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Article *in* Skin pharmacology and physiology · February 2007

DOI: 10.1159/000103204 · Source: PubMed

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Original Paper

Skin Pharmacology and Physiology

Skin Pharmacol Physiol 2007;20:211–218
DOI: 10.1159/000103204

Received: December 21, 2006
Accepted after revision: April 2, 2007
Published online: May 23, 2007

In vivo Assessment of Ectoin: A Randomized, Vehicle-Controlled Clinical Trial

U. Heinrich B. Garbe H. Tronnier

Institute of Experimental Dermatology, University of Witten/Herdecke, Witten, Germany

Key Words
Skin ageing · Skin surface · Ectoin efficacy · In vivo assessment

Abstract
The aim of the study was to assess an Ectoin formulation with regard to the antiageing properties. The study was designed as a monocentric, randomized, double-blind application test, in order to ensure the compatibility and the efficacy of Ectoin in comparison to a vehicle emulsion. A total of 104 voluntary healthy female test subjects were included in the study. Moisturizing properties as well as other parameters of skin ageing, like skin surface structure and skin elasticity, were determined for treatment A (vehicle) and treatment B (with 2% Ectoin) versus an untreated control. Statistical evaluations according to the Wilcoxon rank-sum test indicate a general preference for the Ectoin treatment by the test subjects in both the application and the efficacy tests. None of the participating test subjects had any side effects throughout the study. In terms of antiageing properties, previous in vitro studies could be confirmed by this clinical trial, clarifying that the natural cell protection concept of Ectoin is transferable to skin care with manifold benefits.

Introduction

The human skin is situated at the interface of the organism and its environment and therefore exposed to a variety of environmental assaults. The cumulative effect of external factors like radiation, wind, humidity and temperature extremes leads to skin ageing [1]. In this context the UV irradiation inherent in sunlight is one of the prominent stressors responsible for processes of extrinsic ageing. Photo-aged skin shows increased wrinkling, thickness and laxity as well as a marked dyspigmentation [2–4].

The naturally compatible solute Ectoin has become available as a protective active against skin ageing. Ectoin is produced by micro-organisms and was mainly developed for the use in cosmetic preparations. During the development process several toxicological endpoints have been investigated for Ectoin according to the official Scientific Committee on Consumer Products guidelines for cosmetic ingredients.

Ectoin is a neutral nonionic, strongly water-binding, organic molecule of low molecular weight occurring in halophilic bacteria [5, 6]. In its natural habitat, this micro-organism grows under extreme conditions such as intensive sun irradiation, high temperatures and extreme dryness and protects itself against these stresses by synthesizing Ectoin.

Various investigations underline the outstanding antiageing properties of Ectoin: epidermal dendritic Langerhans cells are the single most important antigen-pre-

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Prof. Dr. Ulrike Heinrich
Institut für Experimentelle Dermatologie, Universität Witten/Herdecke
Alfred-Herrhausen-Str. 44 (FEZ)
DE–58455 Witten (Germany)
Tel. +49 2302 2826 300, Fax +49 2302 2826 326, E-Mail ulrike.heinrich@uni-wh.de

senting cell population in the skin. The number of Langerhans cells decreases significantly in aged skin, whereas the decline in skin exposed to the sun is greater than that in skin protected from the sun [7–9]. Topically applied Ectoin shows an immunoprotective potential on sun-exposed skin of healthy subjects. The UV-induced reduction of Langerhans cells has been prevented by pre-treatment with Ectoin before sun exposure [10].

The exposure of primary human keratinocytes with UVA provokes the formation of ceramide by a singlet-oxygen-mediated mechanism. As a consequence of the increased ceramide level an intracellular signalling cascade is activated, leading to expression of the pro-inflammatory intercellular adhesion molecule-1. These negative effects are successfully prevented by Ectoin due to its singlet-oxygen-quenching properties [11, 12]. Taking into consideration that the activity of antioxidant enzymes and the levels of nonenzymatic antioxidants decline with age [13, 14] Ectoin could prevent such oxidative damage in skin.

Furthermore, it is known that Ectoin prevents UVA-induced large-scale mutations of the mitochondrial DNA, so-called 'common deletion' in human dermal fibroblasts [11, 15, 16]. It has been described that this deletion is accompanied by a marked up-regulation of the matrix-metalloproteinase-1 followed by skin wrinkle formation [17–19].

Despite the multitude of in vitro models characterizing the positive antiageing properties of Ectoin, the in vivo situation has hardly been investigated.

Therefore, the aim of the present study is to assess an Ectoin formulation with regard to the antiageing properties in vivo.

Methods

Study Design

The study was carried out as a monocentric, randomized, double-blind, vehicle-controlled application test. A total of 104 voluntary healthy female test subjects (30–60 years) with normal, dry and sensitive skin were included. The blinding of the tested verum and reference emulsion was maintained throughout the whole study. No differences in the appearance of the creams could be detected. The participants were asked not to change their skin care habits during the study.

All test subjects received detailed information listing every single parameter relevant to the study. Every test subject had to submit a written declaration of consent for their participation. The study design was approved by the ethics committee of the University of Witten/Herdecke.

Pregnancy and breastfeeding, intake of medication, which could influence the outcome of the study, sunbathing or the use of sunbeds were exclusion criteria.

Compatibility Test

Before the clinical trial was started each test product (product B = verum with 2% Ectoin and product A = vehicle), was checked in a pre-test with respect to skin compatibility. For this purpose an epicutaneous test was performed with each product. A collective of 50 female test subjects, ranging in age from 30 to 60 years, participated in the study. Through the utilization of a standard test plaster (Curatest, Lohmann & Rauscher, Germany), the substances to be tested were applied undiluted and fixed on the healthy skin. Test plasters were removed after 48 h and the test areas were assessed. Further assessments took place after 72 h.

Application Test

The study included 30 female test subjects (30–60 years) with normal, dry and sensitive skin. The participating test subjects applied the test products randomly twice daily, as a skin care product for a period of 4 weeks. The use of other skin care products during the course of the study was not allowed. At the end of each application test the subjects received a questionnaire to assess the cosmetic acceptance, the galenic parameters, the efficacy as well as the compatibility of the tested products.

All data were evaluated and recorded as a graphic acceptance profile as shown in figure 1, according to the following scoring: 1 = very good, 2 = good, 3 = fair, 4 = satisfactory, 5 = unsatisfactory, 6 = insufficient.

Efficacy Test

Moisturizing properties as well as other skin parameters of skin ageing, like skin surface structure and skin elasticity, were determined. In order to analyze the efficacy, 24 voluntary healthy female test subjects (30–60 years) with normal, dry and sensitive skin, with skin hydration values of 30–40 arbitrary units (AU), measured by the Corneometer®, according to Beradesca [20] and Heinrich et al. [21], were included in the study.

The duration of the examination was 4 weeks and was carried out on the insides of the forearm of the test subjects. Both test substances, product A (vehicle) and product B (verum) were applied to the inside of the forearm twice daily and compared to an untreated control.

The application was randomized and the test subjects were asked not to use any other cosmetics on the test fields and the untreated control.

For the documentation of the efficacy of both treatments, the following measurements were carried out:
- skin hydration (Corneometer CM 825®);
- skin elasticity (Cutometer SEM 575®);
- skin surface structure by means of the SELS method (surface evaluation of living skin, Visioscan®).

Before treatment was started, the initial values were determined for each of the test subjects. There was a final examination after 4 weeks.

Study Treatment

The following emulsions were tested, as shown in table 1.

Measurements

Skin Hydration (Corneometer). Since 1980 the Corneometer has provided a well-established method to determine the hydration level of the skin surface [20, 21] in a reproducible manner. The measuring principle of the Corneometer CM 825 (Courage

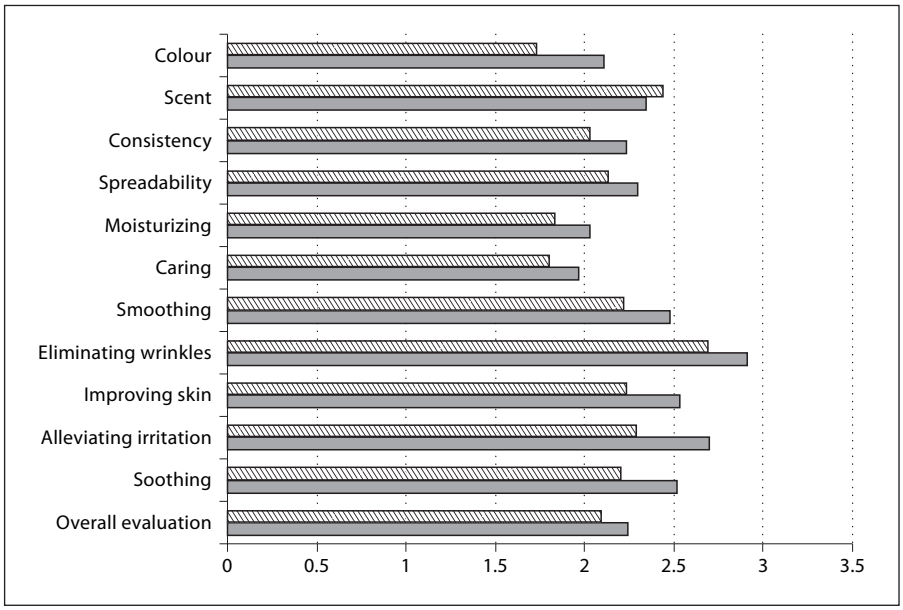

Fig. 1. Comparison of the subjective evaluation of 30 volunteers after 4-week product application for cosmetic acceptance and efficacy of treatment A (vehicle, grey bars) and treatment B (verum with 2% Ectoin, hatched bars); according to the following scoring: 1 = very good; 2 = good; 3 = fair; 4 = satisfactory; 5 = unsatisfactory; 6 = insufficient.

& Khazaka Electronics, Cologne, Germany) is based on capacitance measurement of a dielectric medium. Any change in the dielectric constant due to skin surface hydration variation alters the capacitance of a precision-measuring capacitor. Skin hydration is measured in AU.

Elasticity of Human Skin

The elasticity measurements are carried out with the Cutometer SEM 575 (Courage & Khazaka Electronics). It consists of a micro-processor-driven pneumatic device with electronic measurement and a special measuring probe. During the measurement the skin is sucked into the measuring probe by means of sub-atmospheric pressure. Here, the impression depth is recorded by an optical measuring system without contact.

Prior to each assessment, maximum pressure, measuring time, the time of the pressure rise and fall as well as the number of measuring cycles are set. After the evaluation, the respective measuring curve appears in a coordinate system on the monitor. It shows the impression depth of the skin into the measuring probe during action time and its regression in the time afterwards [22, 23].

The following parameters play a role in the evaluation of elastic properties. The suction phase is represented by the quotient: U_v/U_e = R6 (viscoelastic properties), the regression phase is represented by the quotient: U_r/U_f = R7 (biologic elasticity).

Skin Surface Structure (SELS, Visioscan)

Skin surface analysis according to the SELS method is based on the evaluation of an image of living skin taken under certain illumination; the picture is electronically processed for quantitative analysis. The skin surface is described by 5 different parameters: roughness, scaling, smoothness, wrinkling and volume [24–27].

The measuring device consists of a measuring head containing 2 contra-rotating metal-halogen lamps evenly illuminating a 15 × 17 mm field on the skin. The spectrum of the lamps and their intensity as well as their location permits to analyze the skin surface without interfering reflections from deeper layers. A CCD camera, located in the measuring head, records a picture of the skin, which is then transferred as graded grey values into a bitmap file (software: Skin-Visiometer, Courage & Khazaka). The method permits to differentiate between 256 grey values. By means of the additional software SELS the skin-specific parameters are then calculated.

Table 1. Study treatment product A (vehicle) versus product B (verum with 2% Ectoin)

Product A	Aqua, simmondsia chinensis oil, paraffinum liquidum, glycerin, cetearyl alcohol, pentylene glycol, cetearyl glucoside, sodium lactate, xanthan gum, sodium cetearyl sulfate
Product B	Aqua, ectoin, simmondsia chinensis oil, paraffinum liquidum, glycerin, cetearyl alcohol, pentylene glycol, cetearyl glucoside, sodium lactate, xanthan gum, sodium cetearyl sulfate

Statistical Methods

For all parameters of the efficacy test and all measuring times (day 0 and after 4 weeks) descriptive statistics (mean, standard deviation, minimum, lower quartile, median, upper quartile and maximum) were calculated. For all parameters pre-post differences were calculated and analyzed descriptively.

Within the treatment groups each combination of 2 time points was compared by means of the Wilcoxon signed-rank test. For the pre-post differences each combination of 2 treatment groups was compared with the Wilcoxon rank-sum test. Percentage changes of all measured parameters were calculated (week 0 vs. week 4) and the p values ($p < 0.05$ regarded as statistically significant) were determined at all measuring points.

Results

Compatibility Test

Positive or improbable reactions could be found neither after 48 nor 72 h for any of the 50 voluntary test subjects, indicating that the tested products had no primary irritating effect of the skin. The test did not set off any possibly already existing sensitizations triggered by the substances contained in the products either.

Application Test

The study included 30 female test subjects (30–60 years) with normal, dry and sensitive skin. At the end of each application test the subjects received a questionnaire to assess the cosmetic acceptance, the galenic parameters, the efficacy as well as the compatibility of the tested products. All data were evaluated and recorded as a graphic acceptance profile, as shown in figure 1, according to the following scoring: 1 = very good, 2 = good, 3 = fair, 4 = satisfactory, 5 = unsatisfactory, 6 = insufficient.

Treatment A (Vehicle Reference). With an overall evaluation of 2.25, the test product A (vehicle) showed a satisfying cosmetic acceptance by the test subjects. First of all the galenic parameters, such as colour, scent, consistency and spreadability, were given values between 2.0 and 2.7. Consumption was said to be very economical. The skin feeling was described as pleasant and in addition skin-protective, moisturizing, soothing, antiwrinkling and caring properties (2.5–2.9) were found. None of the participating test subjects had any side effects such as irritation or reddening of the skin.

Treatment B (Verum with 2% Ectoin). With an overall evaluation of 2.09, the test product B (verum) showed good cosmetic acceptance by the test subjects. First of all the galenic parameters, such as colour, scent, consistency and spreadability, were given values between 1.7 and 2.1. Consumption was said to be very economical. The skin feeling was described as pleasant and furthermore skin-protective, moisturizing, soothing, antiwrinkling and caring properties (1.8–2.6) were found.

The subjective evaluation of the test panel showed a clear preference for the verum formulation with 2% Ectoin (hatched bars), as shown in figure 1. This is very remarkable, since the reference/vehicle product was not a placebo substance but a high-quality skin care product. Thus, the test subjects also ascribed subjectively experienced superior properties to the Ectoin emulsion.

None of the participating test subjects had any side effects such as irritation or reddening of the skin.

Table 2. Hydration measurements (in AU) before and after 4-week treatment with product A (vehicle), product B (verum with 2% Ectoin) versus untreated control

Hydration	Week 0	Week 4	%
Product A (vehicle)	39.2	47.6*	+21.6*
Product B (verum)	38.1	49.4*	+29.5*
Untreated control	37.9	38.6	+1.7 (n.s.)

* $p < 0.05$ was regarded as statistically significant. n.s. = Not significant.

Table 3. Elasticity measurements (in AU) before and after 4-week treatment with product A (vehicle), product B (verum with 2% Ectoin) versus untreated control

Elasticity	Week 0	Week 4	%	
Visco-elasticity (R6) ▼				
Product A (vehicle)	0.11	0.12	+3.5	deterioration
Product B (verum)	0.12	0.11	–7.5	improvement
Untreated control	0.14	0.15	+9.1	deterioration
Biologic elasticity (R7) ▲				
Product A (vehicle)	0.85	0.83	–1.6	deterioration
Product B (verum)	0.84	0.86	+2.4	improvement
Untreated control	0.78	0.75	–3.5	deterioration

Positive influences on skin elasticity are accompanied by a decrease of the parameter R6 (visco-elastic properties) and an increase of the parameter R7 (biologic elasticity).

Efficacy Test

Hydration Measurements

By using product B (with Ectoin) skin moisture values could be increased from 38.1 to 49.6 AU in the course of a 4-week treatment. This corresponds to an increase in skin moisture by 29.5%.

In the treatment field of product A (vehicle) skin moisture increased from 39.2 to 47.6 AU, which corresponds to an increase of 21.6%.

In the test field of the untreated control the skin moisture remained unchanged.

The statistical evaluation according to the Wilcoxon signed-rank test showed a significant improvement of skin moisture for both the verum and vehicle emulsion. The untreated control showed no effect (table 2, fig. 2).

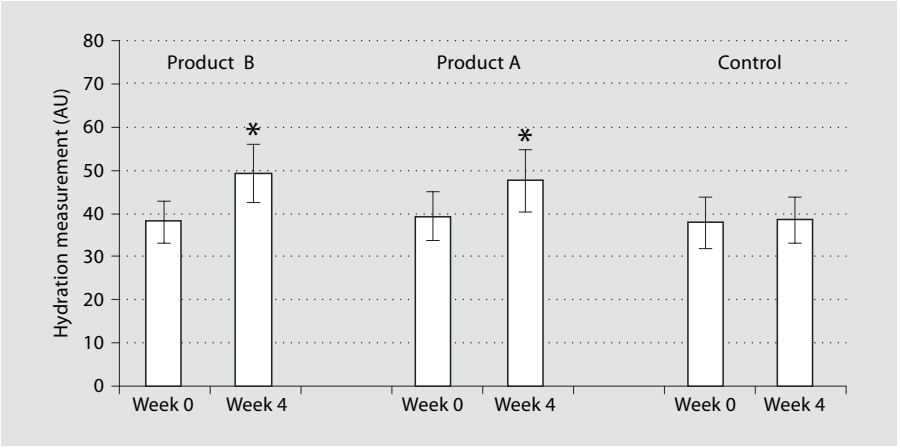

Fig. 2. Hydration measurements with Corneometer CM 825 (n = 24) before and after 4-week treatment with product A (vehicle), product B (verum with 2% Ectoin) versus untreated control; * $p < 0.05$ was regarded as statistically significant.

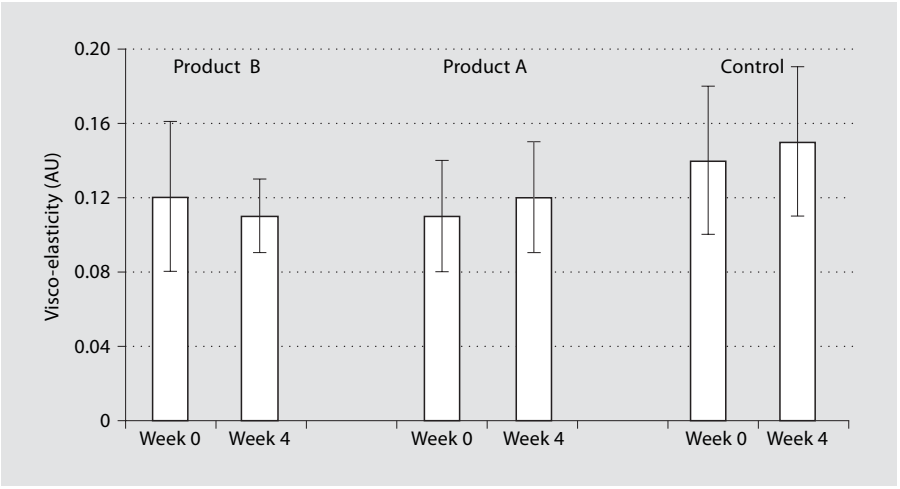

Fig. 3. Visco-elasticity (R6) before and after 4-week treatment with product A (vehicle), product B (2% Ectoin) versus untreated control (n = 24). Positive influences on skin elasticity are accompanied by a decrease of the parameter R6 (visco-elastic properties).

Measurements of Skin Elasticity

When measuring skin elasticity, the parameters R6/visco-elasticity and R7/biologic elasticity were used. Positive influences on skin elasticity are accompanied by a decrease of the parameter R6/visco-elastic properties and an increase of the parameter R7/biologic elasticity.

In the test field of product B (with 2% Ectoin) R6 could be improved by 7.5%. In the test field of product A (vehicle) no positive influence could be found. The untreated control field, however, showed a deterioration of the visco-elastic properties by 9%.

The biological elasticity (R7) could be increased by an average of 2.4% in the test field of product B (with 2% Ectoin). The vehicle treatment (product A) as well as the untreated control did not show any improvement but a decrease of the vehicle treatment and a deterioration of the untreated control. Admittedly the statistical evaluation did not show a significant change in elasticity for any of the above-mentioned parameters (table 3, fig. 3).

Measurements of the Skin Surface according to the SELS Method

According to the methodological description, the SELS procedure differentiates between roughness, scaling and smoothness of the skin. Furthermore, the parameter volume reflects individual deeper structures of the skin (table 4, fig. 4, 5).

In the test field of product B (with 2% Ectoin) skin roughness could be reduced from 0.40 to 0.19 AU. This equals a decrease of roughness of 52%.

In the test field of product A (reference) the decrease of roughness was 33%, in the untreated control a decrease of 14% was measured.

Scaling of the skin could be positively influenced by the treatment with both formulations. Here the decrease in scaling was by 76% for product B and by 70% for product A.

The untreated control showed a decrease in scaling by 21%.

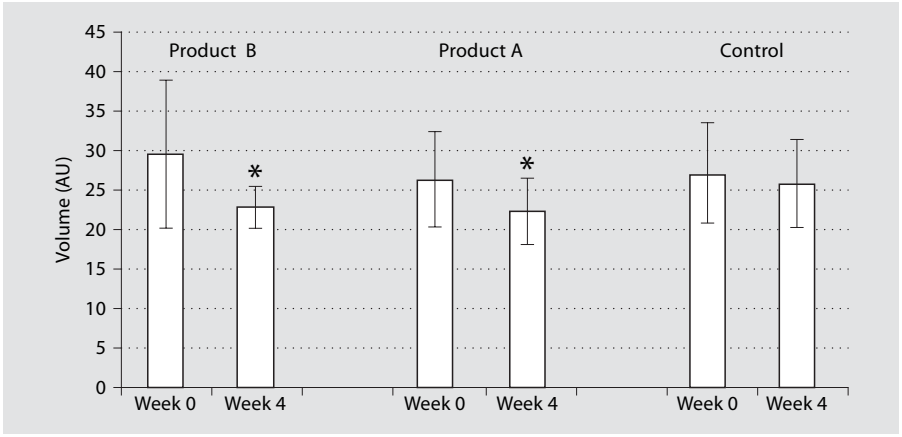

Fig. 4. Skin surface parameter volume by the SELS method (Visioscan) before and after 4-week treatment with product A (vehicle), product B (verum with 2% Ectoin) versus untreated control (n = 24); * $p < 0.05$ was regarded as statistically significant.

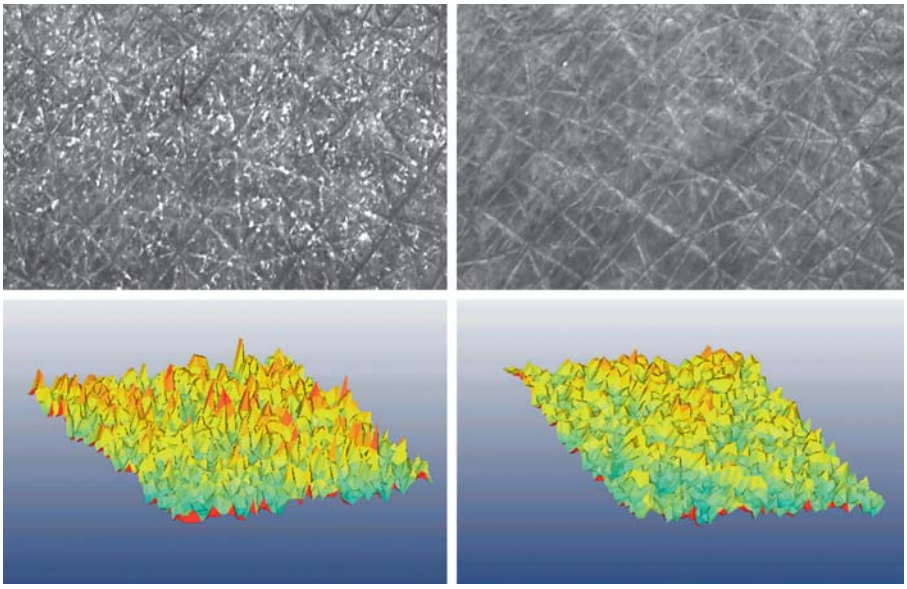

Fig. 5. Skin surface measurements by the SELS method (Visioscan) before (left) and after (right) 4 weeks of treatment with product B (verum with 2% Ectoin).

Skin smoothness could only be slightly improved by product B (with Ectoin) by 2%. No improvement could be found in the test field of product A (vehicle) and the untreated control. Here a decrease of smoothness by 3 and 8%, respectively, was observed.

The parameter volume could be positively influenced by the treatment with both formulations as well. However, the attenuation of volume was measured at 23% for product B and at 16% for product A. The untreated control showed a decrease in volume by 4.8% (fig. 4).

The data of the skin surface measurements demonstrate a course similar to the skin hydration measurements. Both products, verum (B) as well as vehicle (A), showed an improvement of the surface parameters like roughness, scaling, smoothness and volume. However, it has to be pointed out that the Ectoin treatment (B) performed better in all parameters. The differences (improvements) between the 2 treatments were 19.6% in roughness and 7.3% in volume in favour of Ectoin (B).

The differences between the verum treatment (B) and the untreated control were calculated at 38% in roughness and 18.1% after 4 weeks of treatment in favour of treatment (B).

Statistics

Percentage changes of all measured parameters were calculated (week 0 vs. week 4) and the p values ($p < 0.05$, statistically significant) were determined (Wilcoxon signed-rank test).

Significant changes could be evaluated after 4 weeks of treatment for skin hydration as well as for skin roughness, scaling and volume for product A (vehicle) and product B (verum).

Table 4. Skin surface structure measurements (in AU) by the SELS method (Visioscan)

Skin surface structure	Week 0	Week 4	%
Roughness ▼			
Product A (vehicle)	0.43	0.29*	–32.6*
Product B (verum)	0.40	0.19*	–52.2*
Untreated control	0.41	0.36 (n.s.)	–14.2 (n.s.)
Scaling ▼			
Product A (vehicle)	0.28	0.09*	–69.9*
Product B (verum)	0.37	0.09*	–75.9*
Untreated control	0.44	0.35*	–20.6*
Smoothness ▲			
Product A (vehicle)	22.17	21.45	–3.3 (n.s.)
Product B (verum)	21.65	22.05	+1.8 (n.s.)
Untreated control	21.76	20.02 (*)	–7.9 (n.s.)
Volume ▼			
Product A (vehicle)	26.29	22.19*	–15.6*
Product B (verum)	29.51	22.75*	–22.9*
Untreated control	26.99	25.69	–4.8 (n.s.)

Before and after 4-week treatment with product A (vehicle), product B (verum with 2% Ectoin) versus untreated control. * $p < 0.05$ was regarded as statistically significant. n.s. = Not significant.

The untreated control showed only a significant decrease for scaling after 4 weeks, which might have been influenced by climatic changes during the study.

For the pre-post differences (week 4 – week 0) the comparison of product A (vehicle) and product B (verum) showed statistically significant differences in favour of product B (verum) for the parameters skin hydration and biological elasticity (R7).

For the pre-post differences (week 4 – week 0) the comparison of product B (verum) and the untreated control showed statistically significant differences in favour of product B (verum) for all measured parameters according to the Wilcoxon rank-sum test.

Discussion

Ectoin, a natural, vital substance was developed for the use in cosmetic applications. It was discovered in halophilic bacteria, which survive and grow under extreme conditions in salt lakes, sea water and saline deserts. These halophilic bacteria are exposed to a high dosage of UV irradiation, dryness, extreme temperatures and high salinity. They maintain cell stability by synthesizing Ectoin, a special, active substance belonging to the group of compatible solutes.

Efficacy studies in vitro demonstrated that Ectoin counteracts the effects of UVA-induced and -accelerated skin ageing at different cell levels. With the help of a UVA stress model, it was shown that Ectoin protects the skin from the effects of UVA-induced cell damage in a number of different ways [11]. The studies indicated that Ectoin has the potential to protect mitochondria of human fibroblasts in vitro against UVA-radiation-induced mutagenesis. The up-regulation of the expression of matrixmetalloproteinase-1 is followed by skin wrinkle formation. By employing different assay systems, the studies reported on here demonstrate that Ectoin could provide protection against undesired effects such as photo-ageing by UVA radiation on human skin cells in vitro [11, 28, 29].

The studies carried out confirmed that the natural cell protection concept of Ectoin is transferable to skin care with manifold benefits. In order to evaluate the effect of Ectoin on human skin, a cosmetic formulation containing 2% Ectoin was applied in the in vivo assessment as a randomized, vehicle-controlled clinical study. Special parameters which are related to skin ageing, like skin hydration, skin elasticity or skin surface structure, should be examined before and after a 4-week treatment with the Ectoin formulation. As this active ingredient is designed to be applied in high-class cosmetic products, a reference formulation (vehicle, product A) was chosen, which was not a simple basis formulation but also a high-quality skin care emulsion with valuable ingredients such as jojoba oil.

In a pre-test, the compatibility of both test formulations was confirmed, using a single patch test for 48 h under occlusive conditions.

Cosmetic acceptance, efficacy as well as skin compatibility were tested in a randomized 4-week in use test for each product. Here the subjective evaluation of the test panel showed a clear preference for the verum formulation with 2% Ectoin (treatment B, fig. 1, hatched bars). This is very remarkable, since the reference product was not a placebo substance but a high-quality skin care product and used as a vehicle in this study. Thus, the voluntary test subjects also ascribed subjectively experienced superior properties to the Ectoin emulsion. Furthermore, the skin compatibility of the test substance was very good. No side effects were observed, neither in the patch tests nor during the application tests. No dropouts were recorded during the whole study.

The efficacy testings of product B (with Ectoin) and product A (vehicle) versus untreated control showed a very good skin care effect of both formulations. However, it could be demonstrated that the 2% Ectoin formulation

was more effective in terms of skin hydration, skin elasticity and skin surface structure than the vehicle treatment. Statistical evaluations according to the Wilcoxon signed-rank test as well as the Wilcoxon rank-sum test indicate a general preference for the Ectoin treatment. Finally, prior in vitro studies could be confirmed by this clinical trial as far as the antiageing properties of Ectoin are concerned.

Acknowledgements

The authors thank Mrs. M. Wiebusch, Mrs. B. Garbe and Mrs. M. Herling for their assistance, Mrs. Dipl. Stat. A. Grieger for the statistical evaluation of the data and all the volunteers who made this investigation possible. We gratefully acknowledge Merck KGaA, Darmstadt, Germany, for providing the Ectoin.

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