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A novel method to study the skin-lightening effect of topical materials

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Synopsis

Skin without significant dyschromia is an aesthetic requirement for people worldwide. There are several *in vitro* methods to determine the whitening potential of actives; however, the *in vivo* testing of skin whiteners is a long and expensive process. We have designed a rapid clinical method to screen potential skin whiteners using a UV-induced skin tan as a model. Small areas of identical suntan are repeatably induced on the skin, and treatment of these sites allows rapid screening of several skin whiteners within the course of a month. The method provides reproducible results and valuable information about the potential skin-lightening activity of topical preparations.

INTRODUCTION

In Asia, Africa, and South America, the popularity of fair-skinned beauties in the media, as well as the cultural preference towards lighter skin, has fuelled public demand for skin-lightening products (1). The tendency of pigmented skin to be more prone to develop hyperpigmentation has also contributed to this demand (2).

In most instances, sun exposure is the main stimulator of skin hyperpigmentation. A direct effect of UV photons results in activation of tyrosinase, the rate-limiting enzyme in melanin synthesis, as well as an increase in cell surface expression of receptors for at least one of the several known keratinocyte-derived melanogenic factors, MSH (3). The inherent defense mechanism of skin against UV-B allows the transformation of tyrosine to 3,4-dihydroxyphenylalanine in the presence of UV-activated tyrosinase. This is further oxidized to DOPA quinone and then to dopachrome (4). Reduction of dopachrome yields 5,6-dihydroxyindole-2-carboxylic acid, which is ultimately converted via dihydroxyindole to the yellow-colored indole 5,6-quinone. Further, oxidative coupling of this results in the formation of eumelanin. Under normal circumstances eumelanins and pheomelanins are formed simultaneously to lead to the so-called mixed-type melanins. This process requires three to six days before the tan is established (4).

There is a myriad of skin whiteners with variable efficacy (5) readily available for consumers. Modulation of melanogenesis in the melanocytes can be achieved using chemicals that share structural homologies with the substrate tyrosine and as thus competitively

inhibit the catalytic function of tyrosinase (6). Kojic acid inhibits the catecholase activity of tyrosinase, which is the rate-limiting, essential enzyme in the biosynthesis of the skin pigment melanin. Hydroquinone, a hydroxyphenolic chemical, is a bleaching agent that has been used for decades as a skin-lightening agent (7). It acts by inhibiting the enzyme tyrosinase, thereby reducing the conversion of DOPA to melanin. Some of the other mechanisms of action are the destruction of melanocytes, degradation of melanosomes, and the inhibition of the synthesis of DNA and RNA (8).

Strong skin lighteners like clobetasol and hydroquinone can cause complications and significant health problems, especially for individuals with skin of color (9–11). Therefore, there is a constant need to investigate new and diverse potential skin lighteners that are safer and do not have side effects.

Determination of the skin-whitening abilities of actives requires three-to-six-month, in-use, clinical studies. Several techniques are used to assess skin lightening such as clinical inspection, photographs, or the use of surface color measuring devices such as a chromameter. Photographs are obtained at various time points, followed by image analysis (12) and/or visual analysis (13,14). Chromameters have been used to determine skin color before and after treatment, but these measurements require strict controls to account for the suntan during the course of the study. Most of these studies require a large number of subjects and a commitment of several months to screen a sample along with its controls. We developed a simple and quick method to screen several materials for skin lightening that can be carried out within a month.

MATERIALS AND METHODS

MATERIALS

Several actives were tested including chamomile 1% (E.L. Japan), magnesium ascorbyl phosphate 0.1% (Presperse), hydroquinone 2% (Sigma), and kojic acid 2% as standard (Sigma). All these actives were tested in a silicone-in-water formulation base, which was tested by itself as the placebo (vehicle).

In addition, several commercial lightening formulations were tested. These formulations contained various skin lighteners and were coded as whitening mix 1, 2, and so on. Triluma (Galderma) containing 4% hydroquinine was also tested. Triluma also contains fluocinolone acetonide, an anti-inflammatory corticosteroid and tretinoin. In order to determine if exfoliants would have an effect of skin lightening in this protocol, a formulation containing 1% salicylic acid was also tested.

PROCEDURE

The study was conducted at a contract testing laboratory in New City, New York. In each test, eight to ten subjects were recruited from the local population. Males or females ages 21–48 with no evidence of acute or chronic disease, including dermatological or ophthalmologic problems, were enrolled in the study. In order to qualify, the Caucasian subjects were required to be skin type III, who tan readily. The skin of the back was required to be free of warts, nevi, moles, sunburn, suntan, scars, and active dermal lesions. The

subjects were not under treatment with retinoids, tetracycline, nalidixic acid, corticosteroids, antihistamines, or similar agents during the course of the study and two weeks prior to the study's commencement. The subjects expressed willingness to cooperate with the investigator and demonstrated the ability to understand the purpose of the study and the risks associated with participation. Written informed consent was obtained from each volunteer before entering the study.

The source of radiation was a xenon arc Berger Solar Simulator (Solar Light Co., Philadelphia, PA) equipped with an interference filter with a range of 280 nm to 320 nm and a peak of 300 nm, in addition to WG 320 and UG-11 filters. The test site was the backs of the panelists. The minimal erythemal dose (MED) for the panelists was obtained as follows: about 2-cm diameter circles were exposed to UV-B in 25% increments, and erythema was visually graded after 24 hours. The minimal energy level (mJ/cm^2) to induce a slight pink erythema after 24 hours is the MED.

Six to seven distinct areas (approximately 4 cm^2) were marked on the backs of the panelists corresponding to the test materials and an additional untreated irradiated control. The panelists received twice the MED of UV-B on each site, and the materials to be tested were applied after irradiation, as described below.

Setup of experimental conditions. In order to develop the perfect skin lightening protocol, various procedures were investigated:

Part I: Evaluation of tan reduction
Five days after irradiation, when erythema was replaced by a tan, the test materials were applied on their respective sites (2 mg/cm^2) and allowed to dry for ten minutes. Product treatment was continued once a day except Sunday for 21 days. Color measurements were obtained from the test sites twice a week, using a chromameter.

Part II: Reduction of onset and lightening of tan
Treatment with the test materials was commenced immediately after irradiation and continued once a day, except Sunday, for 21 days. Color measurements were obtained from the test sites twice a week, using a chromameter (Minolta, Ramsey, NJ). This device uses a xenon light lamp to flash a light on any surface, and the light reflected from this surface is converted to color co-ordinates where L* values correspond to skin reflectance, a* values to red and yellow color, and b* values to yellow and blue color.

Reproducibility. The reproducibility of the method was determined by testing kojic acid as a control five times in both methods.

Three vs four weeks. Since hydroquinone takes a lot longer than three weeks to exhibit an effect and it did not appear to be significantly effective in this short skin-lightening protocol, the method was extended to four weeks. Several formulations were tested and the skin-lightening factor was compared between three and four weeks.

Data analysis. Untreated unirradiated skin color was subtracted from all values to determine ΔL^* values (decrease in reflectance), normalized for baseline (one day after irradiation) and plotted against all the time points (days), as illustrated in Figure 1. The area under the curve for each test site was calculated for the treated (A_t) site and the untreated (A_c) irradiated site. The "lightening factor" was calculated as the area under the curve of the treated site subtracted from the untreated, irradiated site ($LF = A_c - A_t$).

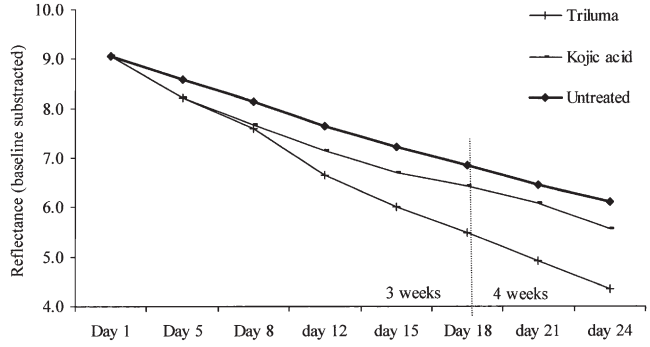

Figure 1. Skin lightening after three and four weeks of treatment. Area under the curve of untreated–treated exhibited the lightening factor, which can be calculated after three- and four-week treatments.

RESULTS

REPRODUCIBILITY

Reproducibility of the method is exhibited in Figure 2. It is clear that kojic acid gave reproducible results in both methods, with the standard deviation within 2–5% of the mean, indicating the validity of this method.

REDUCTION OF TAN VS REDUCTION OF ONSET AND INTENSITY OF TAN

As observed in Figure 3a, the lightening of the onset and the intensity of the tan resulted in a higher lightening factor than the reduction of the tan that was already established. This is to be expected since the anti-inflammatory properties of some of the materials would reduce the intensity of inflammation and thereby the intensity of the initial tan. There was a significant ($p < 0.001$) correlation between the two methods (Figure 3b).

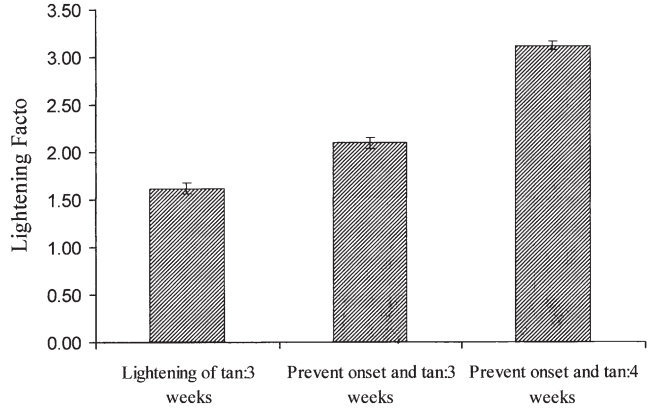

Figure 2. Skin-lightening effect of kojic acid and repeatability of the methods.

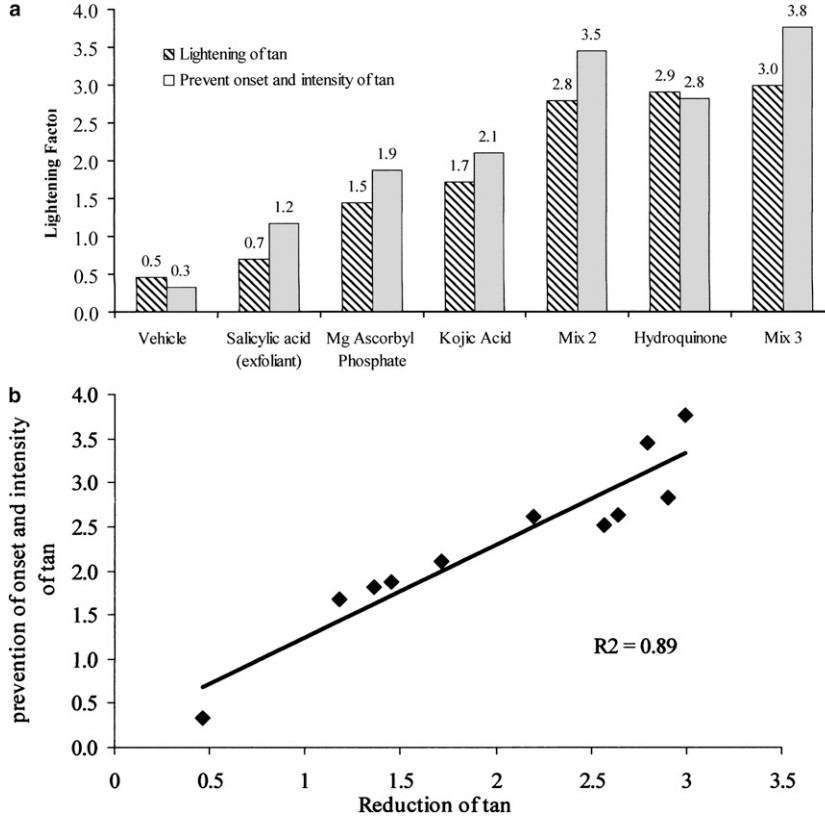

Figure 3. (a) Lightening of tan as well as reduction of the onset and intensity of tan, tested after three weeks. The numbers were slightly higher when the test materials were applied soon after irradiation, probably because the materials reduced inflammation, which is an integral component of skin pigmentation. Hydroquinone can be irritating on skin, especially inflamed skin (8); as a result, it was not that effective when applied soon after irradiation. In addition, this material takes a lot longer than three weeks to induce a significant whitening effect (8). (b) Correlation between reduction of skin tan vs prevention of onset and intensity of tan was significant ($p < 0.001$).

THREE-WEEK VS FOUR-WEEK LIGHTENING

The skin-lightening method was extended to four weeks. Several formulations were tested, and the skin-lightening factor was compared between three and four weeks. As observed in Figure 4, the lightening factor after four weeks was higher than after three weeks probably because the additional time allowed further whitening activity. Hydroquinone can be irritating on skin, especially inflamed skin (8), and as a result it was not that effective when applied soon after irradiation. In addition, this material takes a lot longer than three to four weeks to induce a significant whitening effect (8). As observed in Figure 3b, the correlation between the lightening factor at three and four weeks was significant ($p < 0.001$). In this study Triluma (Galderma), which contains an anti-inflammatory corticosteroid, appeared to exhibit a much higher response, as expected, thus confirming the validity of this method.

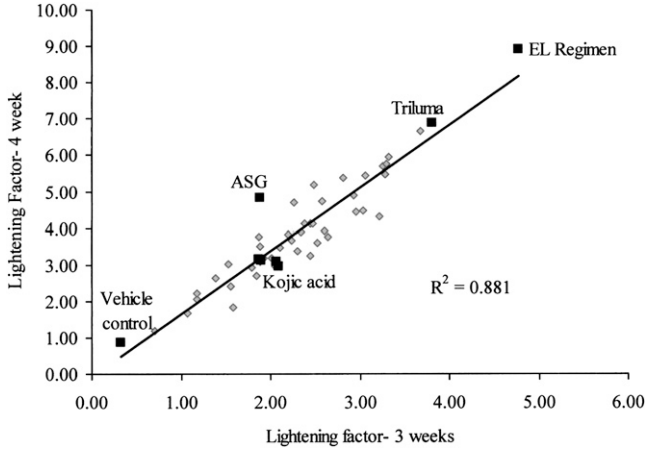

Figure 4. Lightening factor after four weeks was higher than after three weeks. Correlation between lightening factor at three and four weeks was significant ($p < 0.001$).

DISCUSSION

The actual color of skin is determined by the type and amount of melanin synthesized by melanocytes and by its distribution pattern in the surrounding keratinocytes. Melanin forms through a series of oxidative reactions involving the amino acid tyrosine in the presence of the enzyme tyrosinase. Tyrosinase catalyses three different reactions in the biosynthetic pathway of melanin in melanocytes: the hydroxylation of tyrosine to L-DOPA and the oxidation of L-DOPA to dopaquinone; furthermore, in humans, dopaquinone is converted by a series of complex reactions to melanin (15). Production of melanin can sometimes be excessive and uneven, resulting in a dark and discolored skin tone, which is a serious concern in health and beauty maintenance. As a result there is a myriad of skin-lightening and depigmenting products available, which contain actives like magnesium-1-ascorbyl-2-phosphate (MAP), hydroxyanisole, N-acetyl-4-S-cysteaminylphenol, arbutin (hydroquinone-beta-d-glucopyranoside), and hydroquinone (15).

A literature review shows a variety of methods for testing the efficacy of whitening agents, most of which are confined to *in vitro* tests in cell cultures (16–18). These methods deal with blocking various steps of the melanogenesis pathway, such as tyrosinase inhibition (17,19) and inhibition of melanosome transfer (20). These methods are valuable for screening purposes but do not translate to how the materials will act on skin in clinical settings.

There are also several clinical methods available, most involving 8–12-week, in use, clinical trials in which skin color is graded via visual and instrumental assessments (6). Other clinical studies involve the recruitment of subjects with pigmentation disorders like melasma (21), solar lentigos (6), and post-inflammatory hyperpigmentation (22). In these studies, photographs of the lesions are obtained before and after several weeks of treatment, followed by image analysis of the photographs to determine the lightening of the lesions. Fluorescence photography is another noninvasive method that is sensitive in the evaluation and quantification of the distribution and changes of mottled and diffuse hyperpigmentation (23). In addition, the overall skin color is assessed by the subjects and trained technicians. All these current methods are valuable and effective, but extremely time-consuming, mainly because whitening actives take a long time to act.

We have developed quick and simple methods to determine the skin-lightening effect of actives, with various modifications, depending on the objective of the study. If the test material is designed to reduce pre-existing color, the first method, which addresses reduction of tan, can be employed. If the test material contains anti-inflammatories and is designed to reduce the onset and intensity of tan, then the second method can be employed. Both these methods involve the use of UV-B induced tanning as the marker. The effect of overexposure to solar ultraviolet radiation (UVR) on human skin has been well described (24,25). The erythema produced is commonly referred to as "sunburn."

UV irradiation initially elicits an inflammatory reaction (sunburn) that resolves within a few days and converts to a suntan (26). The UV-induced tanning starts to resolve quickly and, depending on the intensity of UV exposure and skin type, is almost gone in four to five weeks. Small areas of identical suntan can be repeatably induced on the skin, and treatment of these sites allows a rapid screening of several skin whiteners within the course of a month. In addition, the lightening factor described in this paper takes into account the whole picture of skin color reduction over the course of the study rather than at individual time points (Figure 1). This method addresses one aspect of skin lightening and by no means reflects the effect of whiteners on lentigos, melasma, and other skin discolorations. However, it is a valuable tool for screening and choosing the best of several materials and concentrations, and must be followed by the 8–12-week, in-use, clinical trials currently in use.

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