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Original article

Inhibition of AGE-induced Melanogenesis in B16 Melanoma Cells by Iridoid-containing Plants

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Abstract

***Background*:** As previously reported, AGE-collagen causes direct melanin production and is one possible cause of skin hyperpigmentation.
***Materials and Methods*:** AGE-collagen-induced melanogenesis tests were performed to investigate the inhibitory activities of extracts from *Morinda citrifolia* (noni; Rubiaceae) fruits, leaves and seeds (MCF, MCL and MCS), *Cornus officinalis* (cornelian cherry; Cornaceae) fruits (COF) and *Olea europaea* (olive; Oleaceae) leaves (OEL).
***Results*:** All samples inhibited melanin production without influencing cell proliferation. MCS exhibited the most potent activity, providing dose-dependent inhibition against melanin production.
***Conclusion*:** The data indicate that noni seeds are useful as a whitening cosmetics material.

***KEY WORDS*:** Advanced glycation end-product (AGE) pigment freckle, AGE-collagen, *Morinda citrifolia*, noni seed extract, *Cornus officinalis*, *Olea europaea*, B16 melanoma cell.

Introduction

Aging deteriorates cellular function and structure and frequently leads to organ dysfunction. Since morphological changes in the skin are obvious, skin aging receives a lot of medicals and cosmetological attention. The skin is often more affected by environmental factors than genetics. Among these factors, chronic UV irradiation is one of the most significant and is responsible for wrinkles, freckles and coarse skin texture [1].

Glycation is the result of the non-enzymatic bonding of a macro molecule, such as a protein or lipid, with a sugar molecule, which eventually form an AGEs (advanced glycation end-products). The binding reaction between AGEs and RAGE (receptor of AGEs) increases inflammation in skin and other organs [2]. Glycative stress is a skin aging factor that promotes inflammatory reactions and tissue damage [2]. The accumulation of glycated collagens causes the skin to become less resilience, more inflexible, and to form wrinkles [3]. Ogura *et al*. reported that lipid peroxidation-induced protein carbonylation was responsible for dullness and yellowish skin tones [4].

We previously reported that glycated collagens stimulate melanongesis without UV exposure, which we described as "AGE pigment freckles" [5]. In this study, we evaluated iridoid-containing herbs—noni (*Morinda citriflia*), cornelian cherry (*Cornus officinalis*), and olive (*Olea europaea*)—for their abilities to prevent AGE pigment freckle formation.

Materials and methods

Plant materials

Noni fruits (MCF), leaves (MCL) and seeds (MCS) were collected in French Polynesia. The fruits were separated into flesh and seeds by hand. The flesh was then freeze-dried. The leaves and seeds were air-dried. Aqueous ethanol extracts of noni fruit flesh, leaves and seeds were prepared as described previously [6]. Cornelian cherry fruit (COF), grown in China, was extracted with aqueous ethanol. Olive leaves (OEL), grown in Morocco, were extracted with ethanol.

Reagents

Synthetic melanin and [Nle4, D-Phe7]-α-melanocyte stimulating hormone trifluoroacetate salt (α-MSH) were purchased from Sigma-Aldrich Japan (Tokyo, Japan).

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Collagen type I (bovine skin, Pepsin-solubilized) was purchased from Nippi, Incorporated (Tokyo, Japan). Other reagents were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

Preparation of AGE-modified collagen

Preparation of AGE-modified collagen (AGE-collagen) was performed according to the method described previously [5]. The reaction solution contained 250 µL of 200 mmol/L phosphate-buffer (PB; pH 7.4), 200 µL of 2 mol/L glucose solution, 200 µL of 3 mg/mL collagen and 350 uL water. Control solutions were prepared by replacing the collagen solution with water. Reaction solutions were incubated at 60°C for 24 h. For the tests, each reaction sample was diluted 5 times in Dullbecco's modified Eagle's medium (DMEM, Sigma-Aldrich).

Cell culture

B16 murine melanoma cells (B16F10) were purchased from RIKEN BioResource Center (Ibaragi, Japan) in Feb. 2015. The B16 cells were cultured in DMEM supplemented with 10% (v/v) fetal bovine serum (FBS, Nichirei Biosciences Inc., Tokyo, Japan) and 1% Pen Strep, a mixture of 10,000 U/mL penicillin and 10,000 µg/mL streptomycin (Life Technologies Japan Ltd., Tokyo, Japan), at 37°C in a humidified, CO_2-controlled (5%) incubator.

Melanin content assay

Melanin synthesis in B16 cells was evaluated according to a method described previously [5]. Briefly, cells (2×10^4 cells/well) were seeded on 24 well-plates with 800 µL in phenol red free DMEM, and at 24 h after seeding, treated with test samples (100 µL) and AGE-collagen (100 µL). Test samples were dissolved in dimethyl sulfoxide (DMSO) and then diluted with DMEM to an appropriate concentration. The DMSO final concentration was 0.1% v/v. In the control and vehicle control groups, DMSO solution was used instead of the sample solution. AGE-collagen was added to the medium in vehicle control and test groups. After additional treatment for 72 h, the culture medium supernatant was collected and transferred to a 96 well-plate for measurement of secreted melanin. The cells were washed with phosphate-buffered saline (PBS, pH 7.4) and lysed with 1N NaOH for 1 h at 60°C. After centrifugation, the supernatant was transferred to a 96 well-plate for measurement of intracellular melanin. The absorbance of the culture medium and lysed cells was measured at 405 nm with a microplate reader (Thermo scientific Varioskan™ Flash Multimode Reader, Thermo Fisher Scientific KK, Kanagawa, Japan). Melanin content was then determined by comparison with absorbance of the synthetic melanin standard.

Cell viability assay

Cell viability was determined by a 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfpphenyl)-*2H*-tetrazolium (WST-8) assay using a Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan). Test samples were dissolved DMEM to an appropriate concentration. In the control group, DMEM solution was used instead of the sample solution. Briefly, 2.8×10^3 cells, in 90 µL medium, were placed in a 96-well plate. After 24 h of incubation at 37°C, 5% CO_2, AGE-collagen or α-MSH (in 10 µL medium) were added. After incubation for a further 72 h, the medium was replaced with a WST solution (WST: culture medium = 1 : 10, 100 µL). After incubation for 1 h, the resulting formazan content was photometrically determined by measuring absorption at 450 nm with a microplate reader. Cell viability was expressed as a percentage of the control group absorbance.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of results obtained from the experiments, as shown in the figures. Intergroup differences were evaluated by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test with Statcel Ver. 3 (OMS Publishing Inc., Saitama, Japan, 2011).

Results and discussion

Inhibition of AGE-induced melanogenesis in B16 Melanoma Cells by iridoid-containing plants

Below 50 µg/mL, MCF, MCL, MCS, COF and OEL exhibited no cytotoxicity towards B16 murine melanoma cells, as determined by the WST-8 method (***Fig. 1***). We quantified melanin formation in the cell culture pellet (intracellular

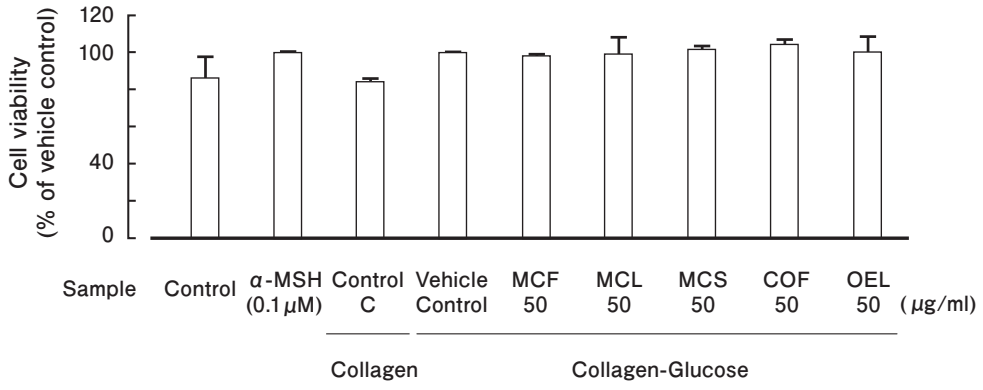

Fig. 1. **Effects of MCF, MCL, MCS, COF and OEL on cell viability.**

The cells were incubated in DMEM in presence of the indicated concentration of sample for 72h. Cell viability was determined by a WST-8 assay. All data is given as mean ± SD of three separate experiments. α-MSH, α-melanocyte stimulating hormone; DMEM, Dullbecco's modified Eagle's medium; MCF, MCL and MCS, *Morinda citrifolia* fruits, leaves and seeds; COF, *Cornus officinalis* fruits; OEL, *Olea europaea* leaves; SD, standard deviation.

melanin) and in the growth medium (extracellular melanin) after 72 hours incubation with AGE-collagen and 50 μg/mL MCF, MCL, MCS, COF or OEL extract. As shown in ***Fig. 2***, melanin production doubled with AGE-collagen, as compared to the control. The stimulant activity of AGE-collagen was as strong as α-MSH, which is commonly used for melanin studies.

Collagen-glucose, without heating, did not simulate melanin formation. On the one hand, AGE-collagen (the collagen which overheated with glucose at 24 h) has much the same effect as α-MSH on melanogenesis. All herbal extracts in this study inhibited AGE-collagen induced melanogenesis to some degree. But MCS had the strongest activity, both intracellular (73% inhibition) and extracellular (65% inhibition). MCS was effective in a dose-dependent manner at 2, 10 and 50 μg /mL (***Fig. 3***).

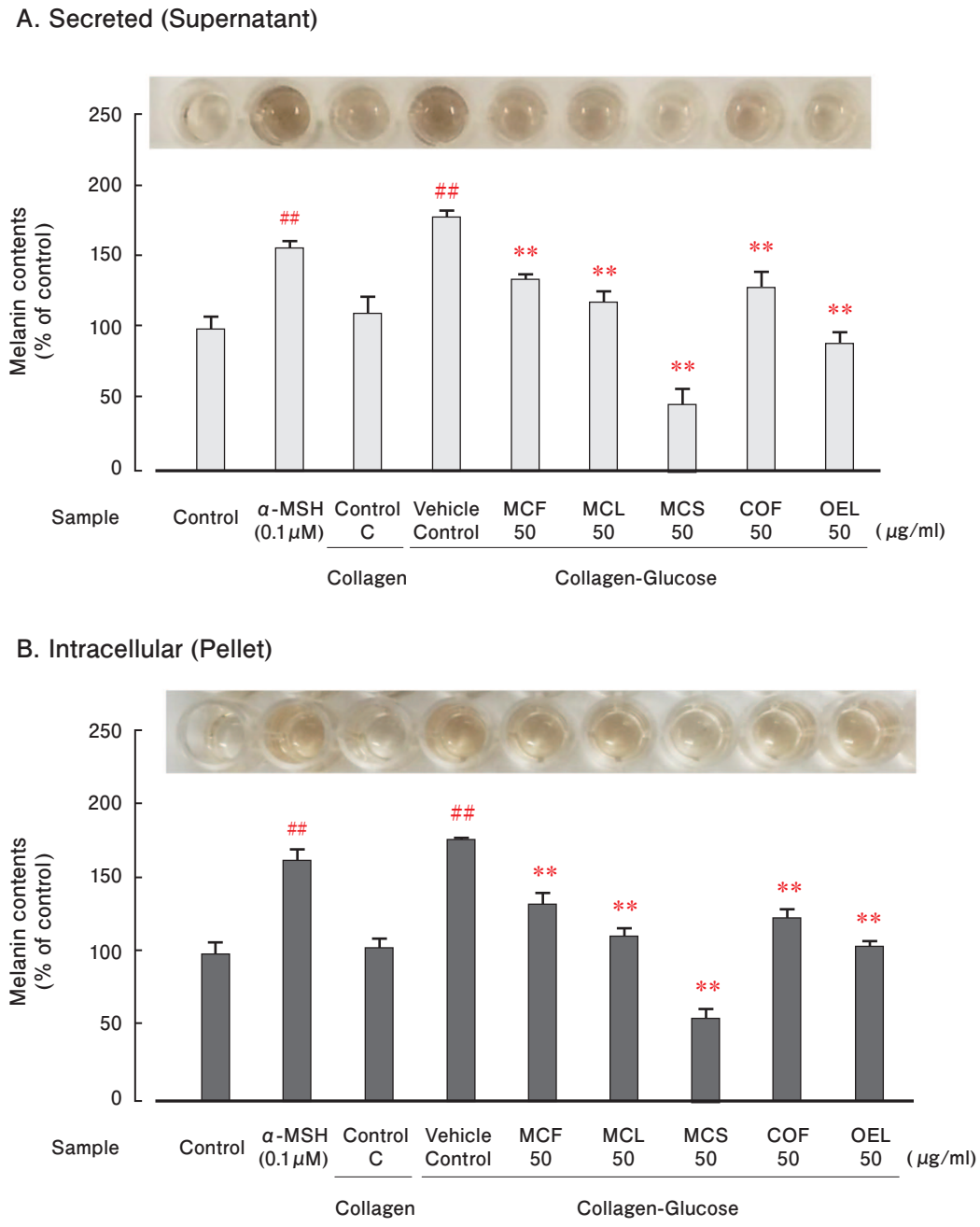

Fig. 2. **Effects of MCF, MCL, MCS, COF and OEL on Melanogenesis in AGE-collagen-stimulated B16 melanoma cells.**

The cells were treated with collagen-glucose and each sample for 72 h. (**A**) Secreted melanin content. (**B**) Intracellular melanin content. Each melanin value represents the mean ± SD of three experiments. Significantly different from the control C group: ##; $p < 0.01$. Significantly different from the vehicle control group: **; $p < 0.01$. α-MSH, α-melanocyte stimulating hormone; MCF, MCL and MCS, *Morinda citrifolia* fruits, leaves and seeds; COF, *Cornus officinalis fruits*; OEL, *Olea europaea leaves*; AGE, advanced glycation end product; SD, standard deviation

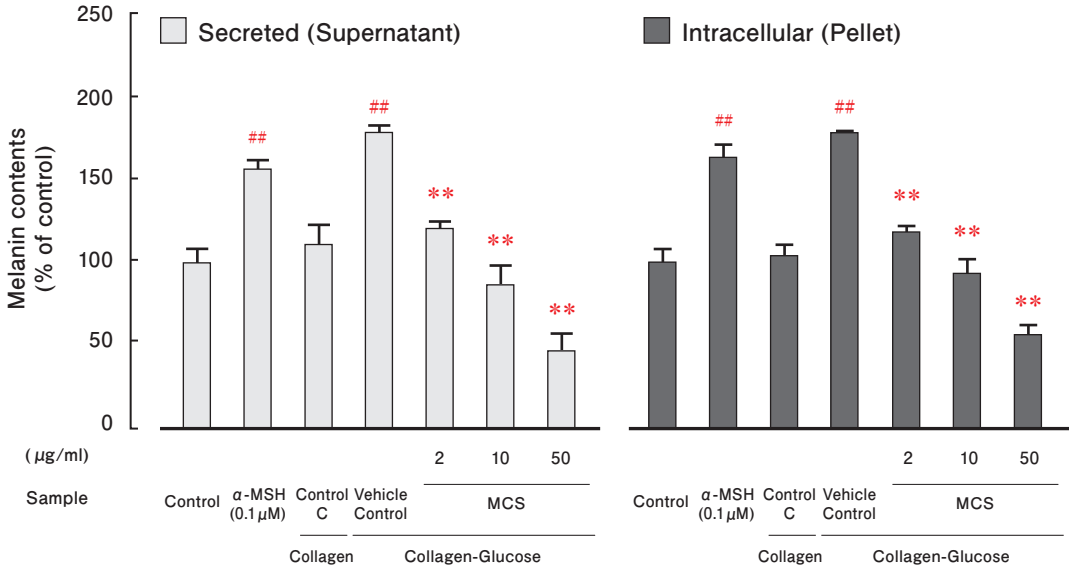

Fig. 3. **Effects of MCS on Melanogenesis in AGE-collagen-stimulated B16 melanoma cells.**

The cells were treated with collagen-glucose and each sample for 72 h. Secreted or intracellular melanin content was determined. Each value represents the mean ± SD of three separate experiments. Significantly different from the control C group: ##; $p < 0.01$. Significantly different from the vehicle control group: **; $p < 0.01$. α-MSH, α-melanocyte stimulating hormone; MCS, Morinda citrifolia seeds; AGE, advanced glycation end product; SD, standard deviation.

MCS was previously reported to be an inhibitor of melanin formation that is induced by α-MSH[7]. In the α-MSH assay, 50 μg/mL noni MCS inhibited melanogenesis by 35%. But in the AGE-collagen assay, its rate of inhibition was 65%. In the previous study, no inhibitory activity by noni fruit or noni leaf, even under 200 μg/mL concentration, was reported. However, the current study reveals that 50 μg/mL noni fruit and noni leaf reduce melanin synthesis by 25% and 42%, respectively. This discrepancy might be due to different melanogenic pathways that are induced by AGE and α-MSH. Further study is necessary to understand the mechanisms involved.

Conclusion

We demonstrated that melanin production inhibitors are present in noni fruit, noni leaf, noni seed, cornelian cherry, and olive leaf — all of which are iridoid-containing herbs. Among these, noni seed extract had the strongest activity. Because noni seed extract also inhibits melanin production that is triggered by α-MSH, it is an ideal cosmetic ingredient candidate. It is not known if AGE-collagen induced melanogenesis shares the same mechanism as α-MSH. Further investigation is necessary to understand how AGEs induce melanin production.

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Conflict of interest statement

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References

1) Yaar M, Gilchrest BA. Photoageing: Mechanism, prevention and therapy. Br J Dermatol. 2007; 157: 874-887.
2) Inagi R. RAGE and glyoxalase in kidney disease. Glycoconj J. 2016; 33: 619-626.
3) Fisher GJ, Datta SC, Talwar HS, et al. Molecular basis of sun-induced premature skin ageing and retinoid antagonism. Nature. 1996; 25: 335-339.
4) Ogura Y, Kuwahara T, Akiyama M, et al. Dermal carbonyl modification is related to the yellowish color change of photo-aged Japanese facial skin. J Dermatol Sci. 2011; 64: 45-52.
5) Abe Y, Takabe W, Yagi M, et al. Melanin synthesis induction by advanced glycation end-products (AGEs) without α-melanocyte stimulating hormone (α-MSH) or UV exposure. Glycative Stress Res. 2016; 3: 229-235.
6) Masuda M, Murata K, Fukuhama A, et al. Inhibitory effect of constituents of *Morinda citrifolia* seed on elastase and tyrosinase. J Nat Med. 2009; 63: 267-273.
7) Masuda M, Itoh K, Murata K, et al. Inhibitory effects of *Morinda citrifolia* extract and its constituents on melanogenesis in murine B16 melanoma cells. Biol Pharm Bull. 2012; 35: 78-83.