Skin-Lightening Formulation: A Comparative In Vivo and In Vitro Study

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Acquired hyperpigmentation, which is characteristic of melasma, postinflammatory melanoderma, solar lentigines, and other skin diseases, may produce relevant psychological and cosmetic problems.[1] Treating skin hyperpigmentation, particularly melasma, is a challenge for dermatologists, as there is no gold standard treatment and recurrences are common. Several treatment strategies have been proposed, including sunscreen use for reducing UV exposure, inhibition of melanogenic activity inside hyperactivated melanocytes, removal of excess melanin, and dispersion of melanin granules. Among chemical skin-lightening formulations, hydroquinone, a hydroxyphenol that acts as a competitive inhibitor of tyrosinase, has long been considered the compound of choice and a standard reference in evaluating the efficacy of other skin-lightening formulations.[2] However, because of its numerous side effects, ranging from irritant or allergic contact dermatitis to confetti leukoderma, caused by the irreversible destruction of melanocytes, hydroquinone has been banned in cosmetics in Europe since 2000 by a directive of the European Commission (24th Dir. 2000/6/EC), and formulations that include hydroquinone are available only by prescription. Thus, in recent years, many efforts have been made to discover new compounds and screen these compounds for clinical effectiveness. Today, a variety of skin-lightening products is commercially available. These products are formulated with 1 or more different active compounds that are able to inhibit melanin production, accumulation, or both by acting under different mechanisms. Because melanogenesis is a multistep process, a combination of compounds acting at different stages of pigmentation should be advantageous for managing acquired hyperpigmentation.

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In this study, the authors evaluated, both in vitro and in vivo, the efficacy and safety of a skin-lightening formulation composed of linoleic acid, an unsaturated fatty acid capable of reducing UV-induced pigmentation in vitro and in vivo, and thioctic acid, a strong antioxidant that is able to inhibit tyrosinase expression and activity.

Materials and Methods

In Vitro Study

The in vitro depigmenting effect of skin-lightening formulations was tested in the MelanoDerm™ skin model MEL-300-B, which contains melanocytes derived from a black donor, and seeded at a keratinocyte-melanocyte ratio of 10:1. Ten microliters of 8 combinations of linoleic acid (1%–8%) and thioctic acid 5% in dimethylisosorbide and 25 μL of kojic acid 1% dissolved in sterile ultrapure water (positive control) and ultrapure water alone (negative control) were applied to the MEL-300-B stratum corneum on days 0, 2, 5, 7, and 9. Before each application, the tissues were rinsed with 1 mL of phosphate-buffered saline (PBS) to remove any residue of tested compounds. Tissues were fixed on days 5, 7, 9, 14, and 16 for macroscopic analysis and microscopic observation of the melanocytes. The melanin content in the tissue was evaluated by melanin assays on frozen duplicate tissues on days 9, 14, and 16.

Cytotoxicity Assay

The tested compounds were preliminarily assayed for cytotoxicity with a modified thiazolyl blue (MTT) tissue-viability test. The test material dissolved in dimethylisosorbide was applied topically to the EpiDerm™ (tissue without melanocytes) and MEL-300-B tissue surfaces. The negative control tissue was exposed to 25 μL ultrapure water. After 72 hours of incubation, the tissues were rinsed with PBS and incubated in MTT solution (0.3 mg MTT per milliliter of test medium) for 3 hours at 37°C with 5% carbon dioxide. Each assay was performed in

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duplicate. Results were expressed as a percentage of cell viability to the control.

Macroscopic Darkening

Macroscopic darkening of the MEL-300-B tissues was observed by the naked eye at days 5, 7, 9, and 14.

Microscopic Observation

The melanocytes within the MEL-300-B may be observed in a nondestructive, nonperturbing manner and without the addition of levodopa because of the active melanogenesis. Top-view microscopic observation, using an inverted microscope, was performed on the cell cultures containing the MEL-300-B tissue placed in a Petri dish with sufficient PBS.

Melanin Assay

Two frozen tissues were pooled and homogenized in 0.45 mL of sodium dodecyl sulfate 0.1% containing 0.05 mmol ethylenediaminetetraacetic acid and 10 mmol Tris hydrochloride (pH 6.8). To each homogenate, 20 µL of proteinase K (5 mg/mL) was added. Digestion proceeded overnight at 45°C and was followed by the addition of 20 µL of proteinase K for 4 hours. Following digestion, sodium carbonate 500 mM (50 µL) and hydrogen peroxide 30% (10 µL) were added, and the samples were incubated at 80°C for 30 minutes. The mixture was extracted with 100 µL of chloroform-methanol (2:1) and centrifuged at 10,000*g* for 10 minutes. The melanin was quantified by measuring the optical density of the supernatant at 405 nm. Melanin content in the samples was calculated by using a standard curve made with scalar concentrations of synthetic melanin.

In Vivo Human Trial

The study involved 15 subjects (aged 20–45 years) affected by melasma, presenting with Fitzpatrick skin types III, IV, and V, and not previously treated with skin-lightening agents or dermocosmetics containing α-hydroxy or β-hydroxy acids for 3 months or more. All the subjects gave signed and informed consent before participating in the trial.

In the study subjects, 2 melasma lesions with similar characteristics were identified and treated (1 with the active product, the other with placebo). Products were applied twice daily for 1 month. The subjects selected were instructed to avoid extreme sun exposure and to use sunscreens with a high sun protection factor.

The safety evaluation consisted of monitoring and recording all side effects, especially those commonly reported in studies of skin-lightening agents, such as erythema, peeling, burning, and itching.

Instrumental Tests

Mexameter® MX 18 was employed to assess the degree of pigmentation and the erythema level by measuring the specific absorption wavelengths of melanin and hemoglobin. The melanin index is directly correlated to skin melanization, whereas the erythema index quantifies the amount of hemoglobin.

The degree of hyperpigmentation was also assessed clinically via a high-magnification (20) photograph taken with a videomicroscope.

Results

MTT Tissue-Viability Assay

The toxic effect of solvent was tested by applying 10 µL of different dilutions of the solvent in ultrapure water. A concentration of 33% solvent was well tolerated by the tissues; thus, it was chosen for dissolving the test compounds. No significant cytotoxicity was found following the application of 10 µL of the different combinations of linoleic acid and thioctic acid (data not presented).

Macroscopic Examination

Negative control tissues darkened progressively with increased time in culture, whereas the samples treated with the linoleic acid–thioctic acid combination did not appear to darken significantly (Figure 1). The employed mixtures were composed of a variable amount of linoleic acid (1%–8%) and thioctic acid 5% and showed a similar lightening effect on the reconstructed epidermis, suggesting that thioctic acid was the main lightening agent. The positive and negative control tissues were perceptively darker than the tissue treated with the test compound on days 9 and 14 (data not presented).

Melanin Assay

The melanin assay data confirmed the macroscopic darkening examination. On days 9 and 14, negative control tissues had the highest melanin content (Table). The treatment with kojic acid 1% (positive control) on days 9

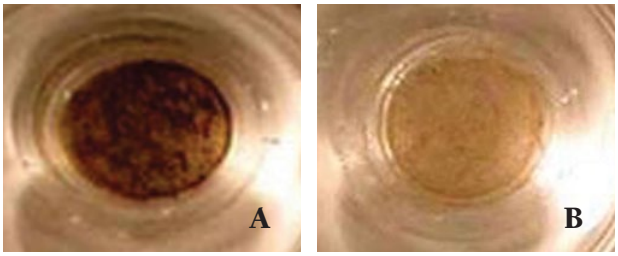

Figure 1. Macroscopic views of melanocytes treated with ultrapure water (A) and linoleic acid 1% and thioctic acid 5% in dimethylisosorbide (B) after 14 days.

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Effect of Linoleic Acid–Thioctic Acid Combination Treatment on Melanin Content in MelanoDerm™ Skin Model MEL-300-B*

Treatment	Melanin Content (µg/2 tissues)	Decrease Vs Negative Control, %
LX1		
Day 9	21.52±3.722	70.1
Day 14	23.6±1.175	84.1
LX2		
Day 9	18.62±4.309	74.1
Day 14	26.23±3.33	82.3
LX3		
Day 9	20.55±0.392	71.4
Day 14	25.68±3.33	82.7
LX4		
Day 9	17.09±0.979	76.2
Day 14	22.49±1.567	84.8
LX5		
Day 9	20.0±0.784.0	72.2
Day 14	19.58±3.667	86.8
LX6		
Day 9	18.06±1.175	74.9
Day 14	19.86±2.546	86.6
LX7		
Day 9	17.09±0.196	76.2
Day 14	26.09±0.392	82.4
LX8		
Day 9	14.04±1.371	80.5
Day 14	18.2±3.722	87.7
Kojic acid 1%†		
Day 9	49.36±6.072	31.4
Day 14	117.5±8.423	20.7
Ultrapure water‡		
Day 9	71.94±8.227	0.0
Day 14	148.1±7.052	0.0

* LX1 indicates linoleic acid 1% plus thioctic acid 5%; LX2, linoleic acid 2% plus thioctic acid 5%; LX3, linoleic acid 3% plus thioctic acid 5%; LX4, linoleic acid 4% plus thioctic acid 5%; LX5, linoleic acid 5% plus thioctic acid 5%; LX6, linoleic acid 6% plus thioctic acid 5%; LX7, linoleic acid 7% plus thioctic acid 5%; LX8, linoleic acid 8% plus thioctic acid 5%.

† Positive control.

‡ Negative control.

and 14 significantly reduced melanin amount compared with the negative control tissues (31.4% and 20.7% decrease, respectively). At the same time, the samples treated with the tested compound combinations showed a stronger reduction in melanin content compared with the negative and positive controls. In fact, the examination of the skin-lightening effect exerted by the different combinations of active compounds revealed that on days 9 and 14, all the combinations of linoleic acid and thioctic acid 5% induced a significant, stable reduction of melanin production compared with the negative control (70%–87.7% decrease compared with the ultrapure water negative controls). However, no significant differences in the used combinations were found, and the results in this group are within the normal fluctuation of the melanin assay.

Microscopic Examinations

The top-view microscopy showed highly pigmented melanocytes that remained dendritic throughout the study in the positive and negative control tissues. On day 14, the treated tissues showed melanocytes that largely were not dendritic (data not presented). However, on day 16, melanocytes in the tissues treated with the linoleic acid–thioctic acid combination recovered and were again dendritic, even though their color was light brown compared with the darker brown or black color in the positive and negative control tissues (Figure 2). This evidence suggests that the treatment inhibited melanin synthesis but did not cause irreversible damage to or cytotoxicity in the melanocytes.

Clinical Efficacy

In the clinical trial, all subjects completed the study. At the end of treatment, there was an improved clinical picture and reduced pigmentation of the spots treated with the active product in 7 of 10 subjects. The colorimetric assay revealed a decrement of the mean value of the pigmentation index from 290 to 180 nm (Figure 3). The depigmentation was also documented with videomicroscopic photographs (Figure 4). No side effects were recorded in the active-treated and the placebo-treated sites. Compliance was good, despite a slight burning sensation

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Figure 2. Microscopic views of melanocytes treated with ultrapure water (A), kojic acid 1% (B), and linoleic acid 1% and thioctic acid 5% (C) after 16 days.

lasting, in some cases, a few minutes postapplication of the active product.

Comment

Our data demonstrate that the linoleic acid–thioctic acid combination was found to be an effective, safe skin-lightening formulation. This compound combination was chosen because data in the literature reported an effect on melanogenesis from both linoleic acid and thioctic acid. Thioctic acid, also known as lipoic acid, is a natural compound, synthesized by all mammals, including humans.[3] When administered topically, it absorbs easily and converts to dihydrothioctic acid, its reduced form. The existence of this equilibrium between the oxidased and the reduced forms of thioctic acid has been confirmed by an in vitro study in which normal mammalian cells appeared to be capable of taking up α-thioctic acid and reducing it to dihydroxy-lipoic acid.[4]

Thioctic acid and dihydrothioctic acid in combination are strong antioxidants, known for their ability to prevent UV-induced photo-oxidative damage.[5] Because of its particular chemical structure, thioctic acid can participate in redox reactions or act as an electron transporter. Moreover, it is able to exert its free radical scavenger properties toward both hydrophilic cellular components, such as proteins, nuclear acids, or enzymes, and lipophilic cell compartments, such as cell membranes.

Thioctic acid is effective in scavenging hydroxyl and hypochlorous radicals and singlet oxygen, whereas dihydrothioctic acid exhibits a slightly higher antioxidant activity by also reacting with superoxide radicals and peroxyl radicals. The reduced form is capable of giving 1 electron to oxidased endogenous antioxidants, such as glutathione disulfide and dehydroascorbic acid, leading to the regeneration of their full, active reduced forms. Thioctic acid appears to be capable of chelating certain metals, such as copper, manganese, and zinc, and may possibly interfere with the activity of several enzymes by interacting with metal ions of the active sites.[6] Data in literature show that dihydrolipoic acid and thioctic acid are able to inhibit tyrosinase activity by reducing microphthalmia-associated transcription factor and tyrosinase promoter activities.[7] These agents also inhibit the forskolin- and UVB-stimulated promoter activities of these genes and significantly reduce tyrosinase activity in melanocyte cultures, resulting in depigmentation. Such lightening activity has also been confirmed, using histologic techniques, in dark-skinned Yucatan swine. Linoleic acid is able to inhibit melanin synthesis through the reduction of

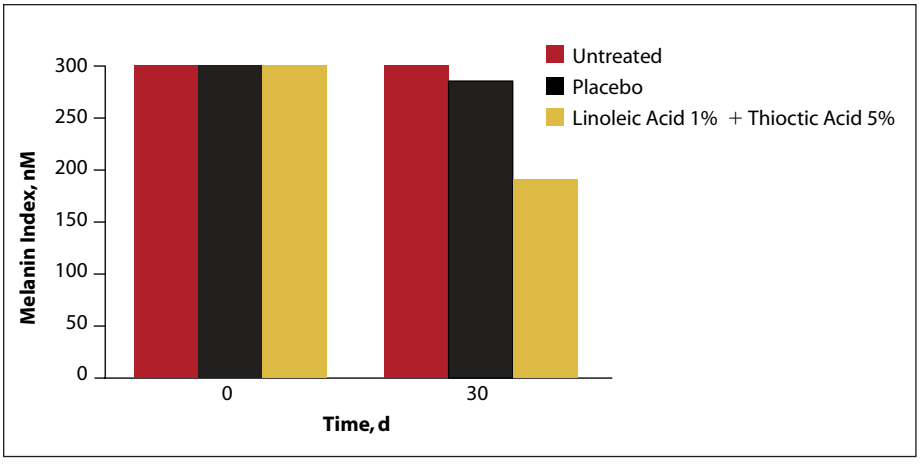

Figure 3. Melanin index used to measure changes in pigmentation in control subjects and subjects given placebo and treated with linoleic acid 1% and thioctic acid 5%.

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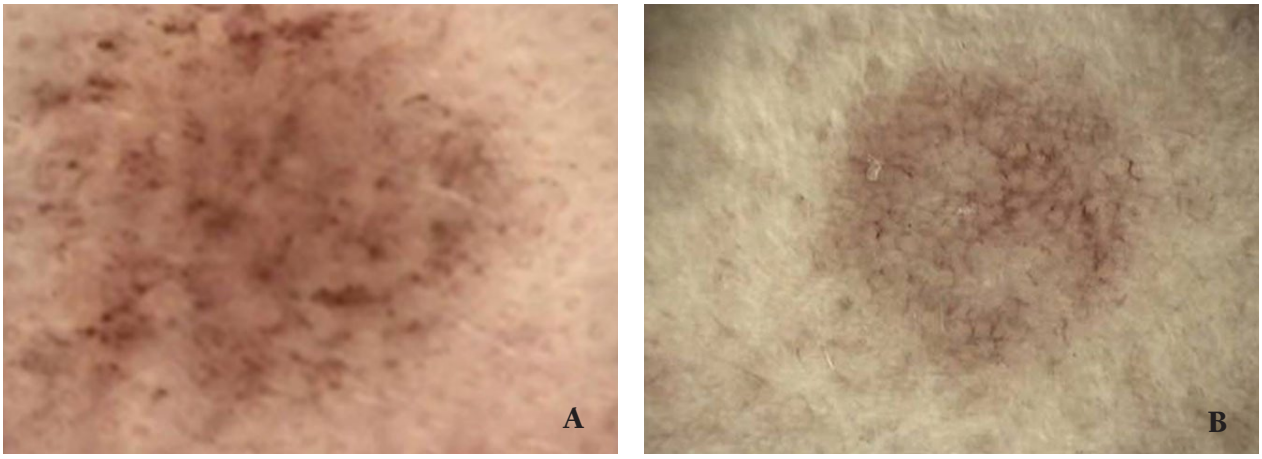

Figure 4. Videomicroscopic photographic evaluation of clinical effectiveness of linoleic acid 1% and thioctic acid 5% in reducing melanin before (A) and after (B) twice-daily treatment for 1 month.

tyrosinase bioavailability and the enhancement of the rate of its proteolytic degradation.[8,9] Linoleic acid has also been reported to be effective in reducing UVB-induced hyperpigmentation in brown guinea pigs and melanin synthesis in B16 melanoma cells.[10] Hwang et al[11] have recently demonstrated that linoleic acid and cystamine reduce melanin synthesis in vitro and in vivo. Our data demonstrate that the linoleic acid–thioctic acid combination is able to significantly reduce in vitro and in vivo melanogenesis, as shown by the inhibition of melanin production and both macroscopic and microscopic darkening in the epidermis analogs. Moreover, our in vivo study showed a significant reduction of excessive pigmentation in subjects treated with the linoleic acid–thioctic acid combination formulations compared with placebo-treated subjects, further suggesting that the combination is clinically effective and safe as skin-lightening treatment. Interestingly, on day 16 of the clinical trial, the melanocytes completely recovered their morphology and functionality, suggesting that the lightening activity is not associated with cytotoxicity.

Conclusion

Our in vitro and in vivo data confirm that the linoleic acid–thioctic acid combination is able to inhibit melanin production, suggesting that the combination can act in a synergistic manner by interfering at different steps of melanogenesis pathway. From our clinical results, we conclude that the combination could be proposed as an effective, safe treatment for melasma.

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