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Inhibitory Effect of 2-Aza-8-oxohypoxanthine on Tyrosinase Activity and Melanin Production

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Abstract: A fairy chemical, 2-aza-8-oxohypoxanthine, has exhibited broad effects on skin barrier function, leading to its launch as a cosmetic ingredient. A clinical trial on a lotion containing 0.1% 2-aza-8-oxohypoxanthine demonstrated the ability of this chemical to increase skin lightness based on the analysis of L^* values. In the present study, to elucidate the mechanism underlying the increase in skin lightness, we aimed to investigate the effect of 2-aza-8-oxohypoxanthine on murine melanoma cell lines, focusing on its influence on tyrosinase activity and melanin production. Our findings revealed that the addition of 2-aza-8-oxohypoxanthine inhibited tyrosinase activity by 13% compared with the untreated control. Similarly, melanin production was suppressed by 36% compared with the control. These results strongly suggest that the inhibition of tyrosinase effectively suppressed melanin production. Thus, 2-aza-8-oxohypoxanthine acts by inhibiting tyrosinase and melanin production to promote skin lightening. This study provides novel insights into the skin-lightening mechanism of 2-aza-8-oxohypoxanthine, demonstrating its ability to inhibit melanin production through the suppression of pro-inflammatory factors, thereby highlighting its potential as an effective cosmetic ingredient for lightening skin tone.

Keywords: fairy chemicals; 2-aza-8-oxohypoxanthine; tyrosinase; melanin

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1. Introduction

Chemical compounds such as 2-azahypoxanthin (AHX), imidazole-4-carboxamide (ICA), and 2-aza-8-oxohypoxanthine (AOH) (Figure 1) are referred to as "fairy chemicals" (FCs) owing to their ability to form "fairy rings" [1]. In parks and golf courses, turfgrass occasionally grows in the shape of a ring, displaying thicker growth than that of the surrounding grass, often followed by the emergence of mushrooms within the same circle. This phenomenon, known as a "fairy ring", is a part of Western folklore, where it is believed that fairies create these circles and dance within them [2–4]. FCs are endogenous in the edible parts of the three major cereal grains—rice, wheat, and corn—consumed since ancient times. Furthermore, FCs increase the yield of crops, particularly rice and wheat [5,6]. Hence, FCs are considered a new family of plant hormones [7,8].

Among the three types of FCs, AOH is considered a safe functional cosmetic ingredient [9,10]. Studies have verified the safety of AOH (Table 1) using Ames, in vitro eye and skin irritation, in vitro phototoxicity, human patch testing, human phototoxicity and

photosensitization, and human skin sensitization (repeated insult patch test) testing. AOH exhibits a cell-activating effect on normal human epidermal keratinocytes (NHEK). Additionally, a DNA microarray analysis of genes involved in skin aging and diseases using NHEK revealed that exposure to AOH increases the expression levels of genes involved in skin barrier function in a concentration-dependent manner [11].

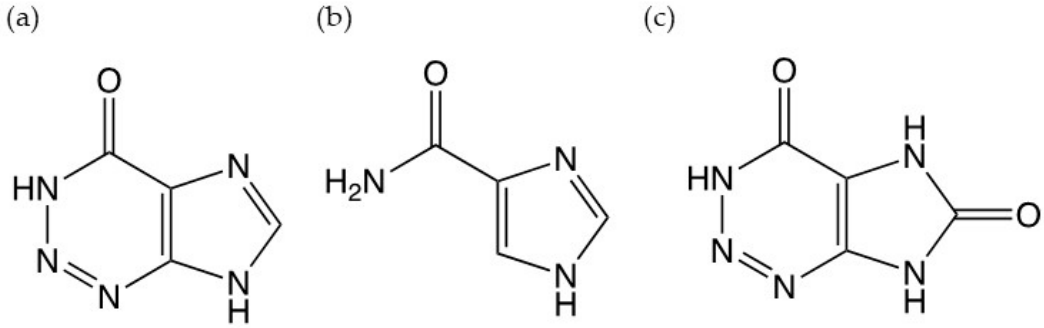

Figure 1. Structures of fairy chemicals (**a**) 2-azahypoxanthin (AHX), (**b**) imidazole-4-carboxamide (ICA), and (**c**) 2-aza-8-oxohypoxanthine (AOH).

Table 1. Comparative results of various AOH safety tests.

Safety Test	Guideline	Result
Ames test	OECD TG471	No mutagenicity (max 5000 μg/mL)
In vitro skin irritation	OECD TG439	No irritation
In vitro eye irritation (Skinethic™ HCE)	OECD TG492	No irritation
In vitro phototoxicity (Skinethic skin irritation)	OECD TG432	No phototoxicity
In vitro skin sensitization (DPRA)	OECD TG442C	No sensitization
In vitro skin sensitization (KeratinoSens)	OECD TG442D	No sensitization
In vitro skin sensitization (h-CLAT)	OECD TG442E	No sensitization
Human skin patch test *	-	Negative (skin irritation index: 3.7)
Human phototoxicity and photosensitization test *	-	Negative
Repeated insult patch test (RIPT) *	-	Negative

* 0.1% AOH aqueous solution.

In melanin synthesis, L-tyrosine is oxidized to L-3,4-dihydroxyphenylalanine (DOPA) and then to DOPAquinone through tyrosinase activity [12–15]. DOPAquinone undergoes auto-oxidation to produce indole compounds, which then combine to form eumelanin. In the presence of cysteine at this stage, DOPAquinone combines with cysteine to form 5-S-cysteinyl DOPA, which subsequently polymerizes to synthesize pheomelanin. Notably, the enzymatic reaction catalyzed by tyrosinase is the rate-limiting reaction in the melanin biosynthesis pathway. Therefore, tyrosinase is a valuable target in dermatology and cosmetology for pharmacologically controlling the enhancement or suppression of skin pigmentation [16,17].

AOH has been demonstrated to increase the moisture content of the skin in a clinical trial focused on assessing the effect of AOH on skin barrier function by evaluating the moisture content of the stratum corneum before and 8 weeks after the application of a 0.1% AOH lotion [18]. This study strongly indicates that AOH effectively regulates skin barrier function. Another clinical trial entailed the application of a lotion containing 0.1% AOH for 8 weeks, followed by the determination of the L* values. The results demonstrated that the L* values of the AOH-applied group significantly increased between 0 and 8 weeks, indicating that AOH increased skin lightness [19]. However, at the cellular level, limited information is available on the effect of AOH on melanin production and the underlying mechanism. Therefore, in the present study, to further evaluate the role of AOH in skin

brightening, we examined its effects on tyrosinase activity and melanin production using murine melanoma cell lines.

2. Materials and Methods

2.1. Chemicals and Reagents

The compound 5-aminoimidazole-4-carboxamide (Sigma-Aldrich, St. Louis, MO, USA) was used to synthesize AOH as previously described [6,20]. AOH was obtained through an oxidation reaction between its precursor AHX and xanthine oxidase, derived from *Burkholderia contaminans* CH-1. The purity of the produced AOH was over 99.9%. B16 melanoma 4A5 (B164A5) cells were purchased from RIKEN Cell Bank (RCB0557). Other reagents used included Dulbecco's modified eagle medium (DMEM) (Nissui Seiyaku, Tokyo, Japan), arbutin, sodium hydrogen carbonate, streptomycin sulfate, trypsin, 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA), Triton X-100, sodium hydroxide, and dimethyl sulfoxide (DMSO) (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan); phenol red-free DMEM (Sigma-Aldrich); L(+)-glutamine (MP Biomedicals Inc., Santa Ana, CA, USA); fetal bovine serum (FBS) (Biowest, Nuaillé, France); phosphate-buffered saline (PBS(–)) (Nacalai Tesque, Kyoto, Japan); and 2-(4-lodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium; and monosodium salt (WST-1) (Dojindo Laboratories, Kumamoto, Japan) were also used.

2.2. Cell Viability Assay

B164A5 cells were seeded at a density of 5.0×10^3 cells/well in 96-well plates and incubated in DMEM medium containing 10% FBS (10%/FBS/DMEM) at 37 °C and 5% CO_2 for 24 h. The cells were then incubated in 10%/FBS/DMEM containing AOH (0 to 1.63 mmol/L) for 24 h. DMEM medium was used as a positive control. Cell viability was evaluated using the WST-1 assay. Cultured cells were replaced with 100 µL of 10%/FBS/DMEM containing 0.03% WST-1 reagent and incubated at 37 °C for 90 min. Cell viability was evaluated by measuring absorbances at 450 nm using FLUOstar OPTIMA (BMG Labtech, Offenburg, Germany). Cell viability was indicated as a percentage (%) relative to the absorbance of the positive control.

2.3. Cell Culture and Tyrosinase Activity Assay

Cells were seeded at a density of 5.0×10^3 cells/well in 96-well plates and incubated at 37 °C for 24 h under 5% CO_2 conditions. Synthesized AOH and 10% FBS/DMEM were used to prepare a stock solution of AOH (1.63 mmol/L concentration) for all subsequent experiments. An AOH concentration of 0.98 mmol/L was prepared via dilution in 10% FBS/DMEM. Cells were treated with 10%/FBS/DMEM containing AOH (0, 0.98, or 1.63 mmol/L) (100 µL) and incubated at 37 °C for 24 h under 5% CO_2 conditions. Untreated cells were used as controls. After removing the medium from each well, the cells were rinsed with PBS(−) (200 µL), 1% Triton X-100 (100 µL) was added to each well, and the plates were allowed to stand for 5 min. The wells were then agitated with a mixer for 1 min, 5 mM L-DOPA (100 µL) was added, and after incubation for 1 h at 37 °C and under 5% CO_2, absorbances at 490 nm were measured using Varioskan Flash 2.4 (Thermo Fisher Scientific, Waltham, MA, USA).

2.4. Cell Culture and Measurement of Melanin

B164A5 cells were seeded at 3.6×10^5 cells/well in 60 mm dishes and incubated at 37 °C for 24 h under 5% CO_2 conditions. Next, 10% FBS/DMEM or 250 µg/mL AOH (5 mL) was added to each dish and incubated for 24 h under 5% CO_2 (37 °C). Arbutin (500 µmol/L) was used as a positive control. This procedure was repeated twice, with an interval of 24 h. The AOH solution was removed, and the cells were trypsinized, scraped, and collected via centrifugation (1200 rpm, 4 °C, 3 min). Then, the supernatant was removed, 2N NaOH (400 µL) was added, and the cells were lysed via sonication (30 min) and pipetting. One

hundred microliters of the extracted solution was added into each well of a 96-well plate, and the absorbances at 405 nm were measured using FLUOstar OPTIMA.

2.5. Statistical Analysis

Data are presented as means ± standard deviations. Statistical analyses (Dunnett's test) were performed using Excel statistics (Bell Curve for Excel, ver. 4.03). Statistical significance was set at <5%.

3. Results

3.1. Cell Viability after AOH Treatment in B164A5 Cells

The WST-1 assay was used to evaluate the effect of AOH on B164A5 cell proliferation. B164A5 cells were treated with AOH (0 to 1.63 mmol/L) and incubated at 37 °C for 48 h. As shown in Figure 2, AOH did not exhibit cytotoxicity in B164A5 cells at concentrations of 0.026–1.63 mmol/L. There was no significant difference in cell viability at any AOH concentration compared with that of the control (0 mmol/L).

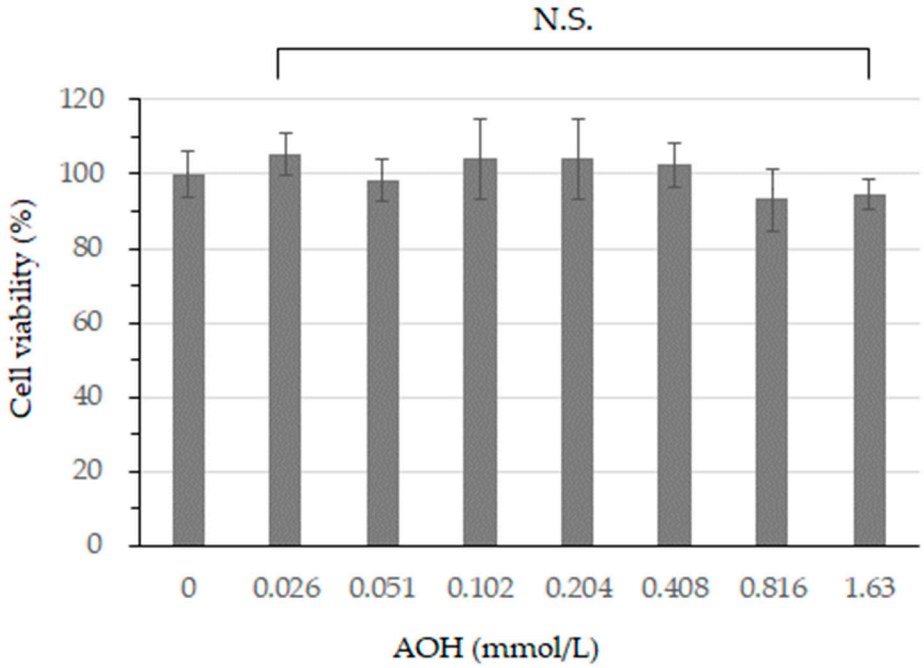

Figure 2. Effect of AOH on cell viability in B164A5 cells. Cell viability was determined using a WST-1 assay. The cell viability percentage was calculated relative to that of the untreated control. N.S.: not significant (vs. control).

3.2. AOH Inhibits Tyrosinase Activity

To evaluate skin brightening, we investigated the effect of AOH on tyrosinase activity. The relative tyrosinase activity we observed is shown in Figure 3. Treatment with AOH (1.63 mmol/L) inhibited tyrosinase activity by 18.9% compared with that of the control ($p < 0.01$). AOH (0.98 mmol/L) also inhibited tyrosinase activity by 16.2% compared with that of the control ($p < 0.05$).

3.3. AOH Inhibits Melanin Production

To assess whether or not AOH affects melanin production by inhibiting tyrosinase activity, we measured melanin content. The relative melanin content we observed is shown in Figure 4. AOH (1.63 mmol/L) significantly inhibited melanin production by 36% compared with the control ($p < 0.01$). Arbutin is a hydroquinone derivative that is β-linked to hydroquinone and glucose and that is found in plants such as cowberry. Arbutin is registered as a quasi-drug, as a substance that prevents pigmentation and that

is used in numerous medicated cosmetics. Arbutin suppresses melanin production by inhibiting the activity of tyrosinase [21–24]. Therefore, we used arbutin as a comparison compound. Under the same test conditions, arbutin (500 μmol/L) also inhibited melanin production by 44% compared with the control. These results indicate that AOH inhibits melanin production, although not to the same extent as arbutin. Microscopic images of B164A5 cells were captured using an optical microscope (Nikon, ECLIPSE, TE2000-S), as shown in Figure 5. Treatment with AOH in B164A5 cells resulted in markedly less melanin production compared with the untreated controls.

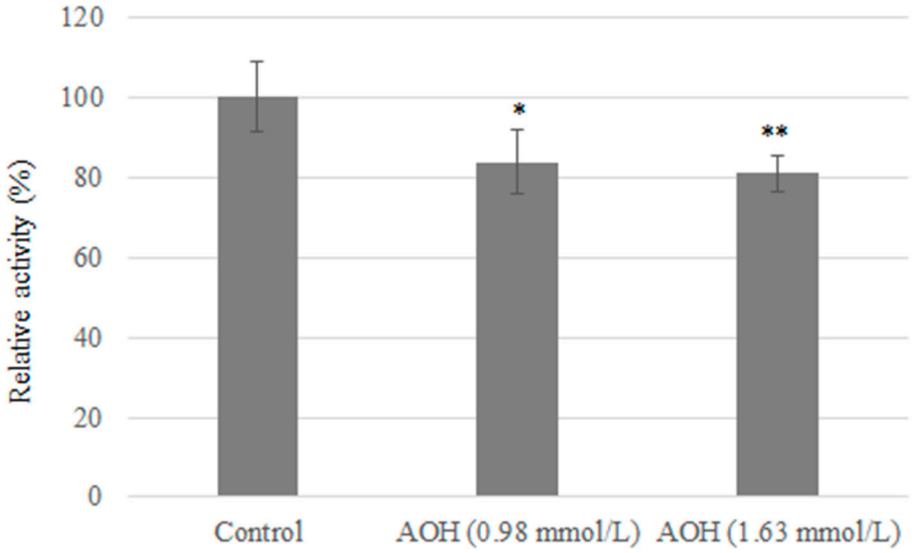

Figure 3. Inhibition of tyrosinase activity following the addition of AOH to B164A5 cells. Tyrosinase activity was calculated as a percentage relative to that of the untreated control. * or ** indicates statistically significant differences compared with the control (* $p < 0.05$, ** $p < 0.01$, and Student's *t*-test).

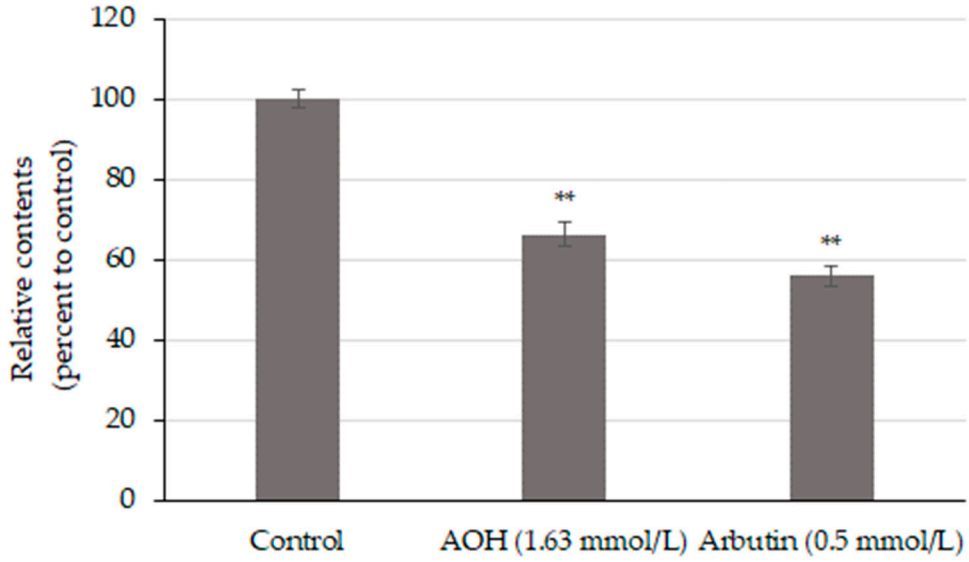

Figure 4. Reduction in melanin production by B164A5 cells after the addition of AOH (1.63 mmol/L) and arbutin (0.5 mmol/L). Melanin content was calculated as a percentage relative to that of the untreated control. ** indicates a statistically significant difference compared with the control (** $p < 0.01$, Student's *t*-test).

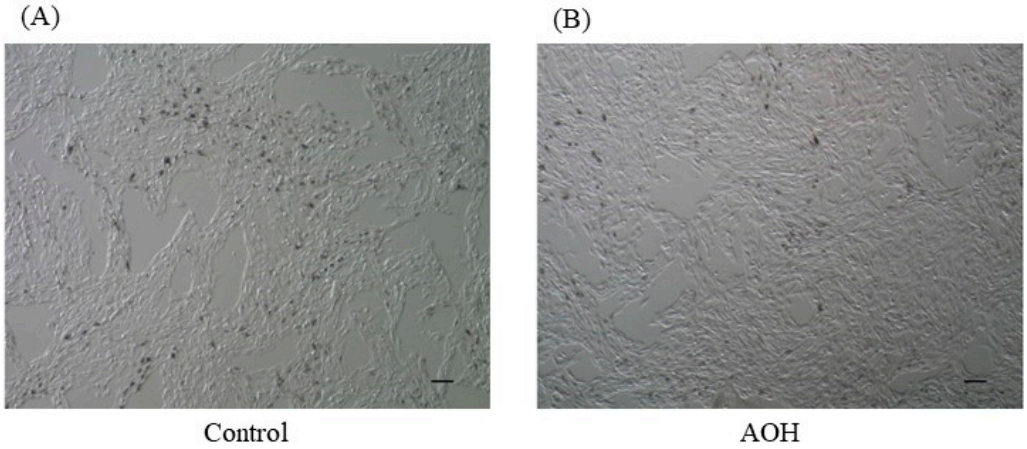

Figure 5. Microscopic images of B164A5 cells captured via optical microscopy. (**A**) Control; (**B**) AOH (1.63 mmol/L). Melanin production was reduced when AOH was added compared with that in the controls. The scale bar represents 100 μm.

4. Discussion

In our previous clinical study, we observed a significant improvement in skin lightness after the application of a 0.1% AOH lotion for 8 weeks [18]. Skin lightening induced by the addition of AOH was attributed to enhanced skin turnover and barrier function based on DNA microarray results [19]. The formation of hyperpigmentation induced by ultraviolet light (UVB) can be divided into three major processes: the proliferation of melanocytes [25], the synthesis [26] and activation of tyrosinase, the rate-limiting enzyme of melanin synthesis [27,28], and melanosome transport from melanocytes to keratinocytes [29]. In this study, we focused on tyrosinase activity and melanin. The color tone of human skin is mainly determined by the amount of melanin. Melanin is biosynthesized in melanocytes in the basal layer of the epidermis by an enzymatic oxidative polymerization reaction using tyrosine, an amino acid, as a starting material [30]. Therefore, skin-lightening effects can be evaluated by measuring the inhibition of tyrosinase activity or melanin levels. In the present study, we quantified tyrosinase activity and melanin levels to verify the lightening effect of AOH. We confirmed that AOH inhibited tyrosinase activity and suppressed melanin production, suggesting that AOH exerts its lightning effect by inhibiting tyrosinase activity and melanin production.

Prostaglandin E_2 (PGE_2) synthase (PGES) and cyclooxygenase 2 (COX-2) are inflammatory cytokines that increase tyrosinase expression [31,32]. The exposure of skin to ultraviolet-B radiation increases reactive oxygen species (ROS) levels in keratinocytes [33]. ROS are involved in the activation of the arachidonic acid cascade; fatty acids (arachidonic acid) excised from plasma membrane phospholipids by phospholipase A2 are converted by COX-2 into the inflammatory chemical mediator PGE_2. In turn, PGE_2 contributes to melanocyte proliferation and dendrite elongation by binding to its receptor on melanocytes (Figure 6) [34]. Notably, a DNA microarray analysis performed on AOH-treated NHEK cells indicated suppressed expression of PGES and COX-2, inflammatory cytokines known to induce melanin pigmentation (unpublished data). In line with the results of the present study, AOH is hypothesized to inhibit tyrosinase activity by suppressing the expression of inflammatory cytokines such as PGES and COX-2. However, this study has some limitations. The quantification of protein levels and a measurement of the enzyme activity of PGES and COX-2 are required to verify this hypothesis. Further studies are needed to elucidate the mechanism by which AOH suppresses the expression of inflammatory cytokines and verify the inhibitory effect of AOH on melanin production under melanogenesis-inducing conditions such as ultraviolet radiation and the addition of a melanocyte-stimulating hormone.

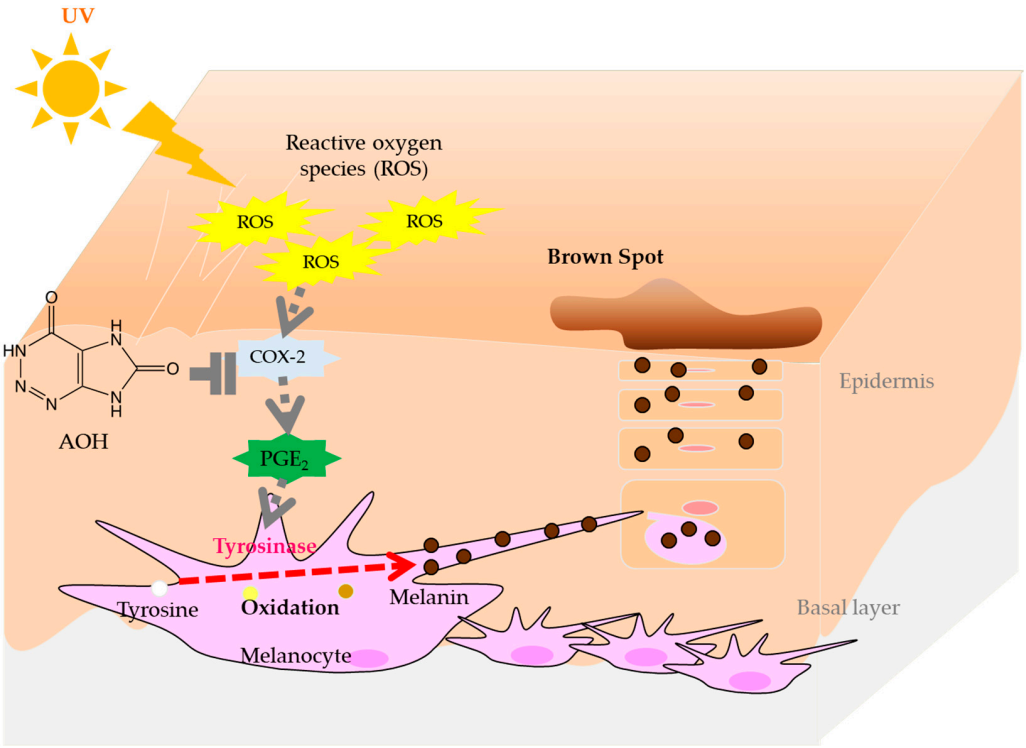

Figure 6. Mechanism of skin lightening by AOH. AOH inhibits tyrosinase activity and suppresses melanin production by reducing COX-2 and PGES expression.

5. Conclusions

To the best of our knowledge, this study is the first to investigate the skin-lightening effect of AOH. Our findings indicate that AOH inhibited tyrosinase activity and suppressed melanin production in B164A5 cells. However, the extent of inhibition of melanin production by AOH was not comparable to that by arbutin, a known brightening agent. Previous clinical trials have demonstrated that AOH application improves skin lightness [19], likely due to the promotion of cell turnover and reduction in inflammatory cytokines by AOH. In our previous study, we reported that AOH suppressed the gene expression levels of PGES and COX-2, which are pro-inflammatory factors involved in the formation of melanin. These results suggest that AOH increased skin lightness by suppressing melanin production through the inhibition of tyrosinase activity following the suppression of PGES and COX-2 expression. However, a direct tyrosinase-inhibitory effect of AOH was also suggested, warranting future studies to elucidate the mechanism underlying the skin-whitening effect of AOH.

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