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N-nicotinoyl tyramine, a novel niacinamide derivative, inhibits melanogenesis by suppressing MITF gene expression

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ABSTRACT

We synthesized and investigated the inhibitory effects of a novel niacinamide derivative, N-nicotinoyltyramine (NNT) on melanogenesis. NNT inhibited melanin production in B16F10 murine melanoma cells stimulated with α-melanocyte stimulating hormone (α-MSH), in human melanocyte and in three-dimensional cultured human skin model. NNT did not affect the catalytic activity of tyrosinase, but acted as an inhibitor of microphthalmia-associated transcription factor (MITF) and tyrosinase expressions in B16F10 cells. These findings suggest that the hypopigmentary effect of NNT results from the down-regulation of MITF and subsequently of tyrosinase, although NNT did not directly inhibit tyrosinase activity. In addition, safety of NNT was verified through performing neural stem cell morphology assay and Human repeated insult patch test as whitening agent. Our findings indicate that NNT may be a potential and non-skin irritant whitening agent for use in cosmetics and in the medical treatment of pigmentary disorders.

1. Introduction

When skin is exposed to ultraviolet radiation (UVR), α-melanocye stimulating hormone (α-MSH) is secreted from keratinocytes. α-MSH, a physiological ligand that binds to the melanocortin 1 receptor (melanocortin MC_1 receptor), induces expression of microphthalmia-associated transcription factor (MITF) through the elevation of cyclic adenosine monophosphate (cAMP) levels, leading to increased expression of tyrosinase (Buscá and Ballotti, 2000). The synthesis of melanin begins with the conversion of tyrosine to dihydroxyphenylalanine (DOPA) quinone by tyrosinase. Excessive accumulation of DOPA quinone forms DOPA chrome and resulted in accumulation of black-brown eumelanin and red-yellow pheomelanin (Vachtenheim and Borovanský, 2010). Therefore, tyrosinase is the key enzyme in the synthesis of the two types of melanin such as eumelanin and pheomelanin, also skin pigmentation is determined by the amount and the type of melanin synthesized by the melanocytes and its transfer to the neighbouring keratinocytes (Minwalla et al., 2001).

MITF is one of the most critical factors for the regulation of melanocyte function including melanocyte differentiation, pigmentation, proliferation, and survival (such as BCL2, p21, p16, CDK2, TBX2) (Liu and Fisher, 2010). In addition, MITF has roles as an essential regulator for expression of 25 enzymes and structural proteins involved in melanin production (such as tyrosinase, tyrosinase-related protein) (Vachtenheim and Borovanský, 2010). The MITF promoter is regulated by various other transcription factors, including paired box gene 3 (PAX3, which is a neural crest associated transcription factor and is associated with WS1 and WS3), sex-determining region Y-box (SOX) 9, SOX10 (mutations in which result in WS4), lymphoid enhancer-binding factor 1 (LEF-1/TCF, a downstream regulator of the Wnt/β-catenin signaling pathway), cAMP-responsive element-binding protein (CREB), one cut domain 2 (ONECUT-2) (Liu and Fisher, 2010). Also, it was known that various physiological factors from keratinocytes, fibroblasts and other sources regulated the expression and function of MITF (Steingrimsson et al., 2004; Passeron et al., 2007). Therefore, new materials that can modulate MITF level might be able to regulate melanogenesis.

Recent studies have noted the beneficial effects of topical niacinamide (NA, the amide form of vitamin B3) in aging skin, including improved barrier function, decreased appearance of signs of photoaging, and reduced sebum production (Hakozaki et al., 2002; Draelos et al., 2005). And it is known as an effective skin whitening compound that works by inhibiting melanosome transfer from melanocytes to keratinocytes (Hakozaki et al., 2002). In previous study, we synthesized N-nicotinoyl dopamine (NND) by reaction of nicotinic acid and dopamine hydrochloride and

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investigated skin whitening mechanisms related with the pigmentation process (Kim et al., 2011). NND retained high antioxidant activity and reduced the degree of cutaneous pigmentation. In addition, the inhibition of the melanocyte melanosome transfer into keratinocytes had suggested a mechanism for skin whitening (Kim et al., 2011). In this study, we synthesized a new NA derivative, N-nicotinoyl tyramine (NNT) to find out novel whitening material and examined the mechanism of skin whitening related to skin pigmentation disorders. We report here that NNT may be used for cosmetics and drugs for improvement of skin whitening.

2. Materials and methods

2.1. Preparation of N-nicotinoyl tyramine synthesis

Dicyclohexylcarbodiimide (Alfa Aesar, Karlsruhe, Germany) was added to Pyridine (Sigma-Aldrich Co., St. Louis, MO, USA) solution containing tyramine hydrochloride (Sigma-Aldrich) and Nicotinic acid (Sigma-Aldrich). The reaction mixture was stirred at room temperature for 12 h and filtered. The solvent was removed by distillation in vacuum condition. The residue was rinsed with water and purified by medium pressure liquid chromatography (MPLC) (Biotage Isolera, Uppsala, Sweden) (Fig. 1). MPLC separations were carried out on a glass column packed with snap ultra-silica gel at a flow rate of 25 ml/min. Chloroform-Methanol (30:1) was used as mobile phase.

2.2. Cell culture

B16F10 murine melanoma cells (ATCC, Manassas, VA, USA) were maintained in Dulbecco Modified Eagle Medium (DMEM) (Gibco, Grand Island, NY, USA), supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco) and 1% penicillin–streptomycin (10,000 U and 10,000 μg/ml) (Gibco) in 5% CO_2 at 37 °C. Normal human melanocytes (Invitrogen, Carlsbad, CA, USA) were purchased and maintained Medium 254 with human melanocyte growth supplements (Invitrogen, Carlsbad, CA, USA) in 5% CO_2 at 37 °C.

2.3. Primary rat neural stem cell culture

Primary rat neural stem cells were isolated from the cerebral cortex of E14 Sprague-Dawley (SD) rats (KOATECH, Gyeonggi, South Korea). The isolated cells were plated in dishes coated with 15 μg/ml poly-L-ornithine and 10 μg/ml fibronectin (Sigma-Aldrich), grown in N2 medium [DMEM:F12 (1:1) (Invitrogen) containing 100 μM putrescine, 30 nM selenite, 20 nM progesterone, 1.55 mg/ml D-(+)-glucose, 25 μg/ml insulin, 0.1 μg/ml apo-transferrin (Sigma-Aldrich), 0.5 mM Glutamax, 100 IU/ml penicillin, and 100 μg/ml streptomycin] containing 10 ng/ml bFGF (basic Fibroblast Growth Factor; Invitrogen, Carlsbad, CA, USA), or valproic acid (VPA) as an inducer of neural tube defects and incubated in 5% (v/v) CO_2 at 37 °C. To monitor toxicity of NA and NNT, the cells were plated at 2×10^5 cells per each well in a 6-well plate and treated with 1 mM NA or 500 μM NNT for 48 h. The animal experimental procedures were approved by the committee for the Care and Use of Laboratory Animals, Yonsei University College of Medicine and were performed in accordance with the Committee's Guidelines and Regulations for Animal Care.

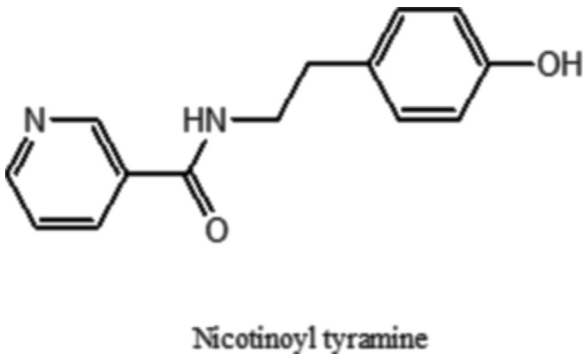

Fig. 1. The structure of Nicotinoyl tyramine.

2.4. Cell viability assay

Cell viability was determined using the CellTiter 96® AQ_{ueous} One Solution Cell proliferation assay kit (Promega, Madison, WI, USA). The B16F10 melanoma cells were seeded into 96-well plate and cultured in the presence of NNT. After 1 day incubation, 20 μl of (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) (MTS) was added to 200 μl of cell culture in the plates. The cells were incubated at 37 °C for 4 h and the absorbance at 490 nm was measured in a microplate ELISA reader (Beckman, Brea, CA, USA).

2.5. Measurement of melanin secretion in B16F10 melanoma cells

Extracellular melanin release was measured as previously described (Siegrist and Eberle, 1986). Briefly, B16F10 cells were incubated at a density of 1.5×10^5 cells in six-well plates overnight. α-MSH (5 nM) was then added and cells were treated with increasing concentrations of NNT (10–250 μM) in phenol red free DMEM for 3 days. 200 μl aliquots of media were then placed in 96-well plates and optical densities (OD) were measured at 405 nm using an ELISA reader (Beckman, Brea, CA, USA). Melanin productions were expressed as percentages of those of untreated controls.

2.6. Measurement of melanin contents in normal human melanocytes

Normal human melanocytes were plated on to 60 mm culture plates at a density of 5×10^5 cells. After 1 day of culture, the medium was changed to fresh medium every other day for a total of 7 days. Cell pellets were then dissolved in 0.4 ml of 1 N NaOH: DMSO: D.W=7: 2: 1 solution at 37 °C for 3 h. Optical densities (OD) of the supernatants were measured at 405 nm using an ELISA reader (Beckman, Brea, CA, USA).

2.7. Tyrosinase activity assay

The tyrosinase activity assay was performed with mushroom tyrosinase because of its ready availability. Each sample was dissolved in DMSO and used for the experiment at 100 times dilution. 0.1 M potassium phosphate buffer (pH 6.8), 3 mM L-tyrosine solution with or without a sample chemical and 2000 units/ml tyrosinase in aqueous solution were mixed. The mixture incubated at 37 °C for 10 min and the reaction was monitored at 475 nm. In addition, quinone is likely to occur from NNT by the oxidation of tyrosinase (Ramsden and Riley, 2014). Therefore, the experimental conditions were planning to eliminate the influence of quinone. S standard reaction was conducted without only sample solution and C control reaction was conducted without only L-tyrosine solution. The percentage of activity of tyrosinase was calculated as follows: [1-(B-C)/S] × 100, where B represents represent the difference in the absorbance of the test sample. Kojic acid was used as references which are well-known tyrosinase inhibitor.

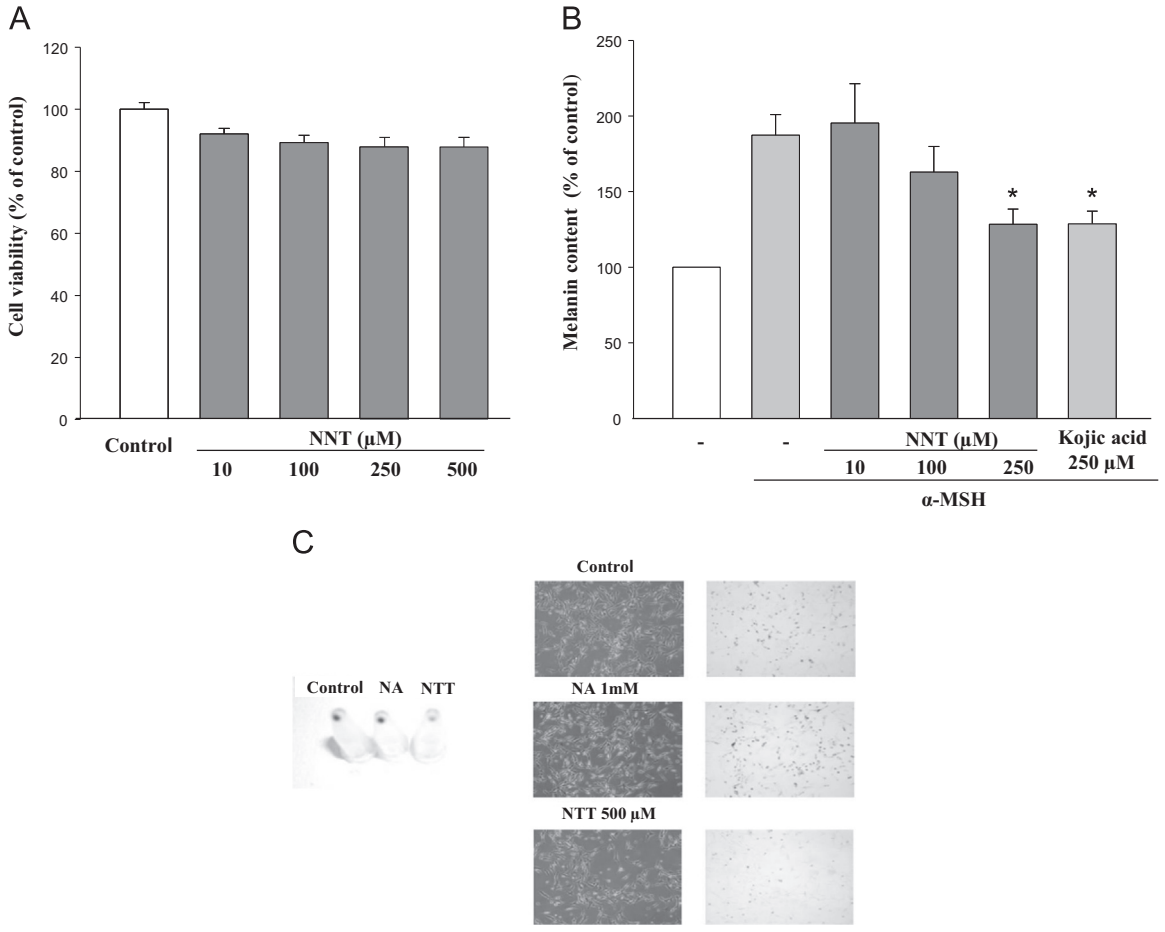

Fig. 2. Determination of cell viability and melanin content in B16F10 melanoma cells. (A) Cells were treated with various concentrations (10–500 μM) of NNT. Cell viabilities were determined using MTS assay kit. (B) Cells were treated with α-MSH of 5 nM in presence or absence of NNT at the indicated concentrations for 3 days. Melanin content in medium was measured, as described in Material and Methods. (C) Photomicrograph of B16F10 cells. The data denote the mean ± S.D. derived from three determinations. $^{*}P < 0.05$ compared to the α-MSH treated control group.

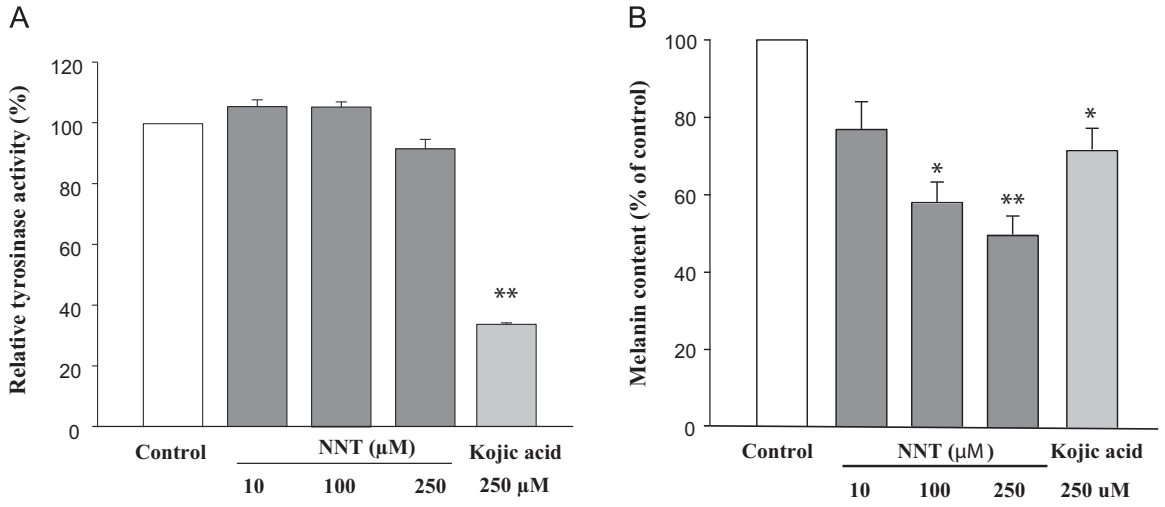

Fig. 3. Inhibitory effect on tyrosinase and melanogenesis in normal human melanocytes. (A) The tyrosinase activity assay was performed with mushroom tyrosinase. (B) Cells were treated with various concentrations (10–250 μM) of NNT for a total of 7 days. Melanin content of cells was determined. The data represent the mean ± S.D. derived from three determinations. $^{*}P < 0.05$, $^{**}P < 0.01$ compared to the untreated control group.

2.8. Western blot analysis

To determine the amount of tyrosinase, western blotting analysis was performed. B16F10 melanoma cells were treated with NNT for 2 days. After the treatment, the cells were collected and washed twice with PBS. The cells were then lysed in RIPA buffer and kept on ice for 1 h. Cell lysates were centrifuged at 12,000*g* at 4 °C for 15 min, and the supernatants were stored at − 70 °C until required for analysis. Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA, USA). Aliquots of the

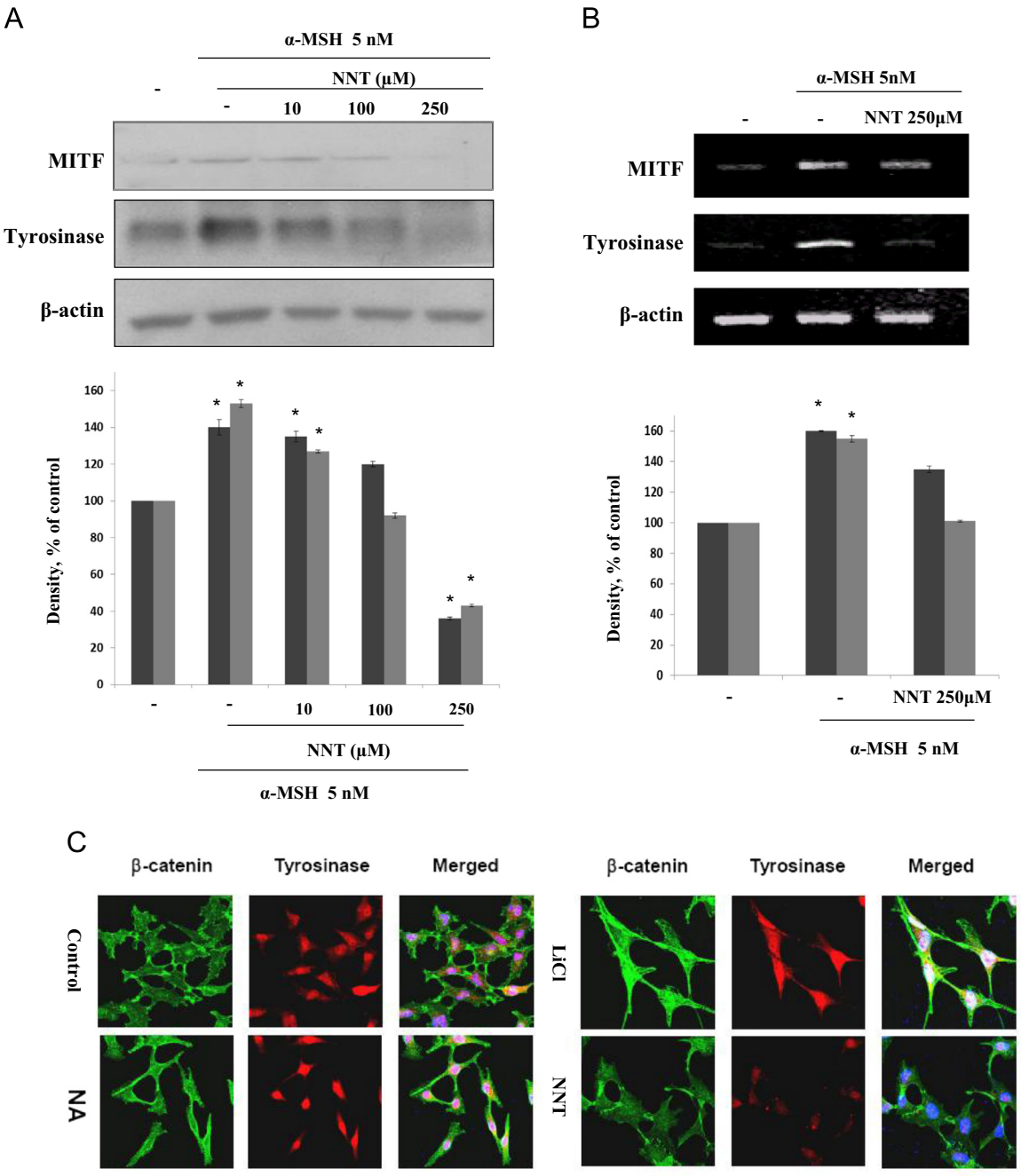

Fig. 4. Effect of NNT on expression of MITF, tyrosinase, and β-catenin in B16F10 melanoma cells treated with NNT. (A) Western blot analysis on expression of MITF and tyrosinase. (B) Expression analysis on genes of MITF and tyrosinase by RT-PCR. Cells were treated with 5 nM α-MSH in presence or absence of NNT at the indicated concentration for 2 days. β-actin was used as an internal standard. Values were normalized to β-actin before calculating changes. Black bars, MITF expression; grey bars, tyrosinase expression. (C) Immunofluorescence analysis of β-catenin and tyrosinase. LiCl was used as a positive control.

lysates (20 μg of protein) were applied to a 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel and transferred onto a polyvinylidene fluoride (PVDF) membrane. After blocking nonspecific sites with 5% non-fat milk powder in Tris-buffered saline with 0.1% Tween-20 (TBST), the membranes were subsequently incubated with specific primary antibodies (mouse anti-MITF: 1:1000, Sigma-Aldrich, St. Louis, MO, USA; mouse anti-tyrosinase: 1:200, Abcam, Cambridge, UK; mouse anti-β-actin: 1:5000, Sigma-Aldrich, St. Louis, MO, USA) for 3 h at room temperature. The membranes were subsequently incubated for 1 h with peroxide-conjugated secondary antibodies (rabbit anti-mouse IgG-HRP: 1:5000, Santa Cruz Biotechnology, Carlsbad, CA, USA). The immunoreactive proteins were detected using an enhanced chemiluminescence (ECL) western blotting detection kit (Amersham Pharmacia Biotech, NY, USA). β-actin was used as the standard for normalizing protein samples. The band intensities were quantified using a Photo-Image System (Molecular Dynamics, Uppsala, Sweden).

2.9. RT-PCR

B16F10 melanoma cells were treated with NNT for 2 days. Cells were washed with PBS, and total RNA was isolated using Trizol reagent according to the manufacturer's instructions (Invitrogen, Carlsbad, CA, USA). Reverse transcription was carried out with 1 μg RNA samples by using reverse transcription master premix (ELPIS

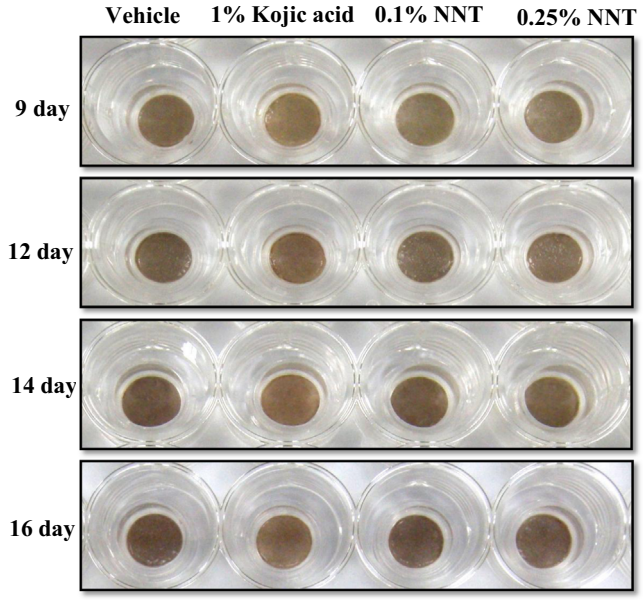

Fig. 5. Macroscopic views of pigmented reconstructed epidermis model treated with NNT. Different concentrations of NNT, kojic acid, and the vehicle emulsion were applied topically on the reconstructed skin and tissues were monitored for up to 16 days.

Table 1
Quantification of melanin amount.

Treatment (21d)	Melanin content (% of control)[a]
Vehicle	100
1% Kojic acid	76.5
0.1% NNT	84.4
0.25% NNT	86.3

[a] Melanin amount was monitored by O.D. 450 nm measurements of the lysates of reconstructed epidermis. Values are data for skin lightness using semi-quantitative image analysis program.

Biotech, Daejeon, South Korea) at 42 °C for 60 min followed by inactivation of the enzyme at 94 °C for 5 min. The PCR was carried out in a 20 μl reaction mixture containing 4 μl of rTaq Plus 5x PCR Premix (ELPIS Biotech), 13 μl of double-distilled DNase-free water, 1 μl of each primer (10 pmol), and 1 μl of reverse transcriptase (RT) product. The polymerase chain reaction (PCR) was performed on a GeneAmp PCR System 9700 system (Applied Biosystems, Carlsbad, CA, USA). The sequences of the specific primers for MITF, tyrosinase, and β-actin are as follows: sense 5′-CCC GTC TCT GGA AAC TTG ATC-3′ and antisense 5′-CTG TAC TCT GAG CAG CAG GTG-3′ for MITF; sense 5′-TTG GCA GAT TGT CTG TAG CC-3′ and antisense 5′-AGG CAT TGT GCA TGC TGC TT-3′ for tyrosinase; sense 5′-TGG AAT CCT GTG GCA TCC ATG AAA C-3′ and antisense 5′-TAA AAC GCA GCT CAG TAA CAG TCC G-3′ for β-actin. Amplification was performed for 28–30 cycles. Each cycle consisted of denaturation for 10 s at 95 °C, annealing for 10 s at the appropriate primer-specific temperature, and extension for 10 sec at 72 °C. An additional incubation at 72 °C for 10 min was executed after the last cycle. The PCR products were subject to electrophoresis on 1.8% agarose gels. The signal intensity of the amplification product was analyzed using UVIband software (UVItec, UK).

Table 2
Human repeated insult patch test.

Sample	Responders	Response	M.I.I.[a]	Sensitization[b]
Placebo	2	1.0/2.0	0.02	–
NNT	2	1.0/2.0	0.02	–

Modified Shelanski method was conducted and evaluated skin sensitization potential of 0.2% NNT emulsion on 52 female subjects.

[a] M.I.I. means Mean Irritation Index.
[b] – means lack of sensitization.

2.10. Immunocytochemistry

Cells were fixed with 4% paraformaldehyde in PBS for 15 min at room temperature and washed with PBS. For permeabilization, cells were treated with 0.1% Triton X-100 for 15 min at room temperature. And cells were blocked with 5% BSA and 1% normal goat serum in PBS for 30 min at room temperature and successively incubated with mouse anti-β-catenin (1:100, BD transduction laboratory, Lexington, KY, USA) and anti-tyrosinase antibody (1:200, Abcam, Cambridge, UK) overnight at 4 °C. Cells were rinsed with PBS and then incubated with Alexa Fluor 488-conjugated

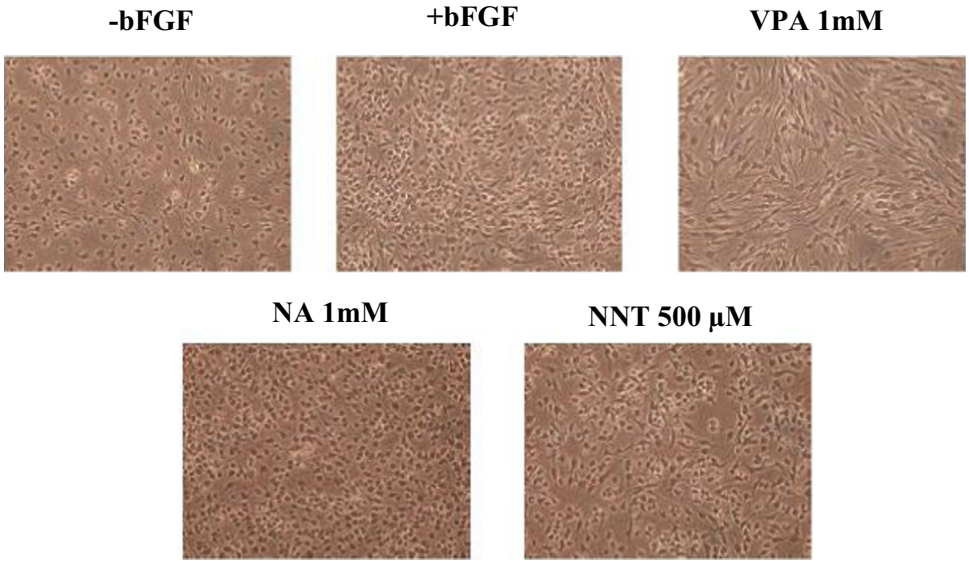

Fig. 6. Morphology of rat neuronal stem cells after treatment of NNT. Bright field microscopy images of rat neuronal stem cells. The cells were treated with 1 mM NA or 500 μM NNT for 48 h in the presence of 10 ng/ml bFGF.

goat anti-mouse antibody (Molecular Probes, Eugene, OR, 1:400) for 1 h at room temperature, counterstained with 4′-6-Diamidino-2-phenylindole (DAPI) (Boehringer Mannheim, Mannheim, Germany, 1:5000), and examined under confocal microscope, LSM510 META (Carl Zeiss, Gottingen, Germany).

2.11. Pigmented reconstructed epidermis

Reconstructed human epidermis MEL-300-B (MatTek, Ashland, MA, USA) consisted of normal human-derived epidermal keratinocytes and normal human epidermal melanocytes that had been cultured to form a multilayered, highly differentiated model. Reconstructed epidermis was placed in 6-well plates and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. The following 25 mg of test emulsions were applied to the apical surface of the tissues: 0.1%, 0.25% NNT, and 1% kojic acid as positive control. Base emulsion formulation contained: water, EDTA-2Na, Glycerin, 1,3-butylene glycol, cetearyl alcohol, stearic acid, ethanol, dimethicone, and PEG-60 hydrogenated castor oil. The tissue was incubated for 19 days, and fed with 5 ml of fresh medium every other day. At indicated time points the samples photographed. Color reflectance of photos has been used to extract the separate components of skin color histogram that occur due to the presence of melanin by image analysis program (Image Pro plus version 4.1, Media Cybernetics, Bethesda, MD, USA). The larger value means that color of epidermis is brighter. Melanin was measured as previously described (Bessou-Touya et al., 1998).

2.12. Human repeated insult patch test

52 female subjects between the ages of 20 and 52 were selected for the study according to the inclusion and exclusion criteria. All subjects signed an informed consent in conformance with the good clinical practice (GCP) and declaration of helsinki. The study conducted by modified shelanski method (Kligman, 1966) and evaluated skin sensitization potential of two test products, NNT 0.2% emulsion and vehicle emulsion, after repeated 24 h epicutaneous applications under occlusive patch. The subjects visited the Research Facility for total of 14 times during 6 weeks. For both induction and challenge phases, approximately 60 μl of the test products with IQ chambers (Chemotechnique Diagnostics, Sweden) were applied to each designated site of the back between the scapulae and the waist, after being washed with 70% ethanol. The patches were allowed to remain in direct skin contact for a period of 24 h. The test products had been studied at the same time on the same subject. A total of 10 applications of the test products were made. All experimental protocols were developed according to the principles of ethics and animal welfare designated by the Ellead Co., Ltd. The experimental protocols were approved by the Ethics Commission of Ellead Co., Ltd. (number: EL-10070904RZZ01-01; September 02, 2010).

2.13. Statistics

All data are presented as the mean ± standard deviation (S.D.). Comparisons between multiple groups were performed with one-way ANOVA (Analysis of Variance) with Bonferroni's test. The *P*-value of less than 0.05 was considered significant.

3. Results

3.1. Effects of NNT on anti-melanogenesis in B16F10 melanoma cells

We treated B16F10 melanoma cells with NNT to determine whether it has a cytotoxic effect. Cell viability was determined using MTS assays. The results showed that NNT was not cytotoxic at B16F10 cells in the concentration range (10–500 μM) (Fig. 2A). Also, cells were exposed to NNT (10–250 μM) in the presence of α-MSH (5 nM) for 3 days, and extracellular melanin release was measured. As shown in Fig. 2B, NNT reduced α-MSH-induced melanin release in a dose-dependent manner. Additionally, cells treated with 250 μM NNT showed a significant low level of melanin like that seen in kojic acid of the same concentration. In addition, as shown in Fig. 2C, inhibitory effect on melanin synthesis of NNT was confirmed by photomicrograph of B16F10 cells unlike control and niacinamide (NA).

3.2. Effects of NNT on tyrosinase activity and anti-melanogenesis in normal human melanocytes

To evaluate the direct effect of NNT on tyrosinase activity, mushroom tyrosinase assay was performed. NNT had no inhibitory effect on tyrosinase activity, whereas kojic acid of 250 μM (a direct inhibitor of tyrosinase) had a strong inhibitory effect (Fig. 3A). These results indicate that the inhibitory effect of NNT on melanogenesis is not due to its direct inhibition of tyrosinase activity unlike kojic acid. To examine the depigmenting effect of NNT in normal human melanocytes, a melanin content assay was performed. NNT did not affect cell viability in human melanocytes (data not shown). The melanin content of normal human melanocytes was decreased by the addition of NNT at concentrations of 10–250 μM. Additionally, cells treated with 250 μM NNT showed a significant low level of melanin compared to kojic acid of the same concentration (Fig. 3B). These results indicate that NNT has a different mechanism on anti-melanogenesis.

3.3. Inhibitory effect of NNT on MITF and tyrosinase expression in α-MSH stimulated B16F10 melanoma cells

It is known that the levels of MITF and tyrosinase were increased by α-MSH in melanocytes. Firstly, we confirmed changes in the expression of MITF and tyrosinase proteins by stimulation of α-MSH (5 nM) in B16F10 melanoma cells. As shown in Fig. 4A, the levels of MITF and tyrosinase protein treated with α-MSH showed high levels of expression compared with the untreated control group, but NNT significantly suppressed the protein expression of MITF and tyrosinase according to concentration. To assess expression of genes related to MITF and tyrosinase, total RNA was isolated and analyzed by semi-quantitative RT-PCR. As shown in Fig. 4B, the gene expression levels of MITF and tyrosinase increased in the groups treated with α-MSH compared with untreated group but NNT reduced the gene expression of MITF and tyrosinase. These results were consistent with the data of protein expression. It is known that β-catenin plays important roles in melanocyte development and migration (Bellei et al., 2011) and activates MITF (Vachtenheim and Borovanský, 2010). We examined the immunofluorescence analysis. As shown in Fig. 4C, NNT significantly suppressed the protein expression of tyrosinase unlike NA, but NNT had no inhibitory effect on β-catenin expression. These results indicated that NNT reduce melanin synthesis by down-regulation of MITF and tyrosinase directly regardless of β-catenin signaling. NA or kojic acid did not affect tyrosinase expression (data not shown).

3.4. Effect of NNT on melanogenesis in cultured Human skin model

To identify the effects of NNT on natural skin pigmentation, we used three-dimensional cultured human skin models (MelanoDerm™). Different concentrations of NNT, kojic acid, and the vehicle emulsion were applied topically on the reconstructed skin and tissues were monitored for up to 16 days post-

application. Photographs of skin specimens show slightly reduced pigmentation in NNT-treated tissues, but the levels were somewhat lower than that in the group treated with kojic acid (Fig. 5, Table 1).

3.5. Effect of NNT on toxicity in rat neuronal stem cells and human repeated insult patch test

Neural stem cells have been used to screen for compound toxicity (Meli et al., 2014). To monitor toxicity of NA and NNT, we examined the effect of NA and NNT in rat neural stem cells. The cells were treated with 1 mM NA or 500 μM NNT for 48 h in the presence of 10 ng/ml bFGF. Cytotoxicity of NA and NNT was not detected in the rat neural stem cells (Fig. 6). These results indicate that NNT is a safe compound. The human repeated insult patch test (HRIPT) is a confirmatory test in the safety evaluation of skin sensitizers (Basketter, 2009). It is concluded that where there is a specific rationale for testing, for example, to substantiate a no-effect level for a sensitizing chemical or to ensure that matrix effects are not making an unexpected contribution to sensitizing potency, then rigorous independent review may confirm that an HRIPT is ethical and scientifically justifiable. Under the conditions of the human repeated insult patch test (HRIPT), there was no evidence of skin irritation or sensitization in any of the 52 subjects (Table 2). The results provide evidence for the lack of skin sensitization potential for 0.2% NNT.

4. Discussion

Pigmentation disorders, such as melasma, post-inflammatory hyperpigmentation and lentigo senilis, are associated with the abnormal accumulation of melanin pigments (Solano et al., 2006). Also, the regulation of skin pigmentation has been a goal for cosmetics and pharmaceutical applications (Briganti et al., 2003). There are two different mechanisms with respect to inhibition of melanin synthesis in melanocytes. The first is direct inhibition of tyrosinase activity and the second is the suppression of melanogenesis-related factors' expression at the transcriptional level. Most anti-melanogenic compounds such as kojic acid and hydroquinone inhibit tyrosinase activity directly. On the other hand, some agents have anti-melanogenic activity by the suppression of tyrosinase expression (Chung et al., 2009). In our study, N-nicotinoyl tyramine (NNT), a newly synthesized NA derivate, was characterized as a relatively safe and effective skin-whitening reagent. Most studies involving melanogenesis and skin whitening are mostly focused on the inhibition of tyrosinase within the melanosomes of melanocytes (Duval et al., 2002). However, the inhibition of melanin synthesis is not a cause of the depigmentation by NNT as revealed by the absent effects of NNT in the tyrosinase activity. In our previous report, N-nicotinoyl dopamine retained high antioxidant activity and inhibited melanin transfer from melanocytes to keratinocytes (Kim et al., 2011). However, it was not shown to effectively inhibit melanosome transfer in keratinocyte-melanocyte co-culture (Fig. 5). Topical application of 0.1% and 0.25% NNT emulsions resulted in somewhat skin lightening and melanin production was only decreased by 15.6% and 13.7%, respectively. On the other hand, 0.05% and 0.1% NND resulted in 31.1% and 28.5% reduction of melanin, respectively (Kim et al., 2011). Moreover, NNT showed no DPPH radical scavenging activity which indicated the antioxidant effect (data not shown). The structural difference between NND and NNT was only a hydroxyl group derived from dopamine hydrochloride during the synthesis of niacinamide derivatives. Therefore, further studies will be needed to elucidate the main cause of the hydroxyl group affecting different patterns on the suppression of melanogenesis. NNT was found to inhibit the expression of melanogenesis-related factors, including tyrosinase and MITF. Recently, the suppression of melanogenesis-related factors' expression has become an attractive target for the research of melanogenesis and skin whitening (Xu et al., 2000; Park et al., 2009). Therefore, the skin whitening effect of NNT may also be attributed to the inhibition the expression of MITF. The suppression of MITF can be attributed to target genes related to pigment formation in melanocytes (Vachtenheim and Borovanský, 2010).

Two transcription factors such as the Wnt/β-catenin pathway effector LEF-1 and the cAMP pathway effector cAMP response element binding (CREB) were important for the maintenance of MITF levels in melanoma cells (Vachtenheim and Borovanský, 2010). A link between Wnt/β-catenin signaling and melanocyte differentiation has been revealed by the finding that β-catenin, which accumulates with activation of Wnt/β-catenin signaling, forms a complex with lymphocyte enhancer factor-1 to up-regulate expression of the MITF gene (Takeda et al., 2000). Also, β-catenin directly interacts with the MITF protein itself and then activates MITF-specific target genes (Schepsky et al., 2006). However, NNT did not promote the degradation of intracellular β-catenin. Tyrosinase is the first rate-limiting enzyme and transcriptional target of MITF. In other word, transcription of tyrosinase can be triggered by MITF and its level should be reflected by changes in transcriptional activity of MITF gene. It is believed that the inhibition of MITF expression leads to the decreased levels of its target genes such as tyrosinase gene in melanoma cells (Levy et al., 2006). Our study showed that the gene expression of tyrosinase and MITF was decreased so that the levels of tyrosinase and MITF proteins were reduced by NNT. Therefore, it has been considered that the inhibition of MITF expression leads to the decreased levels of the down-stream genes such as tyrosinase (Levy et al., 2006). To evaluate the effects of NNT as a cosmetic ingredient, we treated NNT emulsion on reconstructed skin. Our study indicates that photographs of skin specimens show slightly reduced pigmentation in NNT-treated tissues. Additionally, skin sensitization risk assessment of new ingredients or products is critical before their introduction into the marketplace (Gerberick and Robinson, 2000). We confirmed that NNT was not likely to induce skin irritation by the human repeated insult patch test. These results suggest that NNT may potentially be used for cosmetics or drugs for improvement of skin whitening and therapies related to skin pigmentation disorders, and furthermore, that this compound may have advantages as it is a novel material with no adverse effects on human skin. Moreover, we suggested that NND may be attributed to the inhibition of the melanocyte melanosome transfer into keratinocytes in previous study (Kim et al., 2011), but the factors for the distribution patterns of melanosomes within keratinocytes should be investigated to clarify the anti-melanosome transfer mechanism of NND. On the other hand, we investigated that NNT inhibits melanogenesis by suppressing MITF gene expression therefore the purpose of this study is to propose the mechanism of up-stream level on the inhibition of melanin transfer by NNT.

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