

Cutaneous Biology

The effect of niacinamide on reducing cutaneous pigmentation and suppression of melanosome transfer

T.HAKOZAKI, L.MINWALLA,* J.ZHUANG,† M.CHHOA,† A.MATSUBARA, K.MIYAMOTO, A.GREATENS,* G.G.HILLEBRAND,‡ D.L.BISSETT† AND R.E.BOISSY*

Research & Development Department, Procter & Gamble Far East, Inc., Kobe Technical Center 7F, Naka 1–17, Koyo-cho, Higashinada-ku, Kobe 658-0032, Japan

*Dermatology Department, University of Cincinnati, Cincinnati, OH, U.S.A.

†Skin Technology Division and ‡External Business Development & Corporate Licensing, Procter & Gamble, Cincinnati, OH, U.S.A.

Accepted for publication 31 January 2002

Summary

Background Cutaneous hyperpigmentation occurs in multiple conditions. In addition, many Asian women desire a lighter skin colour. Thus, there is a need for the development of skin lightening agents. Niacinamide is a possible candidate.

Objectives To investigate the effects of niacinamide on melanogenesis *in vitro* and on facial hyperpigmentation and skin colour *in vivo* in Japanese women.

Methods Melanin production was measured in a purified mushroom tyrosinase assay, cultured melanocytes, a keratinocyte/melanocyte coculture model, and a pigmented reconstructed epidermis (PREP) model. The clinical trials included 18 subjects with hyperpigmentation who used 5% niacinamide moisturizer and vehicle moisturizer in a paired design, and 120 subjects with facial tanning who were assigned to two of three treatments: vehicle, sunscreen and 2% niacinamide + sunscreen. Changes in facial hyperpigmentation and skin colour were objectively quantified by computer analysis and visual grading of high-resolution digital images of the face.

Results Niacinamide had no effect on the catalytic activity of mushroom tyrosinase or on melanogenesis in cultured melanocytes. However, niacinamide gave 35–68% inhibition of melanosome transfer in the coculture model and reduced cutaneous pigmentation in the PREP model. In the clinical studies, niacinamide significantly decreased hyperpigmentation and increased skin lightness compared with vehicle alone after 4 weeks of use.

Conclusions The data suggest niacinamide is an effective skin lightening compound that works by inhibiting melanosome transfer from melanocytes to keratinocytes.

Key words: hyperpigmentation, melanogenesis, melanosome transfer, niacinamide, skin lightening, skin pigmentation

In many regions of the world, having a light and even skin colour is highly valued. Approximately 60% of Japanese women and 75% of Chinese women desire to achieve a lighter skin colour (researched by the Procter & Gamble Company). In addition, many hyperpigmentary skin disorders such as melasma, postinflammatory hyperpigmentation and senile lentiginos can be exacerbated by sun exposure. One

effective way to maintain a light and even skin colour and to ameliorate hyperpigmentation is to avoid sun exposure by using sunscreens or by wearing protective clothing. Jonker *et al.*¹ proposed that the regular use of sun protection products may lead to skin lightening, as the primary stimulus for facultative melanin synthesis is excluded and pigment that is present in the epidermis is lost through stratum corneum desquamation.

Numerous types of skin care products containing purported skin lightening agents such as vitamin C

Correspondence: Tomohiro Hakozaki.
E-mail: hakozaki.t.1@pg.com

derivatives, arbutin and kojic acid² are commercially available. These skin lightening products suppress or inhibit melanogenesis in various ways. Niacinamide (also known as nicotinamide, 3-pyridinecarboxamide) is the physiologically active amide of niacin (vitamin B₃). Several reports suggest that niacinamide may have various effects on the skin: it acts as an anti-inflammatory agent in acne,³ as an antioxidant,⁴ prevents photoimmunosuppression and photocarcinogenesis,⁵ and increases intercellular lipid synthesis.⁶ Topical niacinamide has been used to treat cutaneous lesions of dermatitis but has not previously been demonstrated to affect pigmentation. We have investigated the effect of niacinamide on melanogenesis in: (i) a purified mushroom tyrosinase assay; (ii) in cultured melanocytes; (iii) a keratinocyte/melanocyte coculture model; and (iv) a pigmented reconstructed epidermis (PREP) model. We also examined the effect of daily use of a moisturizer containing niacinamide on facial hyperpigmentation and skin colour in comparison with a vehicle moisturizer lacking niacinamide.

Materials and methods

Tyrosinase inhibition assay

To ascertain whether niacinamide interferes with the catalytic activity of tyrosinase, 10 µg mL⁻¹ mushroom tyrosinase (Sigma, St Louis, MO, U.S.A.) was mixed with 1 mmol L⁻¹ L-tyrosine in the presence or absence of various concentrations of niacinamide. Absorbance at 490 nm was read 24 h later to determine the production of melanin.

Melanin assay

Melanocytes were purchased from Cascade Biologicals (Portland, OR, U.S.A.) and cultured according to the supplier's recommendations. To ascertain whether niacinamide interferes with melanin production *in vitro*, 10⁵ human foreskin-derived melanocytes were cultivated in the presence or absence of 1 mmol L⁻¹ niacinamide for 12 days. Cells were then counted and equal numbers of cells collected by centrifugation. Melanocyte pellets were photographed and subsequently solubilized with NaOH. The melanin content of the lysates was quantified by measuring absorbance at 490 nm.

Tyrosine hydroxylase and dihydroxyphenylalanine oxidase assays

Cultured melanocytes were seeded into triplicate T25 culture flasks at 50% confluency, and were treated with different concentrations of niacinamide daily for 7 days. Cells were then detached by trypsinization, washed in ice-cold phosphate-buffered saline (PBS) and subsequently solubilized in 350 µL of 0.5% NP40/PBS by sonication. After centrifugation (3000 *g*) at 4 °C for 10 min, supernatants were collected and used for protein determination and enzymatic assays as described previously.⁷ For the tyrosine hydroxylase assay, the reaction mixture, consisting of 10 µCi mL⁻¹ L-[3,5-³H]tyrosine, 80 µmol L⁻¹ L-dihydroxyphenylalanine (L-DOPA) and 100 µg mL⁻¹ lysate protein, was incubated at 37 °C for 60 min. All samples were tested in triplicate. The radioactivity in the eluate of tritium water released during enzymatic hydroxylation of tyrosine to DOPA was counted in a Packard 1900 CA liquid scintillation analyser. Tyrosine hydroxylase activity was expressed as 10⁶ disintegrations per min h⁻¹ mg⁻¹ protein. For the DOPA oxidase assay, the reaction mixture, consisting of 0.1 mol L⁻¹ phosphate (pH 6.8), 3 mmol L⁻¹ L-DOPA and 100 µg mL⁻¹ cellular protein, was incubated at 37 °C for 60 min. The change in absorbance at 475 nm was measured periodically during this period using a Milton Roy 1001 plus spectrophotometer. Specific activity of DOPA oxidase was expressed as ΔA₄₇₅ h⁻¹ mg⁻¹ protein. Cultured fibroblasts, developed as previously described,⁸ were used as negative controls in these experiments. Cultured keratinocytes were developed as previously described.⁹

Pigmented reconstructed epidermis assay

PREPs were prepared according to the method of Bessou *et al.*¹⁰ Niacinamide 1 mmol L⁻¹ was added to the medium ≈ 48 h after cell seeding. The PREPs were photographed at the end of a 10-day treatment period.

Melanosome transfer in coculture of melanocytes and keratinocytes

Cocultures of melanocytes and keratinocytes were developed as previously described⁹ using one line of African/American-derived melanocytes and two independent lines of Caucasian-derived keratinocytes

(coded no. 560 and no. 660). In short, cultures of normal human melanocytes and keratinocytes were established separately from individual neonatal foreskins obtained from the nursery of University Hospital at the University of Cincinnati College of Medicine after routine circumcisions. Foreskins were incubated in 0.25% trypsin for 2 h at 37 °C. The tissue was gently vortexed for 30 s to separate the dermis from the epidermal cell suspension. The dermis was removed and then the epidermal cells were pelleted by centrifugation and resuspended in either melanocyte or keratinocyte growth medium for seeding in 25-cm² tissue culture flasks. Melanocytes were maintained in M154 basal medium (Cascade Biologicals) supplemented with 4% heat-inactivated fetal bovine serum (FBS), 1% antibiotic/antimycotic solution (Gibco BRL, Grand Island, NY, U.S.A.), 1 µg mL⁻¹ transferrin, 1 µg mL⁻¹ vitamin E, 5 µg mL⁻¹ insulin, 0.6 ng mL⁻¹ human recombinant basic fibroblast growth factor, 10⁻⁸ mol L⁻¹ α-melanocyte stimulating hormone and 10⁻⁹ mol L⁻¹ endothelin-1. Keratinocytes were maintained in M154 basal medium supplemented with human keratinocyte growth supplements (Cascade Biologicals) and 1% antibiotic/antimycotic solution. Established cultures of melanocytes were subsequently stained with the succinimidyl ester of carboxy fluorescein diacetate (CFDA; Molecular Probes, Eugene, OR, U.S.A.) at 2 µmol L⁻¹ in Hanks balanced salt solution for 30 min.¹¹ These melanocytes were then added to keratinocytes at a ratio of 1 : 2 and cocultured in normal melanocyte growth medium/normal keratinocyte growth medium 1 : 2. Cocultures were then maintained in the presence or absence of niacinamide (1.0 mmol L⁻¹) for 6 days. Niacinamide was added to the media of the cocultures every 12 h. On the seventh day, adherent cells were washed with PBS. Cells were washed in 5 mL of PBS containing 0.4% heat-inactivated FBS and 0.2% sodium azide (PFA). Cells were prefixed in 4% paraformaldehyde for 30 min at 4 °C followed by permeabilization in fluorescence-activated cell sorting (FACS) permeabilizing solution (Becton Dickinson, San Jose, CA, U.S.A.) for 10 min at room temperature, and washed twice in PFA. This was followed by a 45-min incubation at room temperature with the primary antibody diluted in 10% normal human serum/PFA (PFAN) at 1 : 300. The primary antibody used in these studies was monoclonal mouse anticytokeratin (Zymed Laboratories, San Francisco, CA, U.S.A.) in order to identify keratinocytes by FACS. Cells were then rinsed three times with PFA and incubated for 30 min at 37 °C with goat antimouse

IgG (secondary antibody) conjugated to phycoerythrin (PE; Caltag Laboratories, Burlingame, CA, U.S.A.) at 1 : 20 dilution in PFAN. Cells were washed twice and postfixed in 1% paraformaldehyde, after which they were scraped from flasks and put into analysis tubes. Cells were analysed by flow cytometry using an EPICSXL flow cytometer (Coulter Cytometry, Coulter Corp., Hialeah, FL, U.S.A.). CFDA and PE were both excited with the 488-nm line of an argon ion laser. Fluorescence emission for CFDA and PE was detected selectively by collection with 525 and 575 nm band-pass filters, respectively. Melanocytes were positive for CFDA only. Keratinocytes were positive for PE. Assessment of melanosome transfer to keratinocytes was determined by recording the expression of CFDA within PE-positive cells. The bivariate histograms generated per coculture group were analysed using Coulter XL software (Coulter Corp.). This experiment was performed twice (once with each of the two different cultured lines of keratinocytes). This flow cytometry technique has been demonstrated to reflect the transfer of melanosomes from melanocytes to keratinocytes in the coculture model system.¹¹

Human clinical study I: reduction of facial hyperpigmentation. This study, performed during May–July 1998 in Kobe, Japan, was a randomized split-face double-blind paired design involving a 2-week normalization period using the vehicle moisturizer on both sides of the face. Eighteen Japanese women aged 25–60 years with multiple types of brown hyperpigmentation were enrolled. Most subjects presented with slight to moderate senile lentigines, melasma or freckles. Subjects applied 5% niacinamide moisturizer to one side of the face and the vehicle moisturizer (without niacinamide, oil-in-water emulsion type) to the other side twice daily (morning and evening) for 8 weeks. All subjects gave written informed consent. The protocol had been approved by the regulatory and safety review committee for human testing of the Procter & Gamble Company.

Human clinical study II: lightening of facial skin colour. This study, performed during August–October 1997 in Kobe, Japan, was a randomized split-face double-blind round-robin design. One hundred and twenty Japanese women aged 18–30 years with moderate to deep facial tan (L*-value < 60 using a Minolta Chromameter Model 200) were enrolled, and assigned to one of three groups of 40 subjects. Three products were tested: (i) vehicle moisturizer (oil-in-water emulsion type);

(ii) ultraviolet (UV) B/UVA sunscreen in the vehicle moisturizer (sun protection factor, SPF 15); and (iii) 2% niacinamide + UVB/UVA sunscreen in the vehicle moisturizer. Subjects applied one test product to one side of their face and another test product to the other side twice daily (morning and evening) for 8 weeks. One group applied products (i) and (ii), another group applied products (ii) and (iii) and the third group applied products (i) and (iii). All subjects gave written informed consent. The protocol had been approved by the regulatory and safety review committee for human testing of the Procter & Gamble Company.

Image analysis of hyperpigmentation and basal skin colour

In these studies, facial hyperpigmentation (total area) and basal skin colour on each side of the face were objectively measured using a customized image analysis technique.¹² Specifically, images of the right and left sides of each subject's face were captured at baseline, 4 and 8 weeks (6 weeks as well for study II) by a high-resolution analogue camera (Sony DXC-537H 3CCD colour TV camera) equipped with a polarized lens (Canon J15 × 9.5 BKRS with a polarizing filter). Facial illumination was provided by two Balcar Flux-Lites equipped with flux tungsten light bulbs (3500 °K) positioned above and below the camera to provide even lighting. Before image capture, subjects equilibrated in a controlled temperature and humidity room for 30 min (23 °C, 50% relative humidity). At the 4-, 6- and 8-week visit, accurate repositioning of the subjects was facilitated by superimposing the live image on the digitally stored image obtained at baseline. A colour chart was used as a standard to calibrate the imaging system each study day. Computer analysis of the video images allowed quantification of basal skin colour (L^* , a^* , b^*) and area of hyperpigmentation (mm^2).

Visual assessment of hyperpigmentation and basal skin colour

Subjective visual grading of the captured images was carried out to compare pretreatment hyperpigmentation (baseline image) vs. post-treatment hyperpigmentation (4-week or 8-week image). On a calibrated video monitor (Barco Type 121), paired pre- and post-treatment images appeared side by side randomly. Seven judges independently viewed pairs of images for each subject. The judges were blind to which image was pre- or post-treatment and to the type of treatment.

The judges indicated which of the two images had fewer areas of hyperpigmentation around the eye and cheek (study I) or had lighter basal skin colour in the eye and cheek region (study II), then rated the magnitude of the difference between images on a scale of 1–4: 1, I think there is a small difference; 2, I know there is a small difference; 3, I know there is a moderate difference; 4, I know there is a big difference. There was no 'no difference' option, to increase the sensitivity of the judges' evaluation. The magnitude rating was assigned a positive (+) or negative (–) sign depending upon whether (+) or not (–) the judge indicated the post-treatment image to be lighter. Thus, positive values indicated efficacy and negative values indicated no efficacy. The mean ratings of the seven judges were used for the statistical analysis.

Self-assessment via questionnaire

Self-assessments were conducted at weeks 4 and 8. Subjects evaluated each side of their face for changes in the number and colour of brown hyperpigmented areas (study I) or basal skin colour (study II) compared with baseline on a five-point scale as follows: +2, decreased/lighter; +1, slightly decreased/slightly lighter; 0, no change; –1, slightly increased/slightly darker; –2, increased/darker.

Data analysis

Image analysis data (area of hyperpigmentation and L^* , a^* , b^*) were analysed by analysis of covariance with baseline measurement as the covariant. Visual assessment data were analysed by a paired *t*-test (study I) or analysis of variance followed by a least significant differences test (study II). Questionnaire data were analysed by the Wilcoxon signed rank test or Kruskal–Wallis test. $P < 0.05$ indicated statistical significance.

Results

In vitro studies

To ascertain whether niacinamide interferes with the catalytic activity of tyrosinase, various dosages of niacinamide were added to a mixture of mushroom tyrosinase and tyrosine and melanin production was assayed. Niacinamide up to 10 mmol L^{-1} did not significantly affect the absorbency at 490 nm, a measure of the amount of melanin produced *in vitro* (Fig. 1).

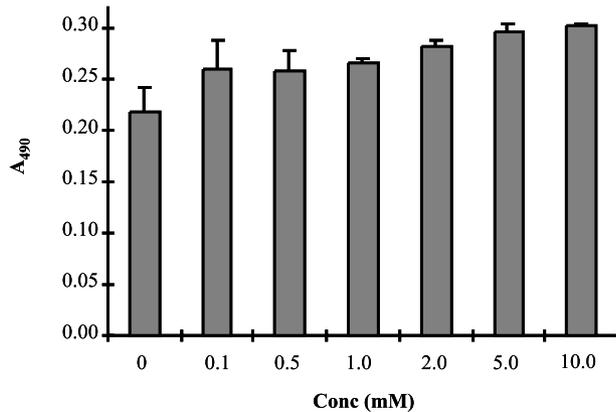


Figure 1. Niacinamide does not inhibit the catalytic activity of mushroom tyrosinase. Niacinamide at doses from 0.1 to 10.0 mmol L⁻¹ were incubated with a reaction mixture containing 10 µg mL⁻¹ mushroom tyrosinase and 1 mmol L⁻¹ L-tyrosine for 24 h. The amount of melanin synthesized was determined by absorbance at 490 nm. Results are given as mean ± SD of triplicate experiments.

To ascertain whether niacinamide interferes with melanin production *in vitro*, cultures of melanocytes were treated with 1.0 mmol L⁻¹ niacinamide for 12 days. Niacinamide did not alter the amount of melanin observed in the cell pellets or quantified spectrophotometrically (Fig. 2).

Next, cultured normal human melanocytes were treated with either 0.1 mmol L⁻¹ or 1.0 µmol L⁻¹ niacinamide for 7 days and assessed for cell number, tyrosinase hydroxylase activity and DOPA oxidase activity. As shown in Table 1, there were no significant differences between either of the two treatment groups and untreated melanocytes for all three criteria. In addition, treatment with 1.0 µmol L⁻¹–1.0 mmol L⁻¹ niacinamide had no significant effect on keratinocyte proliferation.

In the next experiment, six PREPs were constructed. Three were untreated and three were treated with 1.0 mmol L⁻¹ niacinamide for 10 days. All treated PREPs exhibited lighter pigmentation visually than the untreated PREPs (Fig. 3).

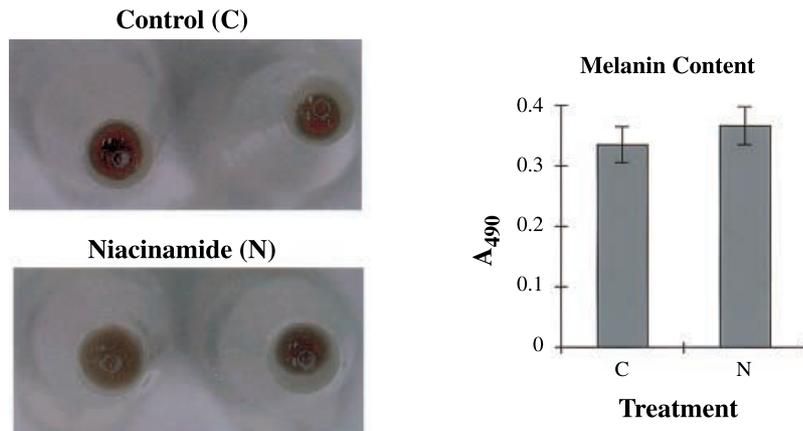


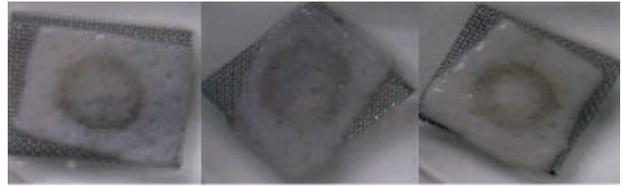
Figure 2. Niacinamide does not affect the amount of melanin synthesized by cultured melanocytes. Melanocyte cultures were treated with 1.0 mmol L⁻¹ niacinamide for 12 days. The amount of melanin in the cell pellets (left) or in the cell lysates (right) was not affected by treatment with niacinamide. Results are given as mean ± SEM of duplicate experiments.

Table 1. Effect of niacinamide on cell number, tyrosinase hydroxylase activity and dihydroxyphenylalanine (DOPA) oxidase activity in cultured cells. Results are given as mean ± SD of triplicate experiments

Cell type and treatment	Cell no. (× 10 ⁻⁴)	Tyrosinase hydroxylase activity (1 × 10 ⁶ disintegrations min ⁻¹ h ⁻¹ mg ⁻¹ protein) ^a	
		DOPA oxidase activity (h ⁻¹ mg ⁻¹ protein) ^a	
Untreated melanocytes (M)	34.00 ± 0.3	0.25 ± 0.06	0.84 ± 0.01
M + 0.1 mmol L ⁻¹ niacinamide	36.50 ± 0.1	0.33 ± 0.04	0.92 ± 0.04
M + 1.0 µmol L ⁻¹ niacinamide	35.50 ± 0.3	0.32 ± 0.07	1.00 ± 0.03
Untreated fibroblasts	ND	0.01 ± 0.00	0.03 ± 0.00
Untreated keratinocytes (K)	18.25 ± 8.3	ND	ND
K + 1.0 mmol L ⁻¹ niacinamide	11.13 ± 7.7	ND	ND
K + 0.1 mmol L ⁻¹ niacinamide	12.25 ± 4.9	ND	ND
K + 1.0 µmol L ⁻¹ niacinamide	25.88 ± 9.2	ND	ND

ND, not done. ^aA paired *t*-test was performed comparing all treated samples with their respective untreated control samples for each of the groups listed in the table. No value was significantly different from its respective control (i.e. *P* > 0.05).

Control



Niacinamide

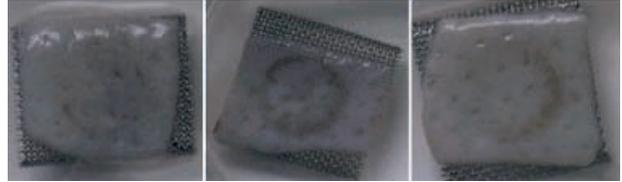


Figure 3. Niacinamide treatment results in lightening of pigmented reconstructed epidermis (PREP) tissue. Separate PREP samples were left untreated (control, upper panel) or treated with 1.0 mmol L⁻¹ niacinamide (lower panel) for 10 days. All treated samples appear visibly less pigmented.

Table 2. Inhibition of melanosome transfer in the coculture of melanocytes and keratinocytes in the presence and absence of 1.0 mmol L⁻¹ niacinamide. Results are given as mean of duplicate experiments

Treatment group	Keratinocyte cell line no. 560		Keratinocyte cell line no. 660	
	Fluorescence	% inhibition	Fluorescence	% inhibition
Keratinocytes alone	0.221	–	0.228	–
Coculture of keratinocytes + melanocytes	22.6	0	0.660	0
Coculture of keratinocytes + melanocytes with niacinamide	14.7	35.3	0.368	67.6

The final experiment showed inhibition of melanosome transfer by niacinamide in coculture of melanocytes and keratinocytes. Table 2 presents results for the flow cytometric analysis of melanosome transfer in two separate tests using different keratinocyte cell lines. In both tests, 6 days of coculture in the presence of 1.0 mmol L⁻¹ niacinamide resulted in inhibition of fluorochrome transfer from melanocytes to keratinocytes. The percentage of inhibition in the two tests was approximately 35% and 68%.

Human clinical study I: reduction of hyperpigmentation by niacinamide

1 Image analysis. Figure 4 presents the image analysis results for the percentage change from baseline in total area of hyperpigmentation for 5% niacinamide and vehicle control treatments. After 4 weeks of treatment, the side of the face receiving niacinamide showed a significant ($P < 0.05$) decrease in total hyperpigmented area vs. the side receiving vehicle. The size of the difference between niacinamide and vehicle treatments did not continue to increase after 4 weeks of treatment, but it remained significantly different throughout the remainder of the study.

2 Visual assessment. Figure 5 presents the mean difference from baseline (positive numbers imply less

hyperpigmentation vs. baseline) for visual grading of severity of hyperpigmentation for 5% niacinamide vs. the vehicle control. After 8 weeks of treatment, the side of the face receiving niacinamide showed a significant reduction in hyperpigmentation vs. the side receiving the vehicle. Representative images are shown in Figure 6.

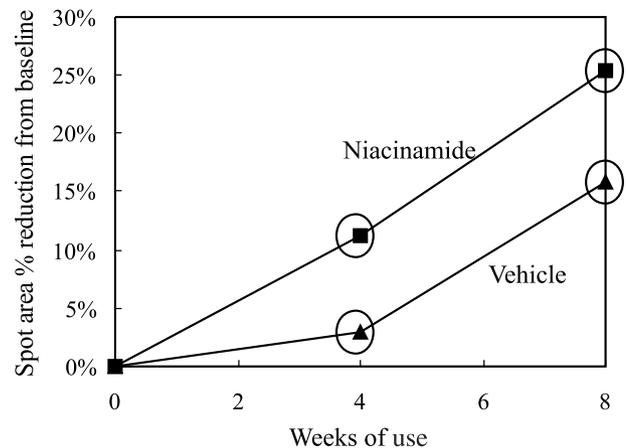


Figure 4. Percentage reduction of area of hyperpigmentation from baseline for niacinamide and vehicle-treated sides of the face. Individual points circled at same week of use indicate significant difference ($P < 0.05$).

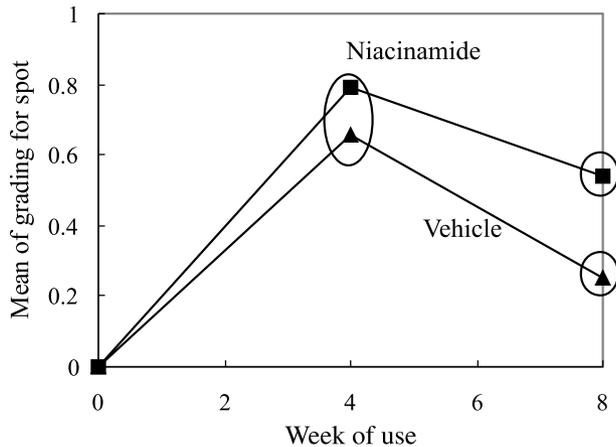


Figure 5. Mean visual grading for reduction of area of hyperpigmentation: pretreatment vs. 4 and 8 weeks post-treatment. Individual points circled at same week of use indicate significant difference ($P < 0.05$); pairs of points circled at same week of use indicate no significant difference.

3 Self-assessment. Tables 3 and 4 show the results of self-assessment of changes in number and colour of areas of hyperpigmentation. In both cases, all assessments were ≥ 0 , i.e. subjects perceived that there were

fewer areas of hyperpigmentation and that their colour became lighter after treatment with both niacinamide and vehicle. Differences between niacinamide and vehicle were significant for colour, but not for number of areas of hyperpigmentation.

Human clinical study II: skin lightening effect of sunscreen and niacinamide

1 Image analysis. Figure 7(a) shows the changes in L^* -value from baseline for niacinamide + sunscreen, sunscreen and vehicle. After 4 weeks of treatment, the side of the face receiving sunscreen showed a significant increase in L^* -value (skin lightness) vs. the side receiving vehicle. The difference between sunscreen and vehicle increased from 4 to 8 weeks and remained significantly different. After 4 weeks of treatment, the side of the face receiving niacinamide + sunscreen showed a significant increase in L^* -value vs. the side receiving sunscreen. The difference in L^* -value between niacinamide + sunscreen and sunscreen remained significant at 6 weeks but not at 8 weeks. There were also significant reductions in a^* -value (redness) on the side of the face receiving either

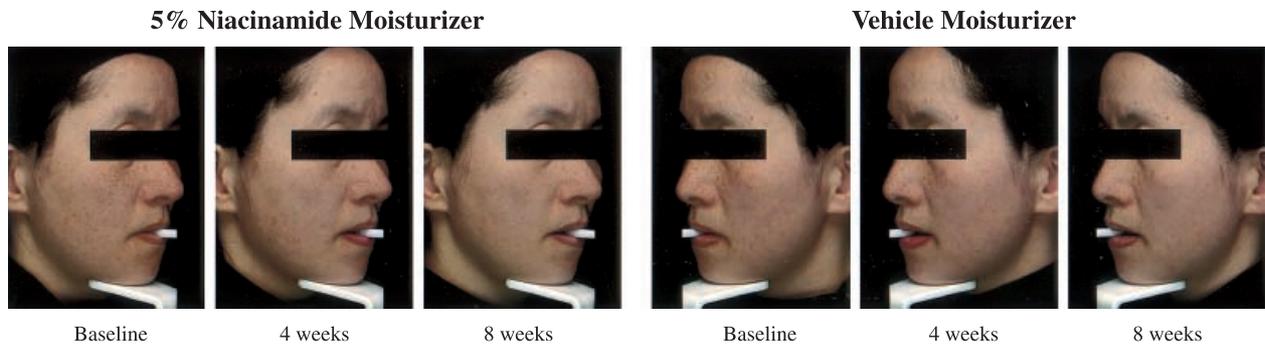


Figure 6. Facial images used for assessment of reduction of hyperpigmentation (human clinical study I). The subject used 5% niacinamide moisturizer on the right side of the face and vehicle moisturizer on the left side.

Table 3. Human study I: self-assessment via questionnaire for changes in numbers of areas of hyperpigmentation. Numbers indicate total number of facial sides

Scale	Wording	4 weeks		8 weeks	
		Niacinamide	Vehicle	Niacinamide	Vehicle
+2	Decreased	2	0	3	0
+1	Slightly decreased	5	6	10	11
0	No change	11	12	5	7
-1	Slightly increased	0	0	0	0
-2	Increased	0	0	0	0

$P = 0.180, NS^a$

$P = 0.059, NS^a$

^aNS, not significant ($P > 0.05$, Wilcoxon signed rank test).

Table 4. Human study I: self-assessment via questionnaire for changes in hyperpigmentation. Numbers indicate total number of facial sides

Scale	Wording	4 weeks		8 weeks	
		Niacinamide	Vehicle	Niacinamide	Vehicle
+2	Lighter	2	0	3	1
+1	Slightly lighter	11	10	12	12
0	No change	5	8	3	5
-1	Slightly darker	0	0	0	0
-2	Darker	0	0	0	0
		$P = 0.025^a$		$P = 0.046^a$	

^aWilcoxon signed rank test.

niacinamide + sunscreen or sunscreen vs. the side receiving vehicle after 4 weeks of treatment (Fig. 7b). However, no significant difference was observed in a*-value between niacinamide + sunscreen and sunscreen at any time point.

Compared with baseline, b*-value (yellowness) decreased in all treatment groups during the course of the study (Fig. 7c). The sunscreen and niacinamide + sunscreen treatments gave a significantly greater decrease in b*-value vs. vehicle at 6 and 8 weeks of treatment, but no significant differences were observed between niacinamide + sunscreen and sunscreen treated sides.

2 Visual assessment. Figure 8 shows the mean of visual grading of facial skin lightness for niacinamide + sunscreen, sunscreen alone and vehicle. After 4 weeks of treatment, the side of the face receiving niacinamide + sunscreen showed a significant increase in skin lightness vs. the side receiving vehicle ($P < 0.05$). The side of the face treated with sunscreen was also lighter than the vehicle-treated side, but the difference was not significant ($P = 0.099$). Representative images are shown in Figure 9.

3 Self-assessment. Table 5 shows the results of self-assessment for changes in basal skin colour. The data suggest that subjects perceived that their skin colour showed no change or became lighter after 4 weeks, regardless of treatment. However, there were no significant differences between treatments.

Discussion

Niacinamide or nicotinamide is a biologically active form of niacin (vitamin B₃)¹³ involved in over 200 enzyme reactions in the form of nicotinamide adenine dinucleotide and nicotinamide adenine dinucleotide phosphate. Niacinamide is well tolerated by the skin, in contrast to other common forms of this vitamin family (e.g. nicotinic acid and its esters), which often induce

uncomfortable skin flushing reactions.^{14,15} Oral niacinamide (or niacin) has been reported to prevent the development of insulin-dependent diabetes mellitus¹⁶ and to cure the niacin deficiency disease pellagra.¹⁴

In contrast to a long history of research on the efficacy of oral niacinamide, research on topical niacinamide is comparatively recent. In 1995, it was reported that topical 4% niacinamide was effective in treating acne when used twice daily for 8 weeks.³ In 1997, Tanno *et al.* reported that niacinamide stimulated biosynthesis of crucial stratum corneum lipids.¹⁷ In a subsequent 4-week human study, the authors reported that topical 2% niacinamide increased ceramide level, cholesterol synthesis and free fatty acid synthesis via an increase in acetyl coenzyme A.⁶ Although various cutaneous effects of topical niacinamide have been reported, an effect on pigmentation or melanogenesis has not been documented.

In this paper, we have shown the effects of niacinamide on melanogenesis and pigmentation *in vitro* and *in vivo*. First, we examined direct inhibition of tyrosinase, which is the first and rate-limiting enzyme in the production of melanin from tyrosine.¹⁸ Most of the currently marketed skin lightening compounds such as arbutin or kojic acid are thought to work by this mechanism.² However, Virador *et al.* reported that niacinamide itself did not show much inhibition of melanogenic activity using purified tyrosinase.¹⁹ Our results also suggested that there was no inhibition of melanin synthesis by niacinamide itself (Fig. 1). We next examined the effect of niacinamide on cultured melanocytes. The results suggested that niacinamide has no effect on tyrosinase activity, melanin synthesis or cell number in this melanocyte monoculture system (Fig. 2, Table 1). In addition, niacinamide has no effect on the proliferation of keratinocytes.

We then focused on the effect of niacinamide on the interaction between keratinocytes and melanocytes with regard to melanogenesis. One well-known inter-

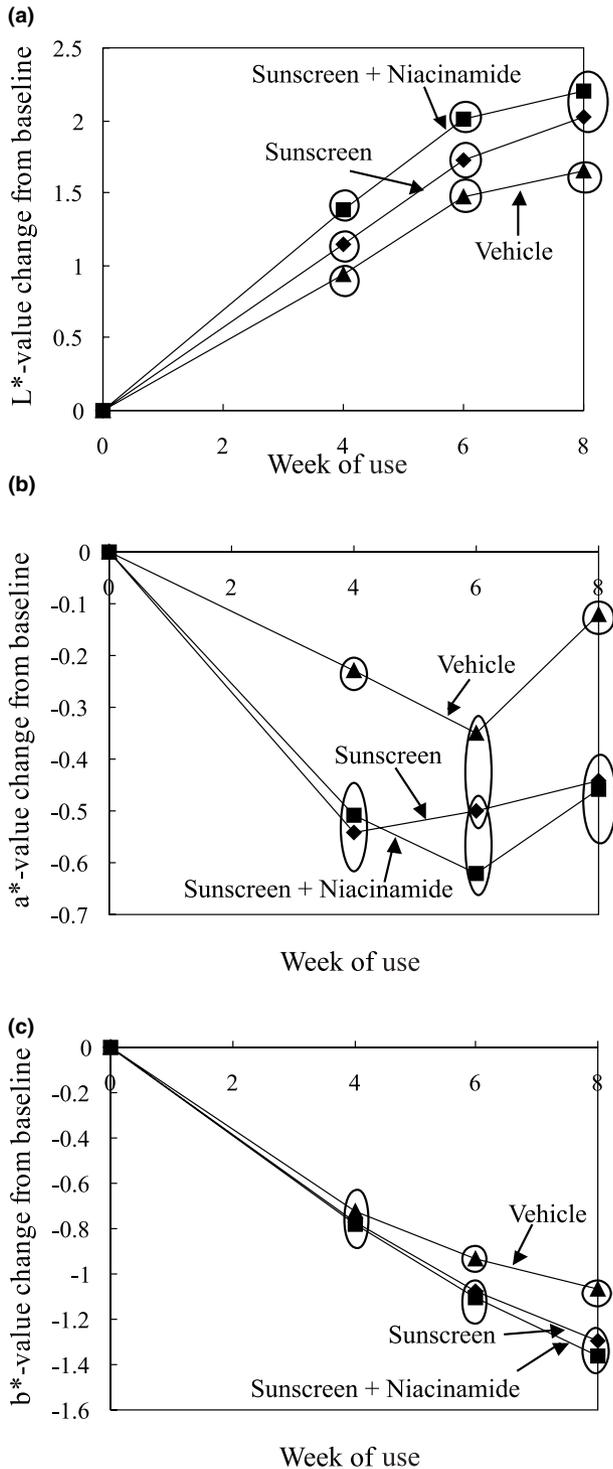


Figure 7. (a) L*-value, (b) a*-value and (c) b*-value changes from baseline for the sunscreen + niacinamide, sunscreen alone and vehicle-treated side of the face. Individual points circled at same week of use indicate significant difference ($P < 0.05$); pairs of points circled at same week of use indicate no significant difference.

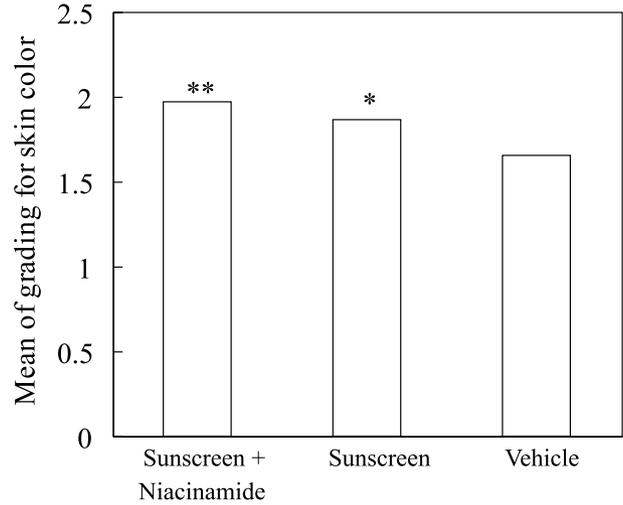


Figure 8. Mean visual grading for skin colour, comparing pretreatment vs. 4 weeks post-treatment. Differences vs. vehicle: ** $P < 0.05$, * $P < 0.1$ (not significant).

action is the cytokine signalling pathway to and from keratinocytes. The roles of keratinocyte-derived cytokines in melanogenesis have been researched extensively in the past decade. Imokawa *et al.* reported that the UV-induced increase in interleukin-1 α in epidermis triggers the release of endothelin-1 from keratinocytes, which in turn stimulates melanin production by melanocytes.²⁰ Here we investigated the effect of niacinamide in the PREP model, which consists of keratinocytes and melanocytes. As shown in Figure 3, addition of niacinamide gave a visible reduction in pigmentation. These data strongly suggested that niacinamide downregulates melanogenesis by interfering with the interaction between keratinocytes and melanocytes.

Besides the cytokine signalling pathway, another important interaction between melanocytes and keratinocytes is the transfer of melanosomes from melanocytes to surrounding keratinocytes. Seiberg *et al.* recently showed that modulation of protease-activated receptor 2 activation affects melanosome transfer into keratinocytes by interfering with keratinocyte phagocytosis, resulting in changes in pigment production and deposition.^{21,22} The authors demonstrated that treatment with a serine protease inhibitor induced a visible skin lightening effect in dark-skinned Yucatan swine. In addition, Minwalla *et al.*¹¹ have recently demonstrated that various lectins and neoglycoproteins can interfere with the transfer of melanosomes into kera-

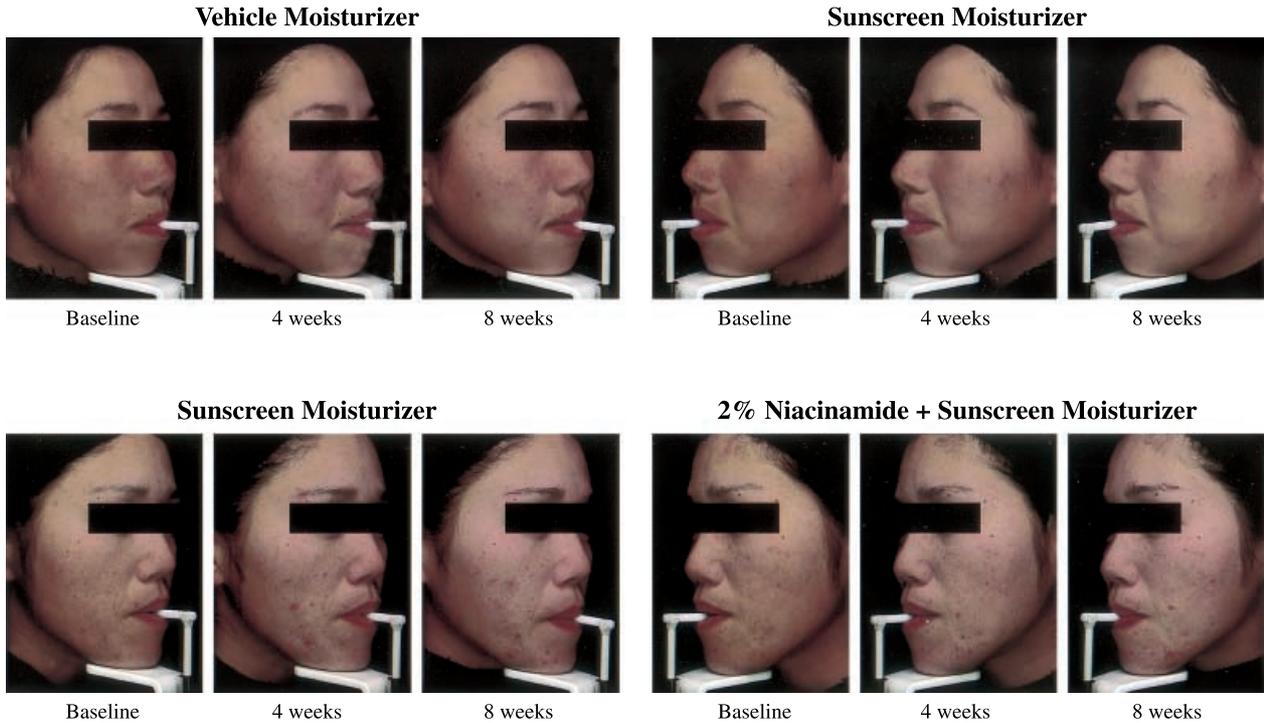


Figure 9. Facial images of two subjects used for assessment of basal skin colour (human clinical study II). Upper row: the subject used sunscreen moisturizer on the left side of the face and vehicle moisturizer on the right side. Lower row: the subject used 2% niacinamide + sunscreen moisturizer on the left side of the face and sunscreen moisturizer on the right side.

tinocytes in a coculture model system. We investigated the influence of niacinamide on melanosome transfer in a coculture model of melanocytes and keratinocytes. In this system, the transfer of fluorochrome, which reflects the transfer of melanosomes, was confirmed visually by confocal microscopy and quantitatively by flow cytometry.¹¹ The results from two experiments in the presence and absence of niacinamide showed that niacinamide downregulated the amount of melanosomes transferred by approximately 35–68% (Table 2).

Next, we clinically evaluated the efficacy of topical niacinamide for decreasing facial hyperpigmentation

and lightening skin colour in vehicle-controlled protocols. Quantitative image analysis of facial images allowed objective determination of treatment effects. The image analysis results demonstrated that niacinamide significantly reduced the total area of hyperpigmentation and increased skin lightness (L^* -value) vs. vehicle after 4 weeks of treatment (Figs 4 and 7a). The size of the difference between niacinamide and vehicle treatments did not continue to increase after 4 weeks of treatment. This plateau in treatment effect is not unique to niacinamide, but is observed for various skin lightening ingredients, such as retinoids.²³ The plateau

Table 5. Human study II: self-assessment via questionnaire for changes in basal skin colour. Numbers indicate total number of facial sides

Scale	Wording	4 weeks			8 weeks				
		Niacinamide + sunscreen	Sunscreen	Vehicle	Niacinamide + sunscreen	Sunscreen	Vehicle		
+2	Lighter	0	2	0	0	2	1		
+1	Slightly lighter	30	37	43	50	42	52		
0	No change	39	31	29	19	28	20		
-1	Slightly darker	0	1	0	0	0	0		
-2	Darker	0	0	0	0	0	0		
				$P = 0.120, NS^a$			$P = 0.351, NS^a$		

^aNS, not significant ($P > 0.05$, Kruskal–Wallis test).

in niacinamide treatment effect could be explained as a balance point between the upregulation of melanogenesis in the hyperpigmented area and the downregulation by niacinamide. Alternatively, the plateau could reflect the fraction of the hyperpigmented area that is sensitive to niacinamide treatment; once niacinamide exerts its effects on this area, no further changes are observed. Subjects themselves perceived that treatment with niacinamide significantly lightened the colour of hyperpigmented areas more than did vehicle (Table 4), while they did not perceive a difference in number of hyperpigmented areas (Table 3).

As part of study II, we tested the effect of regular use of an SPF 15 sunscreen vs. a vehicle lacking sunscreen. We hypothesized that if epidermal melanogenesis were inhibited by regular sunscreen use, then the skin may lighten over time as the epidermal melanin is lost through normal stratum corneum desquamation. After 4 weeks of regular sunscreen use, a significant skin lightening effect was observed (Fig. 7a). However, vehicle treatment also gave skin lightening over the 8-week study period. This is attributed to the seasonal fading in skin colour from the beginning of the study (August) to the end of the study (October).

Niacinamide + sunscreen treatment gave greater skin lightening effects than did sunscreen alone. As shown in Figure 7(a), the magnitude of the difference between niacinamide + sunscreen and sunscreen is similar to the difference between sunscreen and vehicle (approximately 0.25 L* units). Thus, the additional skin lightening afforded by adding 2% niacinamide to an SPF 15 sunscreen is about equal to that delivered by the SPF 15 sunscreen alone. The particular sunscreen tested in this study was specifically formulated so that the 2% niacinamide did not interfere with the sunscreen, and vice versa. Partitioning of the niacinamide into the water phase and the sunscreen into the oil phase helps achieve uniform dispersion. On the other hand, no significant difference in basal skin lightening was perceived by the subjects in study II when comparing niacinamide + sunscreen with sunscreen alone (Table 5). Altogether, subjects only perceived changes in hyperpigmentation, as shown in study I (Table 4).

To confirm that the computer image analysis data reflect actual changes in visible skin features, we developed a semiquantitative method to grade changes in facial hyperpigmentation and skin lightness visually. Judges graded the facial images (before vs. after, shown side-by-side) for the severity of hyperpigmentation. The visual grading data were in general agreement with the computer image analysis data and helped to confirm

that the skin effects of niacinamide observed using image analysis are not artefacts.

In conclusion, we have demonstrated for the first time that niacinamide can effectively reduce cutaneous pigmentation in both the PREP model and in facial skin. This depigmentary effect of niacinamide was not the result of a direct influence on melanin synthesis by melanocytes. We showed that niacinamide reduced melanosome transfer from melanocytes to surrounding keratinocytes in a coculture system. Finally, we have demonstrated that daily use of a niacinamide moisturizer was effective in reducing hyperpigmentation and in increasing lightness of basal skin colour compared with control moisturizer. Although the specific cellular mechanism of niacinamide remains to be elucidated, these data suggest that niacinamide is an effective skin lightening agent that works via a novel mechanism: the suppression of melanosome transfer from melanocytes to keratinocytes.

Acknowledgments

This work was entirely supported by The Procter & Gamble Company.

References

- 1 Jonker DL, Summers RS, Summers B. Constitutive skin tone and sunscreen effects—UV-induced skin darkening in Negroid skin. *Cosmetics Toiletries* 1994; **109**: 51–8.
- 2 Masuda M, Tejima T, Suzuki T *et al.* Skin lighteners. *Cosmetics Toiletries* 1996; **111**: 65–77.
- 3 Shalita AR, Smith JG, Parish LC *et al.* Topical nicotinamide compared with clindamycin gel in the treatment of inflammatory acne vulgaris. *Int J Dermatol* 1995; **34**: 434–7.
- 4 Bowes J, Piper J, Thiemermann C. Inhibitors of the activity of poly (ADP-ribose) synthetase reduce the cell death caused by hydrogen peroxide in human cardiac myoblasts. *Br J Pharmacol* 1998; **124**: 1760–6.
- 5 Gensler HL. Prevention of photoimmunosuppression and photocarcinogenesis by topical nicotinamide. *Nutr Cancer* 1997; **29**: 157–62.
- 6 Tanno O, Oya Y, Kitamura N *et al.* Nicotinamide increases biosynthesis of ceramides as well as other stratum corneum lipids to improve the epidermal permeability barrier. *Br J Dermatol* 2000; **143**: 524–31.
- 7 Zhao H, Boissy RE. Distinguishing between the catalytic potential and apparent expression of tyrosinase activities. *Am J Med Sci* 1994; **308**: 322–30.
- 8 Yang F, Sarangarajan R, Le Poole IC *et al.* The cytotoxicity and apoptosis induced by 4-tertiary butylphenol in human melanocytes is independent of tyrosinase activity. *J Invest Dermatol* 2000; **114**: 157–64.
- 9 Minwalla L, Zhao Y, Le Poole IC *et al.* Keratinocytes play a role in regulating distribution patterns of recipient melanosomes *in vitro*. *J Invest Dermatol* 2001; **117**: 341–7.

- 10 Bessou S, Pain C, Taïeb A. Use of human skin reconstructs in the study of pigment modifiers. *Arch Dermatol* 1997; **133**: 331–6.
- 11 Minwalla L, Zhao Y, Cornelius J *et al*. Inhibition of melanosome transfer from melanocytes to keratinocytes by lectins and neoglycoproteins in an *in vitro* model system. *Pigment Cell Res* 2001; **14**: 185–94.
- 12 Miyamoto K, Hillebrand GG. A sensitive and quantitative split-face clinical protocol to discriminate cosmetic formulations for facial hyperpigmentation reduction efficacy. *J Cosmet Sci* 1998; **49**: 202–4.
- 13 Goldsmith LA, ed. *Physiology, Biochemistry, and Molecular Biology of the Skin*, 2nd edn, Vol. 2. New York: Oxford University Press, 1991: 1315–16.
- 14 Sekine S, Kurata T, Matsumoto K *et al*. *Cosmetic Ingredient Dictionary*. Japan: Cyuo Press Co., 1991: 326–7.
- 15 Draelos ZD. Vitamins and their cutaneous effects. *Cosmet Dermatol* 1999; **12**: 17–20.
- 16 Elliott RB, Pilcher CC, Fergusson DM, Stewart AW. A population based strategy to prevent insulin-dependent diabetes using nicotinamide. *J Pediatr Endocrinol Metab* 1996; **9**: 501–9.
- 17 Tanno O, Ota Y, Kitamura N *et al*. Effects of niacinamide on ceramide biosynthesis and differentiation of cultured human keratinocytes. *3rd Asian Soc Cosm Sci* 1997; **1**: 170–6.
- 18 Prota G. *Melanins and Melanogenesis*. New York: Academic Press, 1992: 4–7.
- 19 Virador VM, Kobayashi N, Matsunaga J, Hearing VJ. A standardized protocol for assessing regulators of pigmentation. *Anal Biochem* 1999; **270**: 207–19.
- 20 Imokawa G, Yada Y, Miyagishi M. Endothelin-1 as a new melanogen: coordinated expression of its gene and the tyrosinase gene in UVB-exposed human epidermis. *J Invest Dermatol* 1995; **105**: 32–7.
- 21 Seiberg M, Paine C, Sharlow E *et al*. The protease-activated receptor-2 regulates pigmentation via keratinocyte–melanocyte interactions. *Exp Cell Res* 2000; **254**: 25–32.
- 22 Seiberg M, Paine C, Sharlow E *et al*. Inhibition of melanosome transfer results in skin lightening. *J Invest Dermatol* 2000; **115**: 162–7.
- 23 Rafal ES, Griffiths CE, Ditre CM *et al*. Topical tretinoin (retinoic acid) treatment for liver spots associated with photodamage. *N Engl J Med* 1992; **326**: 368–74.