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Original Article

Sesamol decreases melanin biosynthesis in melanocyte cells and zebrafish: Possible involvement of MITF via the intracellular cAMP and p38/JNK signalling pathways

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Abstract: The development of antimelanogenic agents is important for the prevention of serious aesthetic problems such as melasma, freckles, age spots and chloasma. The aim of this study was to investigate the antimelanogenic effect of sesamol, an active lignan isolated from *Sesamum indicum*, in melan-a cells. Sesamol strongly inhibited melanin biosynthesis and the activity of intracellular tyrosinase by decreasing cyclic adenosine monophosphate (cAMP) accumulation. Sesamol significantly decreased the expression of melanogenesis-related genes, such as tyrosinase, tyrosinase-related protein-1,2 (TRP-1,2), microphthalmia-associated transcription factor (MITF) and melanocortin 1 receptor (MC1R). In addition, sesamol also induces phosphorylation of p38 mitogen-activated protein kinase (p38 MAPK) and c-Jun N-terminal kinase (JNK). Moreover, sesamol dose-dependently decreased zebrafish pigment formation, tyrosinase activity and expression of melanogenesis-related genes. These findings indicate that sesamol inhibited melanin biosynthesis by down-regulating tyrosinase activity and melanin production via regulation of gene expression of melanogenesis-related proteins through modulation of MITF activity, which promoted phosphorylation of p38 and JNK in melan-a cells. Together, these results suggest that sesamol strongly inhibits melanin biosynthesis, and therefore, sesamol represents a new skin-whitening agent for use in cosmetics.

Key words: antimelanogenic agents – cyclic adenosine monophosphate – microphthalmia-associated transcription factor – sesamol – tyrosinase – zebrafish

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Introduction

Melanin is synthesized as a UV defense mechanism via a complex process involving a series of enzymes and diverse signal transduction pathways (1). Melanogenesis is initiated by the tyrosinase (Tyr)-catalysed oxidation of tyrosinase to dopaquinone via the intermediate 3,4-dihydroxyphenylalanine (2). In the absence of thiols, such as cysteine or glutathione, the second enzyme in the melanin synthesis pathway, tyrosinase-related protein 2 (TRP-2), rapidly converts dopaquinone to dopachrome and subsequently converts dopachrome to 5,6-dihydroxyindole (DHI) or indole 5,6-quinone 2-carboxylic acid (DHICA). The last enzyme involved in melanin synthesis, tyrosinase-related protein 1 (TRP-1), catalyses the oxidation of DHICA and forms eumelanins (3). Tyr contains a binuclear copper centre in its active site and catalyses the hydroxylation of phenols to catechols, as well as the oxidation of catechols to quinones (4,5). Additionally, Tyr is a glycoprotein that requires N-glycan with processing by intracellular α-glucosidase and α-mannosidases, and its glycosylation and degradation play crucial roles on the development of anti-melanogenic agents (6).

Melanogenesis is modulated by several factors, including UV irradiation, alpha-melanocyte-stimulating hormone (α-MSH) (7), forskolin, stem cell factor (SCF) (8), wnt-3a (9) and isobutylmethylxanthine (IBMX) (10), within intracellular melanosomes in the melanocytes (11). Stimulation of the melanocortin 1 receptor (MC1R) by α-MSH activates adenyl cyclase through G protein signalling, which subsequently increases cyclic adenosine monophosphate (cAMP) production. cAMP participates in the expression of microphthalmia-associated transcription factor (MITF), a master transcription factor that plays an important role in melanogenesis by transcriptionally regulating Tyr, TRP-1 and TRP-2 expression (12). Mitogen-activated protein kinase (MAPK) signalling is also a key modulator of melanogenesis through its regulation of MITF activation (13). Phosphorylation of extracellular-signal-regulated kinases (ERK), c-Jun N-terminal kinase (JNK) and p38 MAPK lead to MITF ubiquitination and degradation, and thus inhibit melanin production (14–17).

Sesame seeds (*Sesamun indicum* L.) are widely used as food ingredients in Asian and African countries, and sesame seed oil is more widely used than olive oil in Korea. Sesame oil contains high concentrations of tocopherols, tocotrienols and essential fatty acids, such as linolenic acid and omega-3 fatty acid (18). It has been reported that sesame seed oil produces more health benefits than olive oil (19). Sesame seeds are rich in phytochemicals known as sesame lignans, such as sesamin, sesamolin, sesaminol, pinoresinol and sesamol, which are methylene dioxyphenyl compounds (20). Sesamin affects hyperpigmentation (21), alters osteogenic activity (22), lowers cholesterol (23), influences lipid metabolism (24), reduces tumorigenesis in the liver (25) and protects neuronal cells against oxidative stress (26). Very recently, it was demonstrated that sesamol exhibited significant antioxidative and anti-Tyr activity (27), but the molecular mechanisms through which sesamol inhibits melanogenesis have not been studied. In this study, we investigated the inhibitory activity of sesamol on melanin biosynthesis in melanocyte systems and zebrafish embryos

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and studied the underlying molecular mechanisms of the effects of sesamol in melan-a cells.

Materials and methods

Materials

Arbutin, *N*-phenylthiourea (PTU), tetradecanoyl phorbol acetate (TPA), L-DOPA (L-3,4-dihydroxyphenylalanine), L-tyrosine, Tyr, sodium hydroxide (NaOH), thiazolyl blue tetrazolium bromide (MTT), Tween-20, hydrogen chloride (HCl), tricaine methanesulfonate, synthetic melanin, protease inhibitors, TRIS base, dithiothreitol (DTT), bromophenol blue, glycerol, 2-mercaptoethanol, dimethyl sulfoxide (DMSO), isobutylmethylxanthine (IBMX), rosmarinic acid, rutin hydrate, (-)quinic acid, baicalein, coumaric acid, epicatechin, ferulic acid, sesamol, protocatechuic acid, quercetin, phloretin and esculin were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Anti-Tyr (C-19), anti-TRP1 (G-17), anti-TRP2 (D-18), anti-goat IgG-HRP, anti-mouse IgG-HRP or anti-β-actin antibody (Santa Cruz Biotech., Santa Cruz, CA, USA) was used. All other reagents and chemicals were high-grade and commercially available.

Measurement of Tyr activity

Tyr activity was determined as described previously, with minor modifications (27). The reaction mixture for the determination of mushroom Tyr (EC 1.14.18.1) activity consisted of 150 μl of 0.1 M phosphate buffer (pH 6.5), 3 μl of sample, 36 μl of 1.5 mM L-tyrosine and 7 μl of mushroom Tyr (2100 units/ml;, 0.05 M phosphate buffer, pH 6.5), and the reactions were performed in a 96-well microplate (SPL, Pocheon, Korea). The initial absorbance of the mixture was measured at 490 nm using a microplate reader (Perkin Elmer, Waltham, MA, USA). After incubation at 37°C for 30 min, absorbance was measured at 490 nm and inhibitory activity was calculated.

Cell cultures

The melan-a melanocyte line was purchased from DC Bennett (St George's University of London, London, UK). The cells were cultured in RPMI 1640 medium (Hyclone, Logan, UT, USA) supplemented with 10% foetal bovine serum (FBS; Hyclone), streptomycin–penicillin (100 μg/ml each; Hyclone) and 200 nM TPA at 37°C in 5% CO_2. Cells were passaged every 3 days until a maximal passage number of 40 (28).

Cell viability assay

Cell viability was determined using an MTT assay (29). Various concentrations of sesamol, arbutin or PTU were added to the cells and incubated for 24 h. Next, 100 μl of MTT solution (5 mg/ml MTT in PBS) was added to each well, followed by incubation at 37°C for 1 h. After removal of the MTT solution, 1 ml of dimethyl sulfoxide was added to each well with vigorous mixing. The absorbance of each well at 470 nm was determined with a microplate reader.

Melanization inhibition assay and microscopy on melan-a cells

Cells were seeded into a 24-well plate (BD Falcon, Bedford, MA, USA) at a density of 1×10^5 cells per well and allowed to attach overnight. The medium was replaced with fresh medium containing various concentrations of the test compound. Cells were cultured for 72 h and further incubated for 1 day. After washing the cells with PBS, they were lysed with 250 μl of 1N NaOH and transferred to a 96-well plate. Melanin content was estimated by measuring the absorbance at 405 nm using a microplate reader. Arbutin was used as a positive control (30). After incubation under the conditions mentioned above, phase contrast microscopy photographs observed under a Nikon TE2000-U inverted fluorescence microscope (Nikon Instruments, Tokyo, Japan).

Measurements of intracellular Tyr activity and zymography

Intracellular Tyr activity and zymography were performed as described previously (31). Briefly, the test compound was added to melan-a cells (1×10^5 cells/ml), and the culture cells were harvested with RIPA cell lysis buffer (Elpis Biotech., Taejon, Korea) supplemented with a protease inhibitor. For assay of intracellular Tyr activity, after protein quantification, 100 μl of the reaction mixture contained 50 mM of phosphate buffer (pH 6.8), 5 mM L-DOPA and 250 μg of the supernatant protein was transferred into a 96-well plate and incubated for 1 h at 37°C. Absorbance was measured at 405 nm using microplate reader (Fig. 1b; graph). Zymogram sample proteins (30 μg/well) were subjected to 10% SDS-PAGE and gels stained with 20 mM L-DOPA in 0.1 M sodium phosphate buffer at 37°C for 1 h (Fig. 1b; 1st row). Coomassie blue staining was qualified as the same amounts of protein loading (Fig. 1b; 2nd row).

Measurement of cAMP

The cellular cAMP assay was performed with cell lysates using a cAMP direct immunoassay kit (Biovision, San Francisco, CA, USA). Cells were plated into 6-well plates at 3×10^5 cells/well and incubated overnight at 37°C under normal culture conditions. Cells were treated with the test compound at various concentrations, and the cells were lysed and processed according to the manufacturer's protocol using 0.1N HCl. The termination of

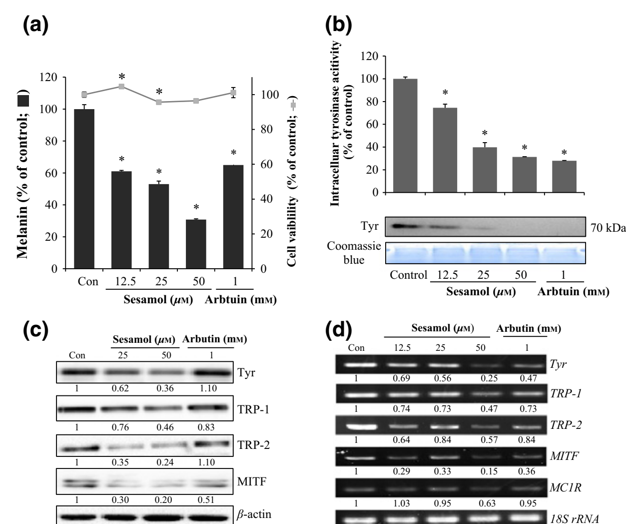

Figure 1. Effect of sesamol on melanogenesis, intracellular tyrosinase and expression of melanogenesis-related protein and genes in melan-a cells. (a) Cells (1×10^5 cells/ml) were cultured for 24 h, and the medium was replaced with fresh medium containing various concentrations of the sesamol and arbutin for 3 days. The cells were collected and lysed with 1N NaOH. Melanin content was estimated by measuring the absorbance at 405 nm. (b) After incubation under the conditions mentioned above, the cells were collected and lysed. The cell lysates were incubated with 20 mM L-DOPA for 1 h. After quantifying protein levels, tyrosinase activity was determined by L-DOPA zymography. Protein was evaluated by Coomassie blue staining. (c) Total cells lysates were extracted and assayed by Western blotting using antibodies against tyrosinase, TRP-1, TRP-2 and MITF. Equal amounts of protein loading were confirmed using β-actin. The band intensity was normalized to the β-actin band using ImageJ software. Cells (2×10^5 cells/ml) were cultured with medium containing various concentrations of the sesamol and arbutin for 24 h. The cells were collected using TRIzol. (d) After quantifying RNA levels, mRNA expression was analysed by RT-PCR. The band intensity was normalized to the 18s rRNA signal using ImageJ software (d). Each determination was made in triplicate, and the data represent the mean ± SD. * $P < 0.05$, Student's *t*-test.

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reactions and cAMP measurements were carried out as described in the cAMP kit manual using a microplate reader.

Western blot analysis

Cell were cultured with or without sesamol and arbutin in six-well plates, and then prepared using a standard protocol. Sample proteins were Western blot analysis was performed as described (32). The Western blot assays were representative of at least three independent experiments.

RT-PCR analysis of mRNA expression

Total RNA was extracted using TRI-zol (Invitrogen Co., Carlsbad, CA, USA) according to the manufacturer's instructions (33). Total RNA (2 μg) was reverse transcribed using reverse transcriptase (MP Biomedicals, Santa Ana, CA, USA) and oligo(dT) primers. cDNA was amplified using a PCR Thermal Cycler Dice TP600 (Takara Bio Inc., Otsu, Shiga, Japan). PCR products were visualized by ethidium bromide staining after electrophoresis (31). The mRNA levels in zebrafish were evaluated as follows: 1 and 2 days after fertilization, embryos (50 per well) were transferred to a 6-well plate with 5 ml of test solution. After 1 day, total RNA was isolated using TRIzol reagent and first-strand cDNA was synthesized using 2 μg of total RNA, 1 μM of oligo-dT_{18} primer and Omniscript Reverse Transcriptase (Qiagen, Valencia, CA, USA). SYBR green-based quantitative PCR was performed using the Applied Biosystems 7500 Fast Real-Time PCR system and Fast SYBR® Green Master Mix (Applied Biosystems, Foster City, CA, USA) as described previously. All reactions were run in triplicate, and data were analysed using the $2^{-\Delta\Delta C_T}$ values method (34). Specific oligonucleotide primers for mouse and zebrafish transcripts were used (Table S1).

Tyr activity and melanin content in zebrafish

Compound treatment and phenotype-based evaluation were measured as previously described (35). Briefly, about 200 zebrafish embryos were treated with melanogenic modulators from 24 to 48 hpf and sonicated in Pro-Prep protein extraction solution (iNtRON Biotechnology, Sungnam, Korea). The lysate was clarified by centrifugation at 9 000 × g for 10 min. After protein quantification, 250 μg of total protein in 100 μl of lysis buffer was transferred into a 96-well plate, and 100 μl of 5 mM L-DOPA was added. The control contained 100 μl of lysis buffer and 100 μl of 5 mM L-DOPA. After incubation for 1 h at 37°C, absorbance was measured at 490 nm. The absorbance of the blank sample was subtracted from each absorbance value, and the final activity was expressed as a percentage of that of the water-exposed control group. PTU was used as a positive control. All experiments were performed in triplicate. For determination of melanin content, extract was prepared at 72 hpf. After centrifugation, the pellet was dissolved in 1 ml of 1 N NaOH at 100°C for 10 min. The optical density of the supernatant was measured at 405 nm, and the result was compared with a standard curve of synthetic melanin.

Statistical analysis

Data are expressed as the mean ± standard deviation. Statistical significance was determined using Student's *t*-test for independent means in Microsoft Excel software (Microsoft Corp., Redmond, WA, USA). The critical level for significance was set at $P < 0.05$ or $P < 0.01$.

Results

Sesamol has anti-melanogenesis activity

To search for natural compounds from plant extracts with anti-melanogenesis effects, we screened 12 single compounds that were isolated from food ingredients for inhibitory effects on melanogenesis using in melan-a cells. From the results of these assays, two compounds with good activity without toxicity were identified: sesamol and quercetin (Table S2). Sesamol was chosen for further evaluation as an anti-melanogenic compound. We examined the effect of sesamol on the activity of Tyr, a key enzyme responsible for melanin synthesis, as well as its copper chelation activity. Sesamol produced a potent inhibitory effect on Tyr with a 50% inhibitory concentration (IC_{50}) of 6.7 μM, and the IC_{50} of arbutin was about 416.6 μM (Fig. S1). Therefore, sesamol was a powerful Tyr inhibitor under the same conditions in which arbutin produced a similar effect. However, sesamol was not found to act as a copper chelator. These findings demonstrate that sesamol suppressed Tyr activity without copper chelating activity (Table S3). Moreover, the capacity of sesamol to inhibit the formation of melanin in *S. bikiniensis* was evaluated by measuring inhibition zones on paper discs, and it was found to produce clear inhibitory activity after 48 h. Sesamol potently inhibited melanin biosynthesis in a dose-dependent manner without influencing the growth of *S. bikiniensis*. Sesamol exhibited inhibition zones of 47 mm at a concentration of 35 μg per disc. Positive control treatment arbutin showed a similar inhibition zone at a concentration of 545 μg per disc. Sesamol had the strongest inhibitory effect on melanin biosynthesis in *S. bikiniensis* (15-fold greater than arbutin) (Table S4).

Sesamol decreased melanin content and inhibited intracellular Tyr activity in melan-a cells

To investigate the melanin content and cytotoxicity of sesamol, after treatment with sesamol at various concentrations for 72 h, cell melanin and viability were measured. Sesamol significantly decreased melanin content in a dose-dependent manner without cytotoxicity (Fig. 1a). Treatment of melan-a melanocyte cells with sesamol (50 μM) or arbutin (1 mM) decreased melanin content to 69 ± 0.5% and 35 ± 3.4%, respectively, than that of the control group (Fig. 1a, 4th and 5th columns). These results indicate that sesamol decreased melanin synthesis without influencing melanocyte viability in melan-a cells. Decreased Tyr activity in melanocytes could be produced either by direct inhibition of Tyr or by repression of Tyr gene expression. Cell lysates prepared from the melan-a cells treated with different concentrations of sesamol were used as a Tyr source. Intracellular Tyr activity was suppressed by treatment with sesamol at concentrations ranging from 12.5 to 50 μM. Sesamol showed a potent anti-Tyr effect with IC_{50} = 97 μM in melan-a cells (Fig. 1b, top). There seem to be little difference in intracellular Tyr inhibitory activity both L-DOPA zymography and intracellular Tyr activity in melan-a cells (Fig. 1b, bottom).

Sesamol inhibited protein levels and expression of melanogenesis-related proteins and genes in melan-a cells

We examined the influence of sesamol on expression of melanogenesis-related proteins Tyr, TRP-1, TRP-2 and MITF, by Western blotting using cell lysates treated with different concentrations of sesamol for 72 h. Sesamol at 50 μM suppressed protein expression of Tyr, TRP-1 and TRP-2 by 65.8 ± 2.8%, 56.4 ± 1.1% and 75.7 ± 4.8%, respectively, in comparison with the control group (Fig. 1c, 1st–3rd rows, 3rd lanes). *MITF* is a key transcription factor that regulates the expression of the most melanogenesis-related genes (13). Sesamol significantly decreased *MITF* protein expression in melanocytes (Fig. 1d, 4th row, 1st–3rd lanes, compared to 4th lane). It has been suggested that down-regulation of melanogenesis-related

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proteins by sesamol could be associated with MITF signalling. To determine whether inhibition of protein expression was associated with decreased mRNA levels of melanogenesis-related genes, the mRNA levels of *Tyr, TRP-1, TRP-2, MITF* and *MC1R* were confirmed using RT-PCR. Sesamol at 50 μM suppressed gene expression of *Tyr, TRP-1* and *TRP-2*, by 74.7 $\pm$ 5.6%, 52.9 $\pm$ 2.2% and 85.3 $\pm$ 4.7%, respectively. Additionally, sesamol significantly decreased mRNA expression of *MITF* and *MC1R* (Fig. 1d). Taken together, these results show that the presence of sesamol significantly decreased the expression of Tyr-related genes, suggesting that it worked by down-regulating *MITF* and *MC1R* transcription.

Sesamol induced Tyr lysosomal and proteasomal degradation in melan-a cells

We next examined whether the decrease in Tyr abundance was due to proteasome-mediated proteolytic degradation using proteolysis inhibitors. To inhibit protein synthesis, cycloheximide (CHX) was added to melan-a cells, which were then pretreated with MG132, a proteasome inhibitor, and/or chloroquine (CQ), a lysosomal proteolysis inhibitor, for 1 h, followed by 6 h of sesamol treatment. As shown in Fig. 2, the decreased Tyr levels that occurred in response to treatment with sesamol were clearly restored by pretreatment with MG132 and CQ. These findings demonstrate that regulation of Tyr protein levels by sesamol results from proteasomal and lysosomal degradation. These results clearly show that sesamol may contribute to the inhibition of melanogenesis by regulating the expression of Tyr-related proteins, as well as MITF, and proteasomal and lysosomal degradation of Tyr.

Sesamol inhibited cAMP-dependent melanogenesis in melan-a cells

Cells were treated with sesamol for 12 h, and intracellular cAMP levels were measured using an ELISA kit. Treatment of melan-a cells with sesamol decreased intracellular cAMP levels in a concentration-dependent manner (Fig. 3a). Furthermore, we elucidated the effects of sesamol on melanogenesis signalling induced by IBMX, an inhibitor of phosphodiesterase. When melan-a cells were incubated with IBMX, the cells formed a black pigment, indicating increased cellular melanogenesis (Fig. 3b). Sesamol dose-dependently decreased IBMX-induced black pigmentation, particularly at a concentration of 50 μM (Fig. S2). Next, we analysed the effects of sesamol on the expression of melanogenesis-related proteins induced by IBMX. Sesamol suppressed the increase in melanogenesis-related proteins induced by IBMX in melanocytes and further suppressed the expression of Tyr, TRP-1, TRP-2 and MITF (Fig. S3). These results revealed that sesamol significantly down-regulated melanogenesis-related proteins through the cAMP signalling pathway.

Sesamol increased phosphorylation of JNK and p38 in melan-a cells

To elucidate the role of MAPK signalling in melanogenesis, the effects of sesamol on JNK, ERK and p38 MAPK phosphorylation were detected by Western blotting. Sesamol increased phosphorylation of JNK and p38 MAPK without affecting phosphorylation of ERK1/2 and did not affect total protein levels of p38 MAPK, ERK or JNK (Fig. 3 c–d). The levels of phosphorylated JNK and p38 were 10 times and 30 times higher than those of the untreated control cells (Fig. 3c, 1st and 3rd lanes). Interestingly, phosphorylation of p38 MAPK and JNK increased in a concentration-dependent manner in 15 min (Fig. 3d, compare 1st–2nd rows to 3rd–4th rows of 1st, 3rd and 5th lanes). These results show that JNK1/2 and p38 MAPK phosphorylation are associated with the effects of sesamol on melanin formation in melanocyte cells.

Sesamol inhibited melanogenesis in zebrafish

Treatment of zebrafish embryos with sesamol for 72 h at concentrations up to 50 μM significantly inhibited skin melanin formation in developed larvae (Fig. 4a). The melanin content of sesamol-treated zebrafish was less than that of untreated zebrafish larva (Fig. 4b). Additionally, we measured total melanin content, Tyr activity and Tyr quantity using whole extracts of zebrafish

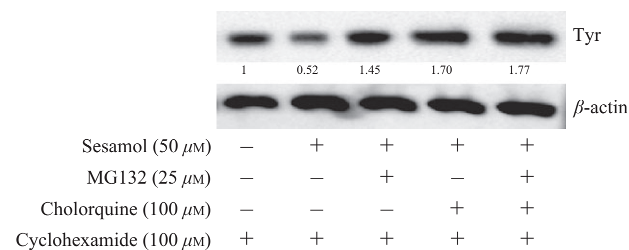

Figure 2. Effect of sesamol on tyrosinase lysosomal and proteasomal degradation in melan-a cells. Cells (3 $\times$ 10^5 cells/ml) were pretreated with 25 μg/ml of cycloheximide, a protein synthesis inhibitor, for 1 h. Cells were also pretreated with 10 μM MG-132, a proteasomal degradation inhibitor, or 50 μM chloroquine, a lysosomal degradation inhibitor, for 1 h, and then treated with sesamol for 6 h. Whole cell lysates were subjected to Western blot analysis using antityrosinase antibodies. Equal protein loading was confirmed using anti-β-actin antibodies.

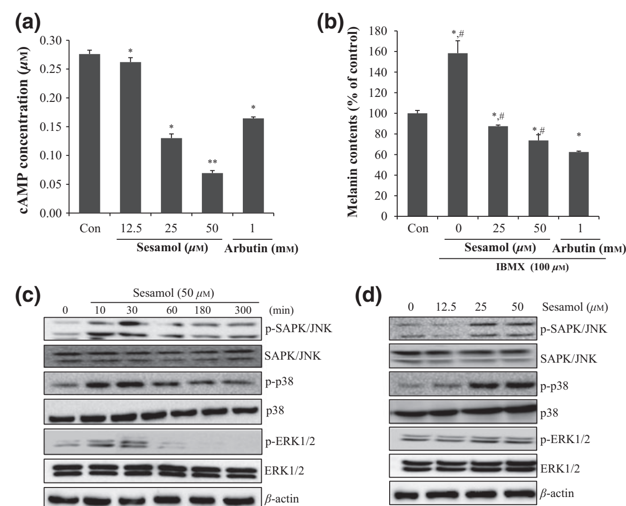

Figure 3. Effect of sesamol on cAMP and MAPkinase-dependent signalling in melan-a cells. (a) Cells (5 $\times$ 10^5 cells/ml) were cultured for 24 h, and the medium was replaced with fresh medium containing various concentrations of test compounds or arbutin for 12 h. Intracellular cAMP levels were measured using a cAMP ELISA kit. (b) Cells (1 $\times$ 10^5 cells/ml) were cultured for 24 h, and the medium was replaced with fresh medium containing various concentrations of sesamol and arbutin, with or without IBMX (a potent inhibitor of phosphodiesterases, 100 μM), for 3 days. Melanin content was estimated by measuring the absorbance at 405 nm. (c and d) Cells (3 $\times$ 10^5 cells/ml) were cultured for 24 h, and the medium was replaced with fresh medium containing various concentrations of sesamol for the indicated times and 15 min. Total protein was extracted and resolved by SDS-PAGE, and the resolved proteins were blotted onto a nitrocellulose membrane. Phosphorylation of JNK, ERK and p38 MAPK was analysed using phospho-specific JNK, ERK and p38 MAPK antibodies. Equal protein loading was checked using β-actin antibodies. Each determination was made in triplicate, and the data represent the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$, versus the control group, and #$P < 0.05$ versus the IBMX-treated group. Student's *t*-test.

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(Fig. 4c). There were considerable decreases in total melanin pigment and Tyr activity after treatment with sesamol. PTU decreased total melanin content and Tyr activity (Fig. 4d). Sesamol at a concentration of 50 μM down-regulated expression of *Tyr, TRP-1, TRP-2* and *MITF* mRNA (Table S5). The results point out that sesamol inhibited the melanogenesis *in vivo*.

Discussion

Sesame oil, used extensively as a traditional health food in Asia, is commonly used in Korea. Sesame seeds are rich in methylene dioxyphenyl phytochemicals called lignans. Sesame lignans, which play an important role in plant defense, are unique phenolic antioxidants with many health benefits (36). Sesamol (3,4-methylene dioxyphenol) is a simple phenolic constituent present in sesame oil that reduces free radicals and prevents certain types of cancer, cardiovascular disease, coronary heart disease, stroke, atherosclerosis and other degenerative diseases related to oxidative stress (37). However, no previous study has examined their antimelanogenesis activity in the context of skin pigment disorders. In the present study, sesamol potently inhibited melanin biosynthesis in melanocytes and zebrafish.

Tyr plays a key role in melanogenesis by catalysing the hydroxylation of tyrosine to L-DOPA and the oxidation of L-DOPA to DOPAquinone. Most skin-whitening agents specifically act to reduce the function of Tyr through several mechanisms. Well-known depigmenting agents, such as hydroquinone, PTU and kojic acid directly inhibit Tyr activity (38). However, arbutin, isolated from bearberry, inhibits both Tyr activity and melanosome maturation (39). Therefore, to determine whether sesamol inhibits Tyr activity, we performed an L-DOPA oxidation assay using mushroom Tyr and intracellular Tyr. Sesamol strongly inhibited both mushroom Tyr and intracellular Tyr activity. Each Tyr molecule contains two copper atoms, and each atom of the binuclear copper cluster is ligated to three histidines. Tyr with different binuclear active site copper structures is involved in the formation of melanin (40). However, sesamol did not show copper chelating activity (Table S3). This result suggests that the inhibitory effect of sesamol on melanogenesis is due to its ability to inhibit cellular Tyr directly without copper chelation. These data were found to be in agreement with previous studies regarding inhibition of mushroom Tyr by sesamol (41). Tyr inhibition by sesamol at the first and second step of melanin biosynthesis is reportedly due to competitive and non-competitive inhibition, respectively (42).

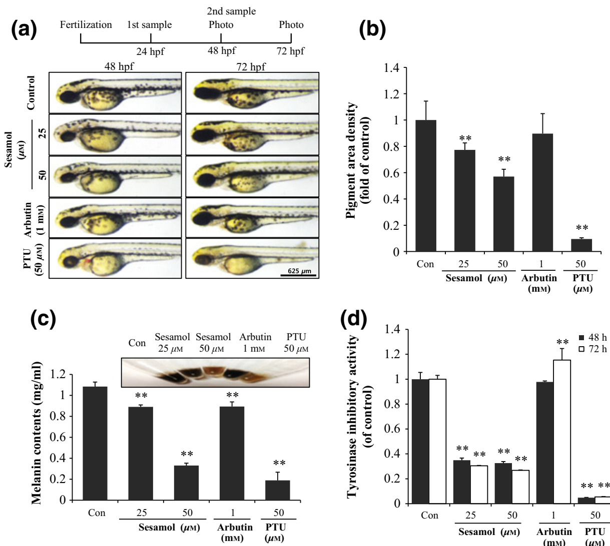

Figure 4. Effects of sesamol on melanogenesis in zebrafish. (a) Synchronized embryos (n = 12) were treated with the test compounds at the indicated concentrations. Test materials were dissolved in 0.1% DMSO and added to the embryo medium. Effects on zebrafish pigmentation were observed under a stereomicroscope (MZ16; Leica Microsystems, Ernst-Leitz-Strasse, Germany) in lateral views of the embryos at 48 and 72 h postfertilization (hpf). (b) The pigment area density was normalized to that of control embryos using ImageJ software. (c) About 200 embryos were collected and dissolved in lysis buffer. After centrifugation, melanin pigment was dissolved in 1N NaOH. Total melanin content was quantified using synthetic melanin. (d) For measurement of tyrosinase activity, 250 μg of total protein was incubated with L-3,4-dihydroxyphenylalanine (final concentration of 5 mM) for 1 h at 37°C and quantified using a spectrometer (d). Values are expressed as mean ± SD of triplicate determinations. **$P < 0.01$ versus the control group.

We directly measured *in vitro* melanin formation and cell toxicity using melan-a cells after sesamol treatment. In comparison with untreated cells, sesamol decreased melanogenesis in melanocyte cells in a concentration-dependent manner (Fig. 1 a–d). In mammals, three major enzymes, Tyr, TRP-1 and TRP-2, directly regulate melanin biosynthesis. Furthermore, MITF is known to be the most important transcription factor involved in melanocyte differentiation and pigmentation (13). To determine the mechanism responsible for the inhibitory effect of sesamol, its effect on melanogenic protein expression was measured. Sesamol decreased protein expression levels of melanogenic proteins and inhibited Tyr, TRP-1 and TRP-2 in melan-a cells. Sesamol also induced down-regulation of MITF protein expression and decreased mRNA levels of Tyr-related genes and *MC1R* (Fig. 1d). α-MSH, which binds to and acts on the MC1R, activates MITF and increases expression of melanogenic proteins, ultimately inducing melanin synthesis (43).

Tyr can be down-regulated by ubiquitin-dependent proteasomal and lysosomal degradation, and Tyr degradation decreases melanogenesis (44,45). Thus, we measured down-regulation of Tyr by sesamol to determine whether it resulted from Tyr degradation. To evaluate this hypothesis, the cells were treated with proteasome inhibitor MG132 and/or lysosomal proteolysis inhibitor CQ. CHX was added to melan-a cells to inhibit protein synthesis, after which the cells were pretreated with MG132 and/or CQ for 1 h followed by 6 h of treatment with sesamol. Tyrosinase protein levels were detected by Western blotting. Changes in Tyr protein abundance in response to treatment with sesamol were restored by pretreatment with MG132 and CQ. Likewise, co-treatment with MG132 and CQ fully blocked Tyr down-regulation (Fig. 2). These results suggest that sesamol leads to Tyr degradation via both lysosomal proteolysis and the ubiquitin–proteasome pathway.

Many reports have suggested that cAMP signalling plays a critical role in the regulation of melanin biosynthesis. Furthermore, cAMP stimulates melanogenesis mainly through activation of MITF and leads to induction of Tyr protein expression (46). Sesamol decreased intracellular cAMP levels in a dose-dependent manner. Furthermore, sesamol inhibited overexpression of melanin biosynthesis by IBMX, which has been shown to increase cellular cAMP and cGMP levels in melan-a cells (Fig. 3b). These results suggest that sesamol decreases melanin biosynthesis via suppression of the MC1R-cAMP-MITF signalling cascade. Moreover, cAMP-induced melanogenesis has been reported to be mediated by a CRE promoter via the binding of CREB family transcription factors that

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are activated by protein kinase A (47). Thus, we will study the effect of sesamol on CREB phosphorylation during melanin biosynthesis in future studies. In addition to cAMP signalling, previous studies have shown that MAPKs such as ERK, p38 MAPK and JNK are also involved in melanogenesis in melanocyte cells (48). We studied the regulation of MAPK phosphorylation by sesamol and found no difference in phospho-ERK levels after sesamol treatment in melan-a cells. On the contrary, phosphorylation of p38 MAPK and JNK was increased by sesamol in a dose- and time-dependent manner (Fig. 3 c–d). These results indicate that sesamol inhibits melanogenesis by activating p38 MAPK and JNK signalling followed by down-regulation of melanogenic proteins.

Because *in vitro* and *in vivo* experiments may give differing results, zebrafish, which have been established as a new *in vivo* model for evaluating the activity of melanogenic regulatory compounds, were evaluated after sesamol treatment (49,50). The zebrafish is an advantageous system for pigment cell chemical biology because the embryos are contained in a large number of transparent eggs that are small enough for easy handling (51). Here, we found that exposure of larvae to sesamol after 48 hpf suppressed pigmentation production even at a low dose, without observable toxicity in the exposed zebrafish. In addition, sesamol markedly decreased melanin content and Tyr activity in comparison with arbutin in zebrafish, and sesamol significantly down-regulated melanogenesis-related gene expression in zebrafish embryos (Fig. 4). Taken together, these results confirm that sesamol suppresses melanin biosynthesis *in vivo* as well as *in vitro*.

In this study, we evaluated the inhibitory effect of sesamol on melanin synthesis in melan-a cells and zebrafish. The present results indicate that sesamol inhibits melanin synthesis in melan-a cells by regulating cAMP, p38 MAP kinase and JNK MAP kinase signalling to suppress MITF, as well as inducing destruction of Tyr via proteasomal and lysosomal degradation. Additionally, sesamol reduced body pigmentation and inhibited intracellular Tyr and melanogenesis-related mRNA transcription in the zebrafish model. Consequently, we suggest that sesamol can be used as a skin-lightening agent for treating hyperpigmentation disorders, and it may be used as an active ingredient in skin care products for preventing the darkening of the skin.

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Author contribution

S.-H. Baek performed the research. S.-H. Baek and S.-H. Lee designed the research, analysed the data and wrote the paper. S.-H. Lee revised the paper.

Conflict of interests

The authors declare no conflict of interests.

Supporting Information

Additional supporting data may be found in the supplementary information of this article.

Figure S1. Effect of sesamol and arbutin on mushroom tyrosinase.
Figure S2. Effect of sesamol on IBMX-induced melanin formation in melan-a cells.
Figure S3. Effect of sesamol in cAMP-dependent protein signalling in melan-a cells.
Table S1. Primer list used for RT-PCR.
Table S2. Inhibitory activity of various compound from natural plant extracts on melanin biosynthesis in melan-a cells.
Table S3. Effect of sesamol against copper chelating activity.
Table S4. Effect of sesamol and arbutin on melanin production in Strptomyces bikiniensis.
Table S5. Effect of sesamol on expression of genes related melanogenesis in zebrafish.

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