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In Vivo Evidence of Ectoin's Enhanced Protective Effects on Skin Against Protein Carbonylation

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1. Introduction

The oxi-proteome is crucial in defining the features of aging. Its increase hampers the organism's ability to eliminate or repair damaged proteins and protect healthy ones through the chaperone system, being itself considered a reliable biomarker of aging. Carbonylated proteins are a key indicator of severe oxidative protein damage, particularly following UV stress, and their accumulation contributes to premature skin aging [1], [2], [3]. The skin is continuously challenged by internal and external exposome factors e.g. metabolic by-products, pollution and UV radiation—which drive extrinsic aging through sustained oxidative stress [4], [5].

Ectoin is a neutral nonionic, strong water-binding, organic molecule of low molecular weight occurring in halophilic bacteria. In their natural habitat, these microorganisms grow under extreme conditions such as intensive sun exposure, high temperatures and extreme dryness, and protect themselves against these harsh conditions by synthetizing Ectoin [6], [7]. Ectoin is a multi-talented ingredient, a natural cell protection factor providing immune system protection, complementary UV protection, super moisturizing and anti-aging properties, as well as protection from oxidative stress and pollution [8].

Previous studies have extensively investigated Ectoin's positive influence on the oxi-proteome and its role in the anti-aging process. Ectoin notably demonstrated strong protective effects against UVA, blue light, chemical, and temperature stress, subsequently reducing carbonylated proteins and protecting proteins from carbonylation in various *in-vitro* and *ex-vivo* models [9]-[11].

However, it remained unclear whether Ectoin's protective effect against protein carbonylation could be also observed on the skin, *in-vivo*, and to the best of our knowledge such results have not been reported in the scientific literature so far.

An *in-vivo* study was conducted to detect and quantify protein carbonylation levels collected from the outermost layers of the skin (*stratum corneum*) using a tape stripping technique and results are reported in this publication.

2. Materials and Methods

Ectoin was the active ingredient tested (INCI: Ectoin) and provided by Merck Electronics KGaA.
Placebo formulation: An o/w formulation was created and utilized without incorporating any active ingredients.
Formulation with the active ingredient: the same o/w formulation was used with the incorporation of 0.3% Ectoin.

In-vivo study and tape stripping for stratum corneum analysis

A 28-day double-blind and placebo controlled *in-vivo* study was undertaken. 31 volunteers applied the placebo and the formulation containing 0.3% Ectoin and were aged 50 to 70 (75% women / 25% men), with all types of skin, phototypes I to IV.
To establish a baseline concentration of carbonylated proteins, volunteers with lifestyles that predisposed them to higher carbonylated proteins levels were included. Thus 25% of smokers were included as well as 17% of volunteers with higher body max index (BMI) (>30). In addition, all volunteers had aging signs (wrinkles and fine lines, lack of firmness and elasticity). Test products were applied twice daily for 28 days.
The aim of this method was to collect cell samples on the skin surface by a non-invasive method via tape stripping [10]. The measuring area was in the face (both hemifaces - malar). Measurements were performed at day 0 and 28.

Carbonyl Score

Tape stripping samples were treated for protein extraction with Oxiproteomics' extraction buffer and optimized method. Then, the carbonylated proteins were labeled with a specific fluorescent probe (Ex= 647nm / Em=650 nm) and then separated by high resolution electrophoresis (SDS-PAGE - gradient 4-20%). After their migration, the total proteins were stained in-gel using fluorogenic SyproRuby™ reagent (Life Technologies, USA). The fluorescence images were collected by iBright system (Thermofisher Scientific, France) in the full range of intensity of signals. Densitometric image analyses were performed using "Image J" software (Rasband, WS, Image J, US National Institutes of Health, Bethesda, Maryland, USA).The quantification of protein carbonylation (carbonyl score) for each sample was obtained by normalizing the specific signal of carbonylation on the fluorescent signal of total proteins. The variations were then converted to fold-change values, where the negative inverse (-1/variation) is taken for

values between 0 and 1 while values greater than 1 are not affected. Statistics were carried out using the “GraphPad” software (La Jolla, California, USA) by using binary student’s t- test (Welch correction, unpaired, one-tailed) or t-test paired comparisons (one-tail), when possible.

***In-situ* detection and visualization of carbonylated proteins**

Fluorescent images were collected with an epi-fluorescent microscope from *in situ* (on corneocytes) labeling (EVOS M5000 Imaging System) using strictly the same acquisition time and resolution per series. The raw source images were collected including the complete range of fluorescence signal intensity (.TIFF 16bit format), then integrated using ImageJ software.

3. Results

The placebo group showed a significant increase observed on day 28, when compared to day 0 (+22%) indicating no effect on preservation of the modulation of carbonylation levels during this study. In contrast, the presence of 0.3% Ectoin in the formula protected the skin from this increase of overall carbonylation levels (-2% at day 28 vs day 0) suggesting its beneficial effect in preserving proteins from oxidative damage (Figure 1).

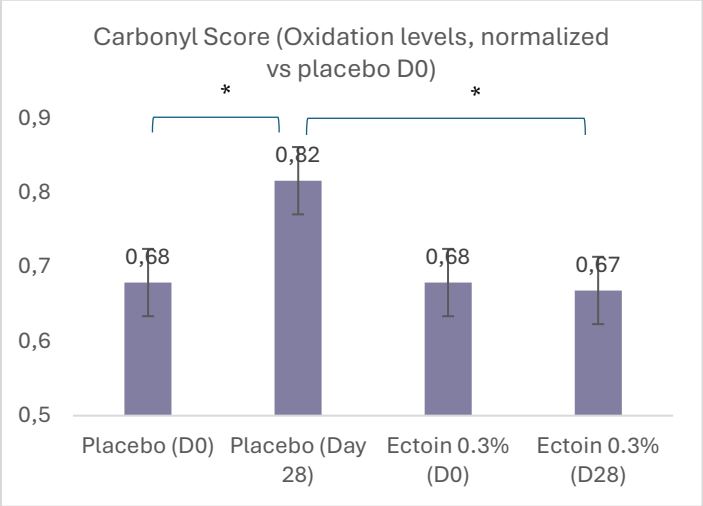

Figure 1: Carbonyl score: effects of placebo and formulation containing 0.3% Ectoin at day 0 and day 28. Comparisons normalized to placebo.

These results are corroborated by the proportion of volunteers presenting reduced carbonylation levels at day 28; it was remarkably higher in the case of the formulation containing 0.3% Ectoin (53%) vs placebo (23%). This confirms Ectoin’s protective effect against protein oxidation (Figure 2).

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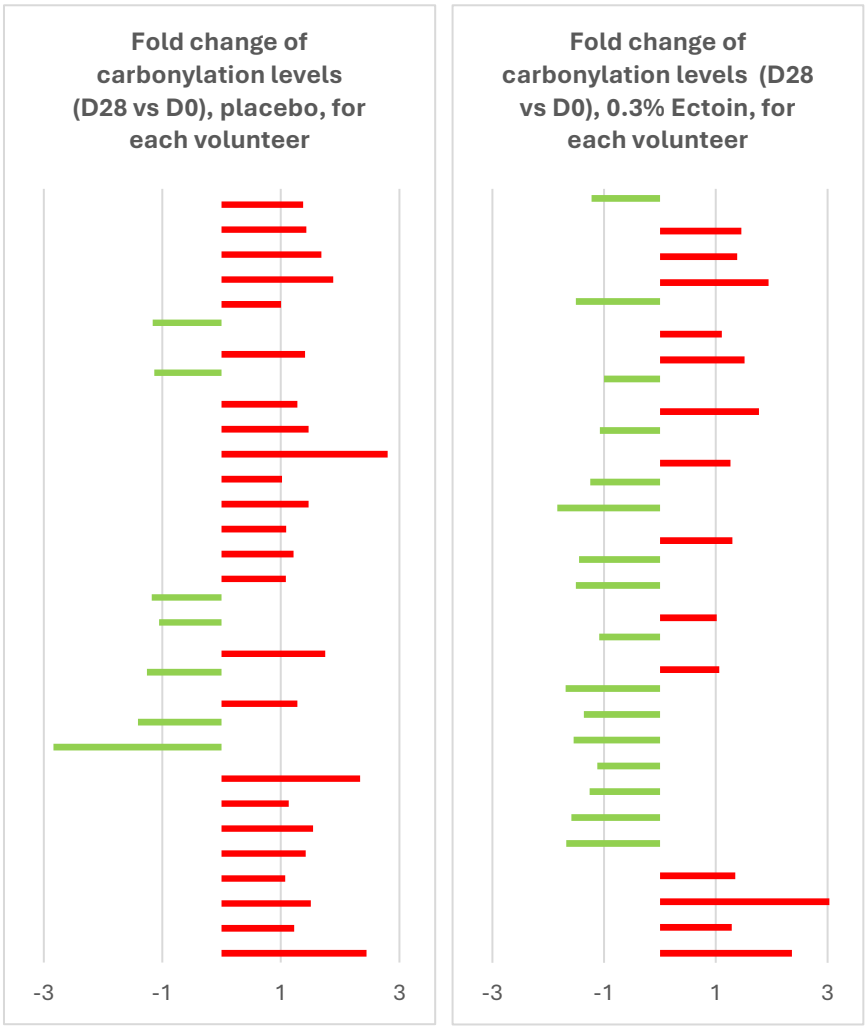


(a) *(b)*

Figure 2: Fold change of carbonylation levels day 28 vs day 0 for each volunteer having applied (a) placebo and (b) formulation containing 0.3% Ectoin. Results are presented as fold change value for each volunteer in green for reduction of carbonylation levels, associated to negative values of fold change and in red for increased of oxidation levels, associated with positive values of fold change.

To better highlight the performance differences between the 2 products, the carbonylation levels at day 28 were directly compared between treatments: the 0.3 % Ectoin formulation showed a significant reduction by 18% ($p<0.05$) in protein carbonylation compared to the placebo formula (Figure 3), and thus confirming the positive effects of Ectoin.

Finally, *in situ* detection (on corneocytes) of protein carbonylation was performed using fluorescence microscopy to visualize the differential effects of both treatments. As shown in Figure 4, the fluorescence intensity (in red) corresponding to carbonylated proteins was noticeably

lower in sampling from the same volunteer treated with the 0.3 % Ectoin formulation than in placebo-treated skin, at day 28. This is a visual confirmation of Ectoin's protective effect.

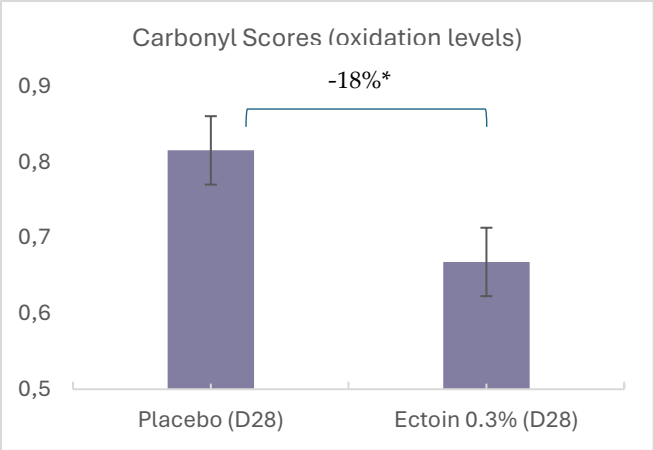
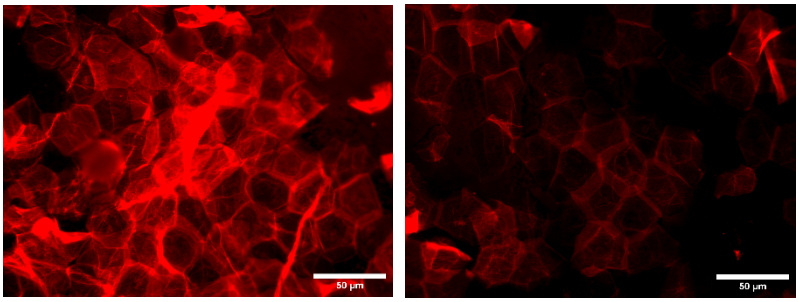
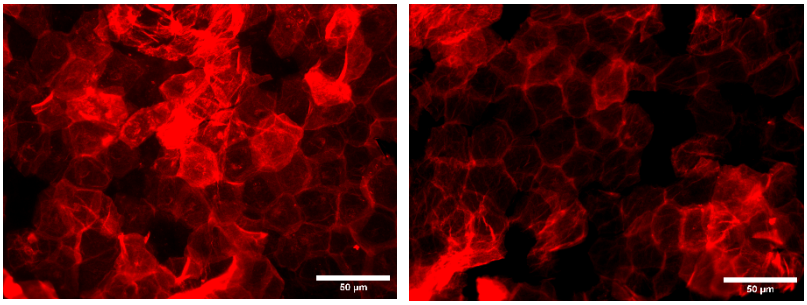


Figure 3 : Carbonyl score and comparison between the 2 test products: effect of formulation containing 0.3% Ectoin vs placebo at day 28 ($p<0.05$).



(a) *(b)*



(c) *(d)*

Figure 4: Illustrations of *in-situ* visualization of carbonylation levels at day 28. Volunteer 4A (60 years old woman) (a) having applied the placebo on left side (b) having applied on right side the formulation containing 0.3% Ectoin. Volunteer 3A (60 years old man) (c) having applied on right side the placebo

(d) having applied on left side the formulation containing 0.3% Ectoin. Oxidized protein signals on skin explants sections are visualized in red.

4. Discussion

A heterogeneous volunteer panel was recruited,including women and men, all skin types and phototypes, smokers and non-smokers, individuals with normal or elevated BMI and with visible signs of aging. Obesity-like conditions, lifestyle (leading to high BMIs) and smoking habits are linked with increased levels of carbonylated proteins [13], [14]. Such a heterogenous panel may represent a challenge to reach significancy, and yet, the results show a significant differential effect thanks to the presence of Ectoin.

These new results also confirm previous findings from *in-vitro* and *ex-vivo* models regarding the efficacy of Ectoin towards protein carbonylation upon UV-A and blue light exposure [9-11]. The current *in-vivo* study further validates that Ectoin is a highly effective skin care ingredient, yielding outcomes with use levels comparable to those observed in previous investigations, although based on *in-vitro* and *ex-vivo* models.

Moreover, the panel of volunteers encompassed a diverse range of skin types—dry, normal, oily, and combination—indicating that Ectoin effectively and significantly reduces carbonylated proteins *in-vivo* across all skin categories and conditions.

Longitudinal analysis (over the 28-day study) of protein carbonylation showed a progressive increase in the placebo group, whereas application of the 0.3 % Ectoin formulation prevented carbonylation levels to grow. In the conditions of the study, this increase of protein carbonylation with the time upon treatment with the placebo may be associated to environmental factors known to be linked to oxidative stress.

The comparison of the effects of the application of both products at day 28 showed a statistically significant benefit of the 0.3 % Ectoin formulation vs placebo, highlighting the efficacy of Ectoin in preventing protein carbonylation in the skin.

5. Conclusion

In this 28-day *in-vivo* study, topical application of a formulation containing 0.3% Ectoin effectively prevented the time-dependent rise in protein carbonylation observed in the placebo group and induced a statistically significant 18 % reduction in carbonylated proteins versus placebo at day 28 .

These results not only confirm previous *in-vitro* and *ex-vivo* evidence of Ectoin’s efficacy against UV- and blue-light-induced protein oxidation, but also establish its ability to preserve protein integrity under real-life environmental stressors.

Ectoin is consequently validated as a powerful ingredient that can help reduce oxidative damage and enhance long-term skin health These findings create thrilling possibilities for early actions against aging process.
Future studies may for instance integrate younger, more homogeneous cohort, to achieve greater statistical significance and but also to demonstrate benefits for younger populations, while also ultimately encouraging them to adopt efficient aging prevention strategies .

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