IFSCC INTERNATIONAL FEDERATION OF SOCIETIES OF COSMETIC CHEMISTS

Increased Keratin Carbonylation Associated with Decreased Fingernail Strength

Andrea Cavagnino[1], Anaïs Bobier[1], Arthur Starck[1], Loïc Pentecouteau[2], Pierre-Yves Morvan[2], Martin Baraibar[1]
[1] OxiProteomics SAS, Paris, France
[2] CODIF International SAS, Saint-Malo, France

Keywords: Nail care, protein carbonylation, keratin oxidation, exposome, anti oxidants

Some of the data of this paper were presented as a poster presentation at the 25th IFSCC Conference, Milan, Italy, September 30 - October 2, 2019.

INTRODUCTION

Fingernails have both an aesthetic and protective function on fingertips, preventing injury of the distal phalanx and surrounding soft tissues. Nail plate brittleness (or fragility) is a common complaint affecting up to 20 % of the population, especially women over 50 years old, with fingernail fragility being more prevalent than toenail fragility [1]. Nail brittleness is characterized by nails that split, flake and crumble, becoming soft and losing elasticity. Nails are composed essentially of a hard, flat, and roughly rectangular nail plate, which is closely connected to the nail bed. During nail plate formation, keratinocytes of the germinal matrix fare flattened due to the fragmentation of cell nuclei and condensation of cytoplasm to form flat, keratinous cells whose cell borders are retained (in contrast to hair) [1]. Nail health results from internal factors (e.g. age, genetic, and nutritional) influencing the nail characteristics when produced by the germinal matrix (matrix unguis) and external factors (environmental stressors) influencing the nail structure and aspect.

At the molecular level, differentiation-associated keratin intermediate filaments form a complex scaffold accumulating in the cytoplasm of terminal differentiated corneocytes and, upon removal of cell organelles, fill the entire cell interior to mainly provide the mechanical strength of nails. Keratins and keratin-associated proteins are structurally organized in overlapping layers by a crosslinking process providing great cohesiveness to the overall nail plate structure. Crosslinked keratin filaments influence the characteristic proprieties of the nail plate, such as stability and resistance. Previous proteomics analyses of nail and hair described a cluster of overlapping proteins, some of them only present in the nail plate (keratins K5, K6A, K14, K17 and junctional proteins DSG1, EPPK1) [2, 3]. Keratins are the fundamental building block components of nails able to self-assemble into filaments. The amino acid sequence on distinct keratins determines various functions in the assembly process, in inter-keratin binding or cross-linking with other proteins. The keratin secondary structure consists of an N-terminal head domain, a central α-helical rod domain and C-terminal tail domain. The tertiary structure is formed

Abstract

Protein carbonylation, a harmful oxidative protein modification, is considered a major hallmark and reliable biomarker of oxidative stress. Fingernails are composed of compact layers of keratinized epithelial cells assuring nail plate strength resulting from its proteinaceous nature, particularly its high content in α-keratins. However, nail keratins are potential targets of detrimental modifications, such as carbonylation, due to their high level of exposure to environmental stressors (pollution, irradiation, chemical reagents), which could explain, at least in part, the molecular basis of physicochemical modifications leading to weaker and brittle nails. To address the molecular basis of nail damage, we firstly developed ex vivo experimental models of human fingernails subjected to the most relevant environmental stressors. Decreased fingernail mechanical strength was observed upon stress exposure; validating our experimental models. Targeted proteomics approaches for evidencing and quantifying carbonylated proteins coupled to image analyses were performed. Chemical or physical stress inflicted oxidative damage on corneocyte structural proteins. A significant increase in protein oxidation (carbonylation) was observed in nail keratins upon UV irradiation or exposure to chemical aggressors. Interestingly, oxidative protein damage was prevented in the presence of antioxidant compounds. Since nails cannot be repaired once damaged, the protection of nail keratins from carbonylation could be an efficient approach for protecting nails against environmentally induced oxidative stress.

by long, coiled heterodimers, aligned in parallel, determining the first building block of the filaments that can generate more complex quaternary structures (i.e. tetramers, octamers) [4].

Attacks on nails from external factors are associated with unaesthetic changes in nail appearance as a consequence of physical and chemical changes of nail structure resulting in weakness and brittleness, discoloration, and formation of striates (Mees lines), among others [5, 6]. Since nail structure (keratins) cannot be repaired and complete nail regeneration takes several weeks, performing nail care products are in high demand by cosmetics consumers to preserve nail strength and function. Although nail care claims are represented more and more in the market, the antioxidant protection of nail proteins is not yet a read-out of choice for efficacy testing due to the absence of sensible and reliable tools for analysis. Traditional methods, such as spectroscopic and microscopic imaging or physical characteristics tests (e.g., strength, color, and water loss) have been used to study photo- and chemical-induced damages. These methods are now mainly used for *in vivo* testing on the whole nail and, eventually, for protective efficacy testing of novel nail-care products [7-13]. However, these approaches are not specific and only a few of them have been used in systemic investigations.

Accumulation of damaged proteins is a hallmark of oxidative stress and cellular and organism aging. This accumulation has been viewed as the combined result of increased production of reactive oxygen species (ROS) and other toxic compounds coming from cellular metabolism and external factors, i.e. UV irradiation and chemicals exposure, as well as the failure of protein maintenance systems (i.e. degradation and repair) with age. In particular, protein carbonylation, an oxidative post-translational modification associated with structural damage, is very detrimental, as the resulting damage can render targeted carbonylated proteins inactive and lead to cellular functional abnormalities [14, 15]. Protein carbonylation is induced either directly by ROS or indirectly by reactions with secondary products of oxidative stress, such as reactive aldehydes as 4-hydroxy-2-nonenal (4-HNE) and malondialdehyde (MDA). These aldehydes are produced by the peroxidation of polyunsaturated fatty acids of membrane lipids and can react with protein to form covalent Michael adducts with the side chains of cysteine, histidine and lysine residues [16]. Proteins can also be carbonylated through the reaction of arginine and lysine side chains with reducing sugars or reactive dicarbonyl compounds, such as glyoxal and methylglyoxal, based on the Maillard reaction [17]. Formation of these lipid peroxidation and glycation adducts is found in many tissues and believed to contribute to aging and a variety of age-associated diseases. Importantly, the nail plate primarily consists of a highly cross-linked keratin network that results in a unique structure, leading to a highly effective physical barrier [2, 18]. The nail's physicochemical properties are the direct result of the arrangement of its structural elements and most significantly the proteins that constitute 90 % of the total nail plate mass [1]. Keratins being the main component of nail plates, protein carbonylation could be a reliable read-out for evaluating nail structural damage.

EXPERIMENTAL

Fingernail preparation

Clipped free-edge fingernails that were free of chemical treatment or visual physical damage were obtained from healthy donors aged 25 to 35 years old. The clippings were washed for 1 hour with 0.1 % of Triton X-100 (Sigma-Aldrich, St. Quentin Fallavier, France), then rinsed with water and dried naturally in ambient conditions, and finally stored in a dark environment at room temperature until analysis.

UV irradiation

Clipped nail plates were placed in a small petri dish and exposed to UVA radiation (365 nm) in an OxiProteomics irradiation chamber at 4 doses (dose 1: 6.8 J/cm^2, dose 2 : 13.6 J/cm^2, dose 3: 27.2 J/cm^2, dose 4: 54.4 J/cm^2).

Incubation with hydrogen peroxide or troclosene sodium

Clipped nail plates were incubated with a solution containing 30 % H_2O_2 (Sigma-Aldrich) in 0.1 M Tris-HCl pH 8.6 for 20, 30 and 60 minutes or in a solution of troclosene sodium (a compound commonly present in house-cleaning products in France) for 30 or 60 minutes.

Antioxidant treatment

Clipped nail plates were incubated with agitation in a solution containing 0.5 % N-acetylcysteine (Sigma-Aldrich) and 50 µM Trolox (a hydrophilic analogue of vitamin E; Sigma-Aldrich) for 1 hour prior to stress exposures.

Nail Strength

This biomechanical property was evaluated by applying a punctual compression to the dorsal face of clipped nails (Nail Meter NM 100, Monaderm, Monaco) and recording their resistance to the compression force. The slope of the linear regression curve (applied force (N) *versus* the tip displacement (mm)) indicates the structural firmness of the nail resulting in the structural strength parameter (S.S.). The average value per experimental condition resulted from four biological replicates. In the analysis, an identical force of 5 N was applied to the different nail plates.

In situ detection of carbonylated proteins

After UVA exposure, the irradiated clipped nail plates were rehydrated in water for 10 minutes and the excess water was absorbed on a paper towel. After chemical stress exposure or nail polish application, the nail plates were directly processed and placed in a cryopreserving solution and flash frozen. Nail slices of 3 µm thickness were generated using a cryostat (Leica Biosystems, Nanterre, France). Then carbonylated proteins were labeled with a specific fluorescent probe *in situ* in nail plate sections. Nail images were collected by Epi-Fluorescence Microscopy (DMi8 – 63X or 5x objectives, Leica Biosystems) and processed using ImageJ software [19].

Identical conditions, i.e., acquisition time, exposure, focus and resolution, were used for all experimental groups.

Carbonylated proteins analysis

Nail proteins were extracted from 5 mg of nail samples by shaking in an extraction buffer containing chaotropic agent, detergents and reductants. The extracts were clarified by centrifugation. The concentration of proteins was determined with the Bradford method using calibrated BSA (Sigma-Aldrich) as the standard [20]. Carbonylated proteins were labeled using functionalized fluorescence probes specific for carbonyl groups, and proteins were resolved by high-resolution electrophoresis. Total proteins were post-stained with SyproRubyTM (Invitrogen, Fisher Scientific, Illkirch, France) protein gel stain. Image acquisition for carbonylated and total proteins was performed using the Ettan® DIGE imager (GE Healthcare, Buc, France). Image processing and densitometric analysis of protein bands were performed using ImageJ [19]. Statistical analyses were performed with GraphPad Software (La Jolla, San Diego, California, USA). The carbonylated protein signal was normalized to the total protein signal for each sample in order to obtain the carbonyl score.

RESULTS AND DISCUSSION

Decreased nail resistance on exposure to environmental stressors

Ex vivo models of human nails have been developed to study the impact of daily stressors. The impact of UV radiation or household products was first evaluated by assessing the structural strength of nails when stressed, which is related to the structural conformation of the free edge of the nail plate. Resistance of the nail to a compression force was evaluated by inducing a punctual compression on the dorsal face and measuring the force applied (N) and the modifications in nail thickness (mm) using a nail meter. Combination of the two measurements results in the structural strength (SS) of the nail. As depicted in ***Figure 1***, the structural strength decreases upon nail exposure to UVA radiation, H_2O_2 or troclosene, indicating a reduced strength of nail plates upon stress.

Increased carbonylation of nails keratins with stress

Although previous proteomics studies [2] investigated the nail plate protein composition, post-translational modifications of the nail proteome have not been studied yet. We focused our attention on the oxidative modifications of nail components using *in situ* detection of carbonylation levels. Due to its irreversible nature, carbonylation could be able to induce structural changes in proteins that have an impact on nail strength. Carbonyl moieties in nails were detected and visualized by epifluorescence optical microscopy after labeling carbonylated proteins with specific fluorescent probes.

The carbonylation-specific signal increases in the presence of environmental stressors (***Figure 2***, from black for low intensity levels to white for maximum intensity), showing differences in nail oxidation patterns as a function of the type of stress exposure. The nail plate is characterized by the superposition of three compartments:

- a dorsal dense and hard outer layer of keratin composed of few cornified cells resulting in a smooth surface;
- an intermediate layer constituting about 75 % of the entire nail's thickness that is composed of fibrillar structures mainly oriented in the direction perpendicular to that of nail growth; and
- a ventral layer comprised of a few layers of cells, which is very thin and was previously implicated in connection with the nail bed. The negative effects of H_2O_2 and troclosene sodium can be seen especially in the external layers of the nail, while UVA irradiation and nail polish treatment induce carbonylation in deeper layers, including the intermediate layer. This approach highlighted distinct effects and impacts on nail structure depending on the type of stressors. This evidence of specific oxidative effects on nail molecular structures prompted us to evaluate the changes in protein carbonylation using in gel detection and quantification.

UV irradiation induces protein carbonylation in a dose-dependent manner

Nail damage is not usually studied at the molecular level but by evaluation of physicochemical properties of the nail plate. These properties are determined with methods applied principally to the whole nail in *in vivo* studies but these tests are neither sensitive nor specific. Keratin oxidation was previously studied only in hair, but not in nails, in the sulfur-containing

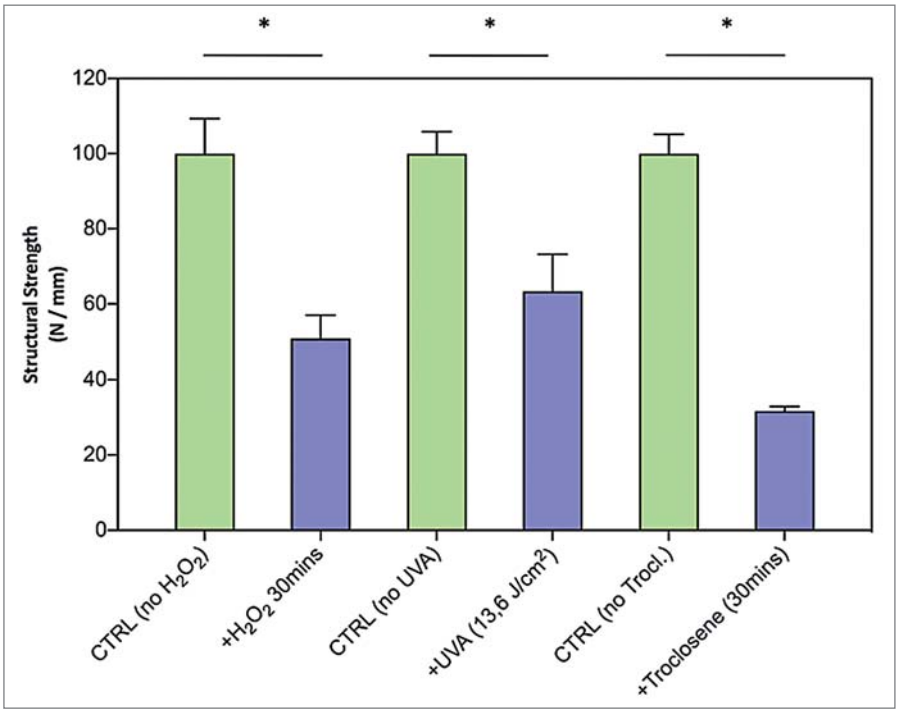

Figure 1 Structural strength (SS) changes upon exposure of nails to stress. Quantification of the structural strength of nail plates by experimental group after scaling of the control value (not exposed) to 100 % of strength. Bars represent the mean of four replicates ± SD. Statistical analyses were performed using a two way Student's t-test: *$p < 0.05$.

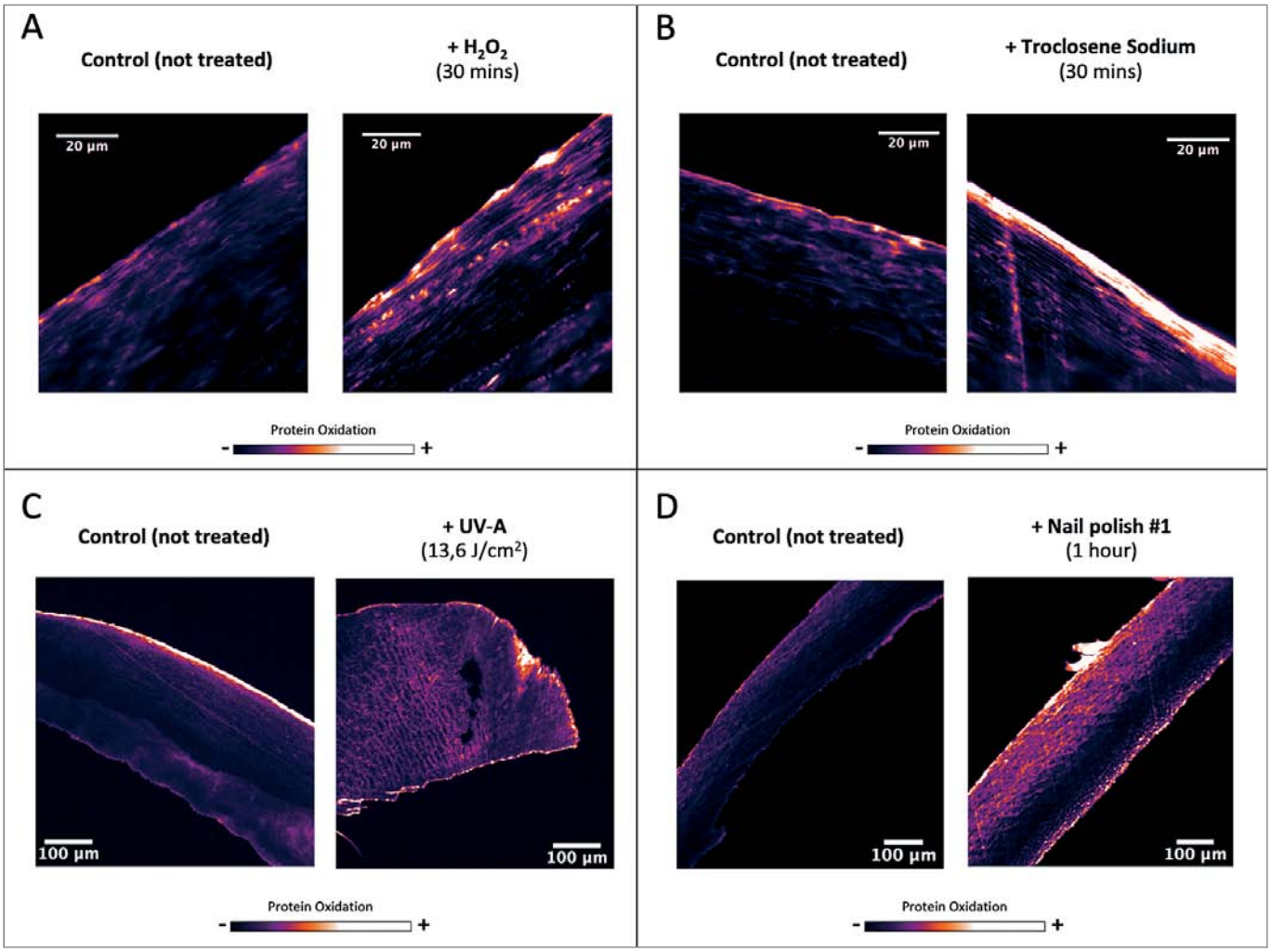

Figure 2 *In situ* visualization of carbonylated proteins upon stress exposure. Carbonylated proteins were labeled *in situ* on nail plate slices (3 µm thickness) with specific fluorescent probes and analyzed by epi-fluorescence microscopy. The fluorescence signal is represented as a continuous histogram of intensity (maximum oxidation level in white and low levels in black). Images were taken with a 630X (A, B) or 50X zoom (C, D).

amino acids methionine and cysteine by labeling their SH group with probes, such as N-(9-acridinyl)-maleimide and N-(7-dimethyl-amino-4-methylcoumarinyl)-maleimide. However, during oxidation the SH group and the S-S bond are changed to cysteic acid (SO_3H), which results in protein damage. As cysteic acid does not react with the above-mentioned dyes, it is assumed that the damage level estimation obtained with these approaches using the SH group as a biomarker for hair damage does not accurately reflect reality [21].

In this study, we developed proteomic approaches for nail damage evaluation based on detection and quantification of carbonylated proteins in nail plates using differential in gel electrophoresis. In addition, we developed a stress model

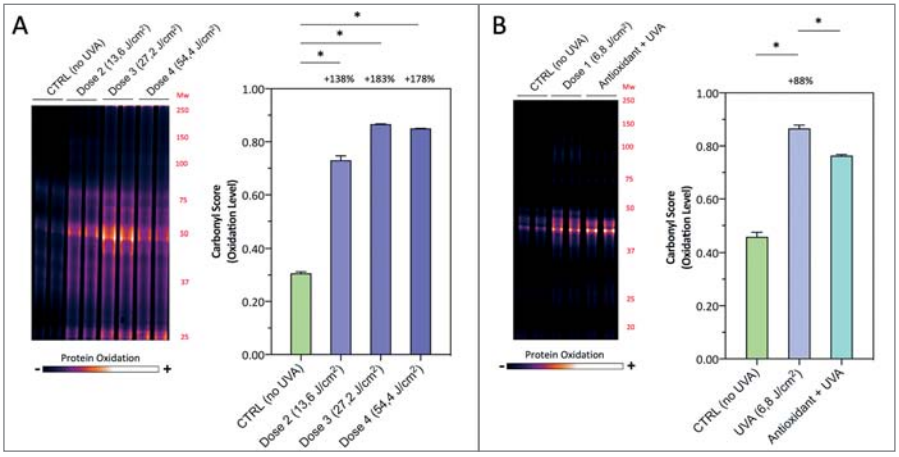

Figure 3 Carbonyl score for different doses of UVA irradiation and antioxidant protection evaluation: Left panels: carbonylated proteins on each sample without normalization relative to the total protein signal represented as a continuous intensity histogram. The maximum intensity levels are in white and lower levels in deep purple/black. Right panels: Quantification of carbonylated proteins by experimental group after normalization by total protein signal; bars represent the mean of three replicates ± SD, statistical analysis (one way ANOVA): *: $p<0.05$.

that is compatible with testing on *ex vivo* (clipped) nail plates.

Previous studies have shown irreversible oxidative damage in hair shafts [22, 23] but so far no evidence has been published about the effects on nail plates. As depicted in ***Figure 3A***, a protein band between 37 kDa and 50 kDa showed high levels of carbonylation on stress exposure. In addition, the level of protein carbonylation increased significantly in a dose-dependent manner (up to 20 J/cm^2) after UV irradiation, which confirms the specificity of the signal. Interestingly, protein carbonylation induced by stressors was prevented in the presence of antioxidants, indicating that carbonylation is mediated by free radical attack. The incubation of nail plates with N-acetylcysteine and trolox, a vitamin E analogue, before stress reduced the levels of carbonylation ***(Figure 3B)***.

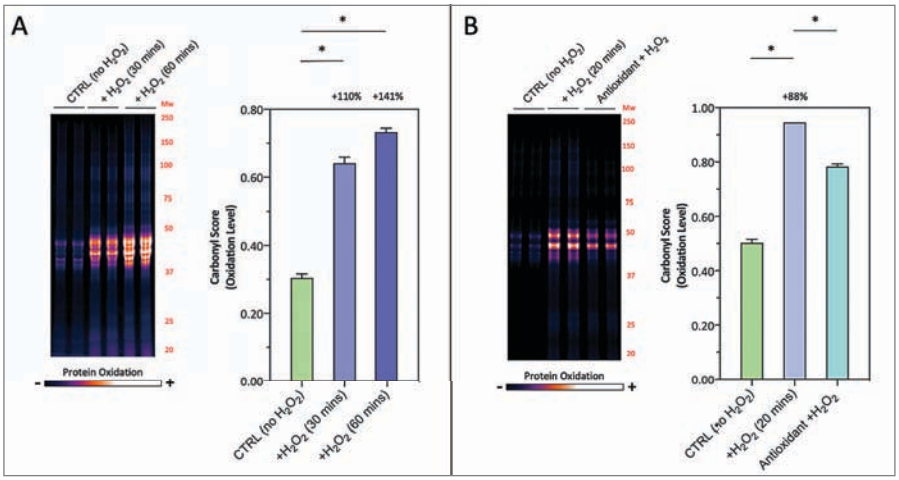

Figure 4 H_20_2-induced carbonylation of nail proteins with different times of contact (A) and with antioxidant N-acetylcysteine + Trolox treatment. (B): Left panels: Carbonylated proteins in each sample without normalization by total protein signal represented as a continuous intensity histogram. Maximum intensity levels are in white and lower levels in deep purple/black. Right panels: Quantification of carbonylated proteins by experimental group after normalization by total protein signal; bars represent the mean of three replicates ± SD, statistical analysis (one way ANOVA): *: $p<0.05$.

Protein oxidation induced by chemical treatments (H_20_2 and troclosene sodium)

Nail plate treatment with hydrogen peroxide is often used to enhance ungual chemical penetration of topically applied drugs overcoming the nail barrier [12, 13]. Troclosene sodium is the salt of a chlorinated hydroxytriazine and is used as a source of free available chlorine in the form of hypochlorous acid for the disinfection of water or commonly present in commercially available house-cleaning products. Also, it is widely used as a stable source of chlorine for the disinfection of swimming pools [24]. Both hydrogen peroxide and troclosene sodium are well-known oxidants.

In this study we developed *ex vivo* models from clipped fingernail plates for the evaluation of nail damage using the detection and quantification of carbonylated proteins as a read-out. We used these models to confirm and validate the relevance of protein carbonylation as a nail damage biomarker upon contact with chemical oxidants. As depicted in ***Figure 4A*** and ***Figure 5A***, alpha keratins were carbonylated upon treatment with H_2O_2 and troclosene, respectively. The level of nail carbonylation increased significantly in a dose-dependent manner with the time of contact with the compounds, confirming the pertinence of this readout for the quantification of nail damage. In addition, pre- and co-treatment of the nail plate with N-acetylcysteine and trolox, a vitamin E analogue, resulted in protection of proteins from carbonylation (***Figure 4B*** and ***Figure 5B***).

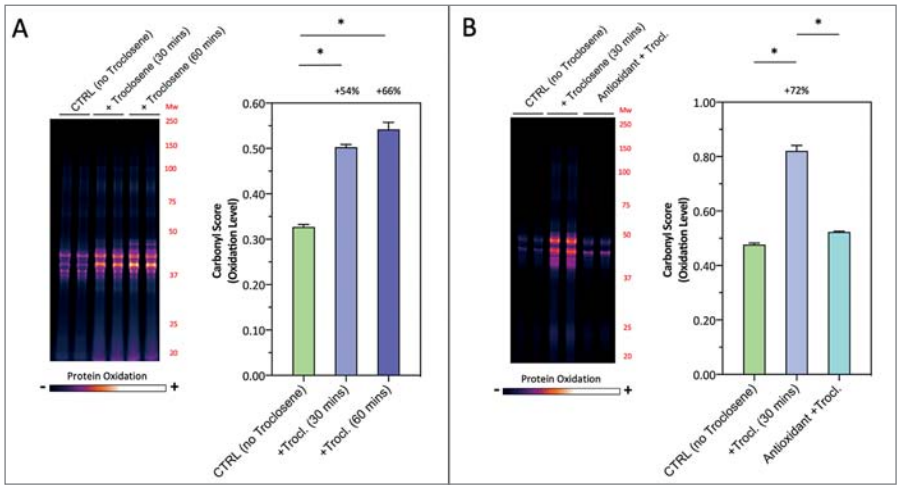

Figure 5 Troclosene-induced carbonylation of nail proteins with increasing times of contact (A) and with antioxidant protection by N-acetylcysteine (NAC) treatment (B). Left panels: Carbonylated proteins in each sample without normalization by total protein signal represented as a continuous intensity histogram. The maximum intensity levels are in white and lower levels in deep purple/black.
Right panels:Quantification of carbonylated proteins by experimental group after normalization by total protein signal. Bars represent the mean of three replicates ± SD, statistical analysis (one way ANOVA): *: $p<0.05$.

CONCLUSIONS

In this study we developed novel *ex vivo* models of stress on nail plates and validated protein carbonylation as a specific biomarker of nail protein oxidative damage. This study suggests that prevention of protein carbonylation could be an efficient approach for nailcare product development and be used as a readout for nail protection evaluation and claims substantiation.

References

[1] *Gupchup G.V., and Zatz J.L.,* Structural characteristics and permeability properties of the human nail: A review, Journal of Cosmetic Science, **50** (1999), 363-385.

[2] *Rice R.H. Xia Y., Alvarado R.J., and Phinney B.S.,* Proteomic analysis of human nail plate, Journal of proteome research, **9** (2010,) 6752-6758.

[3] *Rice R.H.,* Proteomic Analysis of Hair Shaft and Nail Plate. J Cosmet Sci. **62**(2) (2011), 229-236.

[4] *Bragulla H.H., and Homberger D.G.,* Structure and functions of keratin proteins in simple, stratified, keratinized and cornified epithelia., J. Anat., **214** (2009), 516-559.

[5] *Maddy A.J., and Tosti A.,* What's New in Nail Disorders, Dermatologic Clinics, **37**(2) (2019), 143-147.

[6] *Iorizzo M., Piraccini B.M., and Tosti A.,* Back to Basics Nail cosmetics in nail disorders, Journal of Cosmetic Dermatology, **6**(1) (2007) 53–58.

[7] *Chen A.F., Chimento S.M., Hu S., Sanchez M., Zaiac M., and Tosti A.,* Nail damage from gel polish manicure, Journal of Cosmetic Dermatology, **11**(1) (2012) 27-29.

[8] *Romaszkiewicz A., Sławiska M., Sobjanek M., and Nowicki R.J.,* Nail dermoscopy (onychoscopy) is useful in diagnosis and treatment follow-up of the nail mixed infection caused by Pseudomonas aeruginosa and Candida albicans. Postepy. Dermatol. Alergol., **35**(3) (2018), 327-329.

[9] *Pinteala T., Chiriac A.E., Rosca I., Larese Filon F., Pinteala M., Chiriac A., Podoleanu C., Stolnicu S., Coros M.F., and Coroaba A.,* Nail Damage (Severe Onychodystrophy) Induced by Acrylate Glue: Scanning Electron Microscopy and Energy Dispersive X-Ray Investigations, Skin Appendage Disord, **2**(3-4) (2017), 137-142.

[10] Wortsman X., and Jemec G.B.E., Ultrasound Imaging of Nails, Dermatologic Clinics, **24**(3) (2006), 323-328.

[11] *Batory M., Namieci P., and Rotsztejn H.,* Evaluation of structural damage and pH of nail plates of hands after applying different methods of decorating, Int. J. Dermatol., **58**(3) (2019), 311-318.

[12] *Khengar R.H., Brown M.B., Turner R.B., Traynor M.J., Holt K.B., and Jones S.A.,* Free radical facilitated damage of ungual keratin, Free Radical Biology and Medicine, **49**(5) (2010), 865-871.

[13] *Brown M.B., Khengar R.H., Turner R.B., Forbes B., Traynor M.J., Evans C.R.G., and Jones S.A.,* Overcoming the nail barrier: A systematic investigation of ungual chemical penetration enhancement, Int. J. Pharm. **370**(1-2) (2009), 6167.

[14] *Suzuki Y.J., Carini M., and Butterfield D.A.,* Protein Carbonylation, Antioxid. Redox Signal. **12** (201), 323-325.

[15] *Baraibar M.A., Ladouce R., and Friguet B.,* Proteomic quantification and identification of carbonylated proteins upon oxidative stress and during cellular aging, J. Proteomics, **92** (2013), 63-70.

[16] *Esterbauer H., Schaur R.J., and Zollner H.,* Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes, Free Radic. Biol. Med., **11** (1991), 81-128.

[17] *Thornalley P.J., Battah S., Ahmed N., Karachalias N., Agalou S., Babaei-Jadidi R., and Dawnay A.,* Quantitative screening of advanced glycation endproducts in cellular and extracellular proteins by tandem mass spectrometry, Biochem. J., **375** (2003), 581-592.

[18] *De Berker D., Wojnarowska F., Sviland L., Westgate G.E., Dawber R.P., and Leigh I.M.,* Keratin expression in the normal nail unit: markers of regional differentiation, Br. J. Dermatol., **142** (2002), 89-96.

[19] *Schneider C.A., Rasband W.S. and Eliceiri, K.W.,* NIH Image to ImageJ: 25 years of image analysis, Nat. Methods, **9** (2012) 671-675.

[20] *Bradford M.M.,* A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem., **72** (1976), 248-254.

[21] *Fujita H., Kubo S., Yasuda M., Hirao T., and Kawasoe T.,* Method for evaluating hair damage using an oxidized protein in hair as an indicator, U.S. Patent No. US20070110670A1 - Shiseido Co. Ltd. - (2004).

[22] *Grosvenor A.J., Marsh J., Thomas A., Vernon J.A., Harland D.P., Clerens S., and Dyer J.M.,* Oxidative Modification in Human Hair: The Effect of the Levels of Cu (II) Ions, UV Exposure and Hair Pigmentation, Photochem. Photobiol., **92** (2016) 144-149.

[23] *Trüeb R.M.,* The impact of oxidative stress on hair, Int. J. Cosmet. Sci., **37** (2015) 25-30.

[24] Troclosene Sodium, European Chemicals Agency (ECHA) available from: https://echa.europa.eu/fr/substance-information/-/substanceinfo/100.018.880

Corresponding Authors
Martin Baraibar
OxiProteomics SAS
Paris
France
martin.baraibar@oxiproteomics.fr