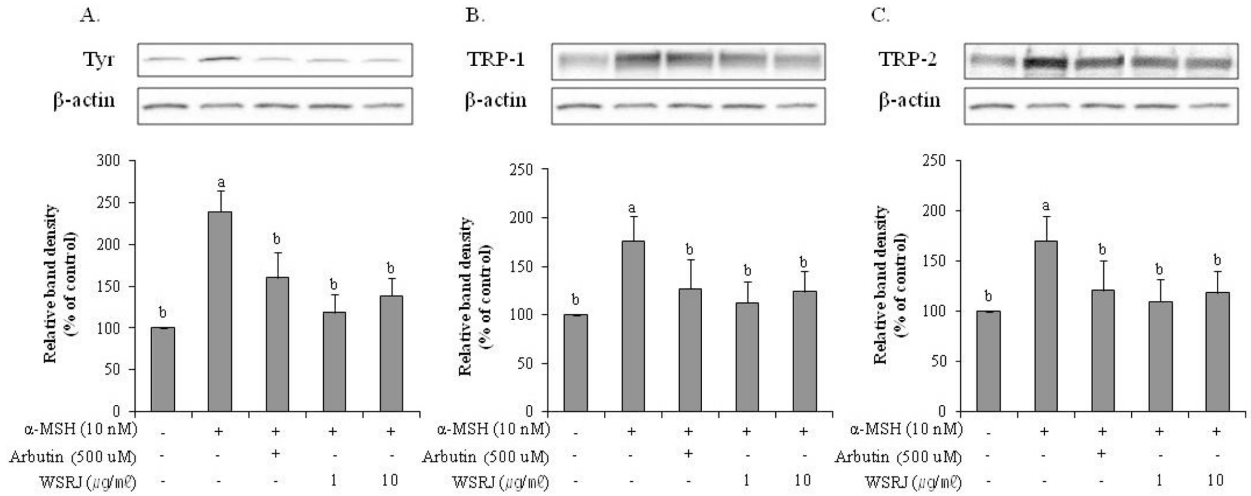

Fig. 4. Suppression of WSRJ on tyrosinase, TRP-1 and TRP-2 protein expression in B16F1 cells. Cells were stimulated with 10 nM α-MSH for 48 h, the medium was replaced with fresh medium with or without various concentrations of WSRJ and the cells were incubated for 24 h. Data are presented as mean±SEM of five independent experiments. Western blot analysis of protein for tyrosinase (A), TRP-1 (B), TRP-2 (C) and β-actin. The relative band intensity of the enzymes was normalized by β-actin. Different letters indicate a significant difference with $p<0.05$.

and TRP-2 expression in α-MSH stimulated B16F1 melanoma cells compared with those of control (approximately 138.7%, 76.3% and 70.0%, respectively, for Fig. 4A, B and C). WSRJ inhibited the protein expression levels of melanogenic enzymes as similar to those of control and arbutin.

Down regulated tyrosinase, TRP-1 and TRP-2 mRNA expression

To investigate the effects of WSRJ on melanogenic gene transcription, RT-PCR analysis was carried out. As shown in Figure 5, tyrosinase, TRP-1 and TRP-2 gene expression were significantly ($p<0.05$) decreased by WSRJ at doses of 1 and 10 μg/mL in α-MSH stimulated B16F1 melanoma cells compared to those of control (approximately 13.6%, 7.7% and 7.7%, respectively, for Fig. 5A, B and C) similar to protein expression of each enzyme. Arbutin inhibited mRNA expression of melanogenic enzymes at levels similar to those of WSRJ. These findings indicate that downregulation of WSRJ on melanogenesis is due to the decreased levels of melanogenic enzymes, tyrosinase, TRP-1 and TRP-2 proteins and mRNA expression in parallel with the inhibition of tyrosinase activity.

Discussion

In the present study, we have demonstrated the inhibitory mechanism of WSRJ in B16F1 melanoma cells. WSRJ did not show any cytotoxicity, WSRJ inhibited melanin synthesis in α-MSH stimulated B16F1 melanoma cells and directly inhibited tyrosinase activity and cellular tyrosinase activity. Also, WSRJ inhibited the mRNA and protein expression of tyrosinase, TRP-1 and TRP- 2 in α-MSH stimulated B16F1 melanoma cells. These results indicate that WSRJ has anti-melanogenic effects through direct inhibition of tyrosinase activity in parallel with the inhibition of melanogenic enzyme activities in α-MSH stimulated B16F1 melanoma cells. To stimulate melanogenesis, we used cAMP inducers such as α-MSH (Imokawa, 2004). α-MSH stimulates melanogenesis by binding to MC1R, resulting in an increase of intracellular cAMP (Busca and Ballotti, 2000).

There are three well-known enzymes such as tyrosinase, TRP-1 and TRP-2, and the upregulation of these enzymes results in an increase in melanin synthesis. Melanogenesis is stimulated by ultraviolet light, X-rays, inflammatory cytokines and hormones. Cyclic AMP activates microphthalmia-associated transcription factor, which leads to upregulation of expression of tyrosinase, TRP-1 and TRP-2 consequently resulting in melanin biosynthesis (Ando *et al.*, 1999). In the present study, we did not test the inhibitory of WSRJ on microphthalmia-associated transcription factor expression and cAMP pathway, anti-melanogenic effects of WSRJ might have been mediated through the downregulation of the cAMP pathway by MC1R and adenylate cyclase activation. Furthermore, WSRJ might have inhibited the expression of microphthalmia-associ-

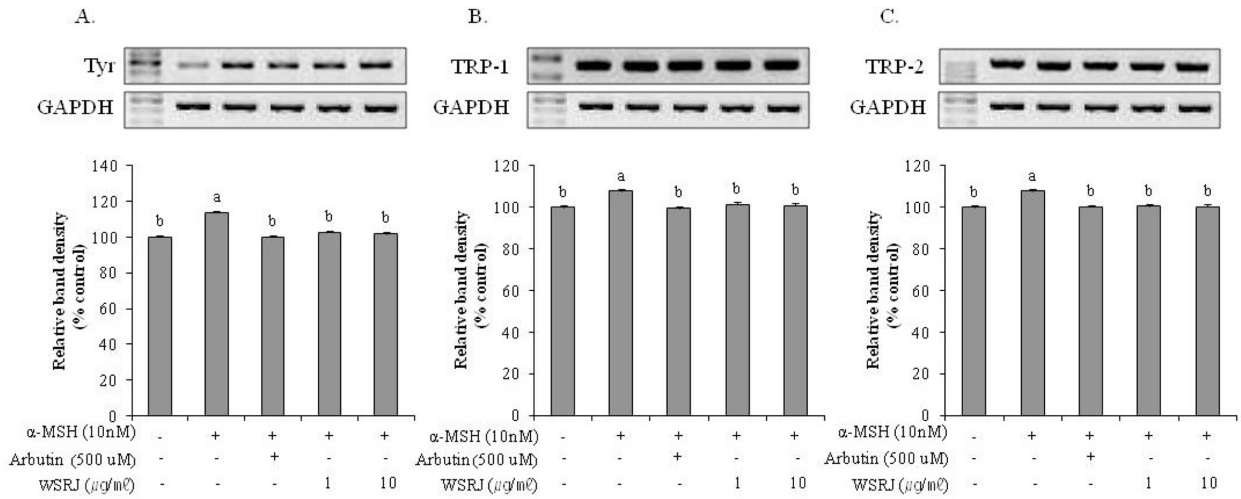

Fig. 5. Down-regulation of WSRJ on tyrosinase, TRP-1 and TRP-2 mRNA expression in B16F1 cells. Cells were stimulated with 10 nM α-MSH for 48 h, the medium was replaced with fresh medium with or without various concentrations of WSRJ and the cells were incubated for 24 h. Data are presented as mean±SEM of five independent experiments. RT-PCR analysis of mRNA for tyrosinase (A), TRP-1 (B), TRP-2 (C) and GAPDH. The relative band intensity of the enzymes was normalized to GAPDH, and the different letters indicate a significant difference with $p<0.05$.

ated transcription factor, which resulted in the downregulation of tyrosinase, TRP-1 and TRP-2 activity. In fact, pigmentation defects in skin, eyes and hair have been observed in patients suffering from mutations of microphthalmia-associated transcription factor (Barsh, 1996). Thus, WSRJ could provide an element for regulating both mRNA and protein expression of tyrosinase, TRP-1 and TRP-2.

Royal jelly (RJ) is a natural food that contains proteins, carbohydrates, fats, free amino acid, vitamins and minerals. As for its bioactive component, 10-HDA is characterized as the principal unsaturated fatty acid in RJ, indicating that low 10-HDA content implies a low RJ activity. According to our preliminary data, 10-HDA content is over eight times higher in WSRJ than that in RJ, with its content being 60-80% in the water-soluble fraction of RJ (Kim *et al.*, 2013). Therefore, 10-HDA may also be a major bioactive component of WSRJ. RJ contains various hydroxyl fatty acids, the molecular structures of which are similar to 10-HDA (Lercker *et al.*, 1982) and they are mostly unsaturated fatty acids such as 10-hydrodecanoic acid and 3-hydroxydecanoic acid. Unsaturated fatty acids are reported to decrease melanin synthesis and tyrosinase activity, leading to downregulation of melanogenesis (Koya-Miyata *et al*., 2004). Although more research is needed to further clarify the mechanisms of the anti-melanogenic effects of WSRJ, we demonstrated the depigmentation effect of WSRJ.

In this report, we demonstrated the depigmentation effect of PQQ. PQQ inhibited cAMP- and IBMX-enhanced melanin production in B16 melanoma cells. Our results show that WSRJ inhibits tyrosinase, TRP-1, and TRP-2 gene transcription and subsequent protein. This result implies that WSRJ directly inhibits transcription of tyrosinase, TRP-1, and TRP-2 to produce the inhibition of melanin synthesis in B16F1 melanoma cells. Further studies are necessary to clarify the mechanism of down-regulation. WSRJ is a useful inhibitor of melanogenesis and it might lead to an effective treatment for hyperpigmentation disorders.

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