Targeted proteomics assessing proteome carbonylation as a reliable biomarker for dermo-cosmetics age management efficacy studies

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Abstract

Background: Accumulation of oxidatively damaged (carbonylated) proteins, is a hallmark of oxidative stress and accelerated skin aging. However, due to technical limitations for their assessment, the quantification of decreased carbonylated skin proteins has not been widely used as a read-out of efficacy in dermo-cosmetics *in vivo* studies.

Methods: An *in vivo* study on 23 subjects using a formulation containing an anti-carbonylation active ingredient compared to a placebo, double-blinded was performed. Protein carbonylation was assessed and biometrological analyses of brown/red spots and skin texture were evidenced using the VISIA positioning system upon 28 days of hemi-face application.

Results: Optimized and validated protocols for protein carbonylation assessment on stratum corneum stripped samples were developed. The beneficial effects of protein carbonylation inhibition were already observed upon 14 days of treatment. At D28 a significant decrease in protein carbonylation was observed when compared to DO. The placebo did not show any beneficial effect. In addition, protein carbonylation inhibition was positively correlated with a significantly decrease in brown spotting. An improvement of skin texture (smoothing effect) in more than 70% of the volunteers (12% on average) was also observed.

Conclusion: A reliable method for protein carbonylation assessment on stratum corneum was validated. These results underscore the prognostic and predictive value of carbonylated protein assessment for *in vivo* dermo-cosmetics efficacy evaluation on aging.

Keywords: Proteomics, Protein Carbonylation, stratum corneum, in vivo, efficacy testing

Introduction

Intrinsic processes, including chronic inflammation, hormonal changes, chronological aging, as well as extrinsic factors, such as chronic sun exposure and urban pollution, contribute to structural and functional deficiencies in the skin. Over time, extrinsic and intrinsic insults result in accumulation of molecular damage leading to wrinkles, fine lines, dryness, brown (age) spots and irregularities in tone and texture [1-4].

The deregulation of protein homeostasis and accumulation of oxidatively-damaged proteins (carbonylation) by reactive oxygen species (ROS) and different processes related to the formation of advanced glycation/lipid peroxidation end products (AGEs and ALEs) are hallmarks of the ageing process in different organs and tissues across different species [5,6]. In the skin, carbonylated proteins has been detected in a higher frequency at sun-exposed sites of the skin in elderly subjects [7]. In addition, previous studies have shown that protein carbonylation in the stratum corneum of skin is associated with decreased hydration such as a negative correlation with skin surface water content, a positive correlation with trans-epidermal water loss, and by reducing bound water [8,9]. Thus, it has been assumed that carbonylation of proteins in corneocytes decreases hydration in the skin.

During recent years, different studies have evidenced that the "Oxi-proteome" (the build-up of carbonylated proteins) during aging and upon oxidative stress is composed only by a limited group of proteins], indicating that not all proteins have the same propensity for accumulation as oxidatively damaged proteins [10,11]. This sub-set of oxidation-prone proteins includes those involved in key cellular functions, such as protein quality control and cellular metabolism [12]. The objective of this research study was to: (i) validate a novel targeted proteomics approach to assess protein carbonylation on the stratum corneum from tape strips, and (ii) to investigate whether absolute quantification of carbonylated proteins could be used during *in vivo* studies studies on volunteers not only to support an anti-oxidant/ proteome protection effect of formulated products, but also to evaluate its prognostic and predictive value by studying its association with known skin physical parameters, such as brown or red spots and skin texture.

Materials and Methods.

Products application. A formulation containing an anti-carbonylation/proteome protectant active ingredient (Product) and the same formulation without the active ingredient (Placebo), were topically hemi-face applied (double-blind), twice a day, for 28 days on 23 healthy volunteers (with their written consent): females, between 38 and 69 years old, phototype II to III, smokers with dull complexion. All the subjects received the same products references. *Stratum corneum sampling*. Skin samples from 23 subjects were removed with D-Squame tape strips (CuDerm Corporation, Dallas, TX, USA) on one defined zone on the cheekbone of each hemi-face at DO, D14 and D28 (two samples at each time point for each zone).

Protein carbonylation detection and quantification. Tape strips were independently processed and subjected to protein extraction using an optimized method for skin surface sampling. Carbonylated proteins were labeled with specific fluorescent probes [13] and resolved by electrophoresis (SDS-PAGE). A pool of samples was mixed and used as internal standard (IS) in all gels for inter-gel normalization of the fluorescence signal. Total proteins were stained in-gel with the SyproRuby™ fluorescent reagent (Life Technologies, USA). Digital image acquisition of carbonylated proteins and total proteins was performed using the "Ettan DIGE imager" system (GE Healthcare). Image processing and analysis were performed using the "ImageJ" software (Rasband, WS, Image J, US National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij /, 1997-2014). The absolute quantification of carbonylated proteins was established after normalization on to the total protein content and the internal standard.

Skin texture and color/homogeneity assessment. Analyses were performed using the Visia Camera System. On D0 and D28, one photograph of each hemi-face was taken under multi-spectral imaging and analysis (normal light, cross-polarized and UV light), then the analysis of skin texture, red and brown spots (for color/homogeneity state) were performed.

Results & Discussion

Decreased levels of protein carbonylation upon product application

Upon extraction from tape strips, carbonylated proteins were labeled with a specific fluorophore [13], and resolved onto 4-20% gradient SDS-PAGE. Proteins were fixed to the gel and carbonylated proteins were evidenced by fluorescence scanning at the emission wavelength of 650 nm. Total proteins were post-stained with SyproRuby^{TM} and the digital images of the gels were collected by differential fluorescence at 595 nm. The signal of the carbonylated proteins (fluorescence units) was normalized with respect to the signal obtained with the total proteins for each sample (Ratio) and finally with respect to the values obtained for the internal standard (IS) in each gel and the reference gel (gel 1) to obtain an absolute value of carbonylation (Carbonyl Score) for each sample. The Carbonyl Score values for each sample are presented in Figure 1 as independent and grouped distribution, as well as by bar graph representation per group. The statistical analysis of the data comparison per longitudinal follow-up (Day 28 versus Day 0 per each treatment) or per treatment (Product and Placebo) were performed by Student test (paired, two-sided).

The hemi-face of volunteers treated with the formulated active ingredient showed a statistically significant decrease in the oxidation (carbonylation) rate of 17% (p-value <0.05) at day 28 when compared to day 0. The placebo did not show a significant effect (Figure 1). Moreover, when compared to the placebo, the Product showed a statistically significant efficacy in decreasing protein carbonylation (at day 28). Based on paired comparison of single values per volunteer, 14 volunteers over 23 showed a more performant efficacy of the treatment A when compared to the product B.

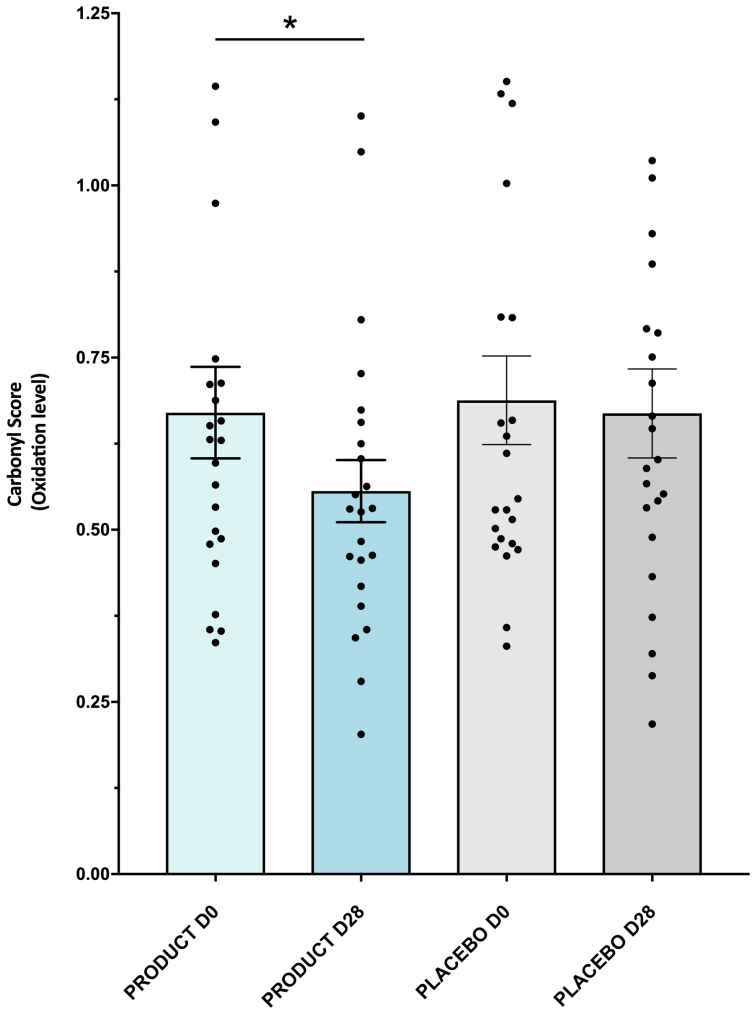

Figure 1. Protein carbonylation on stratum corneum. Quantification of carbonylated proteins and distribution of individual Carbonyl Score values (black dots) *per* experimental group (average values in graph bars with SEM).* *p*-value<0.05 t-test Student *per* experimental group of treatment (paired tests, one-tailed, alpha=0.05)

Decreased protein carbonylation is associated with increased skin texture and decreased number of brown spots.

Skin texture and color/homogeneity was assessed at D0 and D28 using the Visia system. After 28 days of twice daily use on the define hemi-face, the product containing the active ingredient induced a significant decrease of brown spots (9%, p = 0.0002) (Table 1). Less numerous brown spots was observed in 82% of the subjects on D28.

Table 1. Skin texture and color/homogeneity analyses

	Kinetics	Δ D28-D0 (mean ± SEM)	Δ% on the mean	P (T-test)	Subjects presenting an improvement (%)
Brown spots	28 days	-31.2 ± 7.1	-9%	0.0002	82%
Skin texture	28 days	-119.2 ± 57.0	-12%	0.0488	73%
Red spots	28 days	-3.5 ± 5.7	-2%	0.5434	45%

In addition, a significant decrease of skin texture parameter (-12%, p=0.0488) on D28 was observed, indicating a smoothing effect. Smoother skin was observed in 73% of the subjects on D28.However, although a tendency of decreased number of red spots was observed, it does not reach significancy (p> 0.005).

Protein carbonylation as an early event associated with skin smoothing and decreased brown spots.

In order to study the predictive and/or prognostic value of protein carbonylation on consumer benefits an intermediate point at D14 was analyzed. As detailed above the same procedure was used for protein carbonylation assessment. Indeed, the beneficial effects (inhibition of protein carbonylation) of a formulated anti-carbonylation compound was already detected upon only 14 days of topical application on 61% of volunteers. Among them, 70% of subjects showed an increase in skin texture at D28, 80% decreased brown spots, and 60 % decreased red spots.

Conclusions.

In this study, a reliable method for protein carbonylation assessment on stratum corneum has been developed and validated. This method can be used as an early read-out of efficacy in aging studies, since the beneficial effects are already observed upon 14 days of treatment. In addition, the benefits to be gained from increased protection or the stimulation of carbonylated proteins elimination were evidenced by the amelioration of parameters related to skin appearance. Taken together, these results underscore the prognostic and predictive value of carbonylated protein assessment for *in vivo* dermo-cosmetics efficacy evaluation on aging.

Conflict of Interest Statement. NONE.

References

1. Krutmann J, Schikowski T, Morita A, et al (2021) Environmentally-Induced (Extrinsic) Skin Aging: Exposomal Factors and Underlying Mechanisms. Journal of Investigative Dermatology 141:1096-1103.

2. Parrado C, Mercado-Saenz S, Perez-Davo A, et al (2019) Environmental Stressors on Skin Aging. Mechanistic Insights. Frontiers in Pharmacology 10: DOI=10.3389/fphar.2019.00759.

3. Pillai S, Oresajo C, Hayward J. (2005) Ultraviolet radiation and skin aging: roles of reactive oxygen species, inflammation and protease activation, and strategies for prevention of inflammation-induced matrix degradation – a review. International Journal of Cosmetic Science 27(1): 17-34.

4. Ansary TM, Hossain MR, Kamiya K, et al (2021) Inflammatory Molecules Associated with Ultraviolet Radiation-Mediated Skin Aging. International Journal of Molecular Sciences 22, no. 8: 3974. https://doi.org/10.3390/ijms22083974.

5. Stadtman ER, Levine RL (2003). Free radical-mediated oxidation of free amino acids and amino acid residues in proteins. Amino Acids 25, 207–218.

6. Nyström T (2005). Role of oxidative carbonylation in protein quality control and senescence. The EMBO Journal 24:1311-1317.

7. Zucchi H, Pageon H, Asselineau D et al (2022). Assessing the Role of Carbonyl Adducts, Particularly Malondialdehyde Adducts, in the Development of Dermis Yellowing Occurring during Skin Photoaging. Life (Basel) 10;12(3):403.

8. Masaki H, Yamashita Y, Kyotani D et al (2019). Correlations between skin hydration parameters and corneocyte-derived parameters to characterize skin conditions. J Cosmet Dermatol. 18(1):308-314.

9. Yamawaki Y, Mizutani T, Okano Y (2019). The impact of carbonylated proteins on the skin and potential agents to block their effects. Exp Dermatol. 28 Suppl 1:32-37.

10. Le Boulch M, Ahmed EK, Rogowska-Wrzesinska A et al (2018). Proteome oxidative carbonylation during oxidative stress-induced premature senescence of WI-38 human fibroblasts. Mech Ageing Dev.170:59-71.

11. Baraibar MA, Hyzewicz J, Rogowska-Wrzesinska A et al (2016). Impaired energy metabolism of senescent muscle satellite cells is associated with oxidative modifications of glycolytic enzymes. Aging (Albany NY) 8(12):3375-3389.

12. Baraibar MA, Ladouce R, Friguet B (2013). Proteomic quantification and identification of carbonylated proteins upon oxidative stress and during cellular aging. J Proteomics 30;92:63-70.