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Alpha-Tocopherol Protects Human Dermal Fibroblasts by Modulating Nitric Oxide Release, Mitochondrial Function, Redox Status, and Inflammation

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Keywords
α-Tocopherol · Nitric oxide release · Oxidative stress · Mitochondrial function · Reactive oxygen species · Photoaging

Abstract

Background: The altered balance between oxidants/antioxidants and inflammation, changes in nitric oxide (NO) release, and mitochondrial function have a role in skin aging through fibroblast modulation. Tocopherol is promising in counteracting the abovementioned events, but the effective mechanism of action needs to be clarified. ***Objective:*** The aim of this study was to examine the effects of α-tocopherol on cell viability/proliferation, NO release, mitochondrial function, oxidants/antioxidants, and inflammation in human dermal fibroblasts (HDF) subjected to oxidative stress. ***Methods:*** HDF were treated with H_2O_2 in the presence or absence of 1–10 μM α-tocopherol. Cell viability, reactive oxygen species (ROS), NO release, and mitochondrial membrane potential were measured; glutathione (GSH), superoxide dismutase (SOD)-1 and -2, glutathione peroxidase-1 (GPX-1), inducible NO synthase (iNOS), and Ki-67 were evaluated by RT-PCR and immunofluorescence; cell cycle was analyzed using FACS. Pro- and anti-inflammatory cytokine gene expression was analyzed through qRT-PCR. ***Results:*** α-Tocopherol counteracts H_2O_2, although it remains unclear whether this effect is dose dependent. Improvement of cell viability, mitochondrial membrane potential, Ki-67 expression, and G0/G1 and G2/M phases of the cell cycle was observed. These effects were accompanied by the increase of GSH content and the reduction of SOD-1 and -2, GPX-1, and ROS release. Also, iNOS expression and NO release were inhibited, and pro-inflammatory cytokine gene expression was decreased, confirming the putative role of α-tocopherol against inflammation. ***Conclusion:*** α-Tocopherol exerts protective effects in HDF which underwent oxidative stress by modulating the redox status, inflammation, iNOS-dependent NO release, and mitochondrial function. These observations have a potential role in the prevention and treatment of photoaging-related skin cancers.

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Introduction

Skin exposure to chemical and physical pollutants generates reactive oxygen species (ROS), inducing premature skin aging through DNA and mitochondrial damage, lipid peroxidation, inflammatory signaling pathway activation, and protein adducts formation [1]. Skin developed various defense systems against ROS, but their continuous production overcomes the enzymatic and nonenzymatic antioxidant systems, causing the disruption of the extracellular matrix and changing its function and structure. ROS release is physiologically counterbalanced by endogenous defensive micronutrients and enzymes, such as vitamins A, C, and E [2–5], glutathione (GSH) [6], catalase, glutathione peroxidase (GPX), and superoxide dismutase (SOD), which are present in quite high concentration in the skin [7, 8].

Vitamin E, and in particular α-tocopherol, plays a central role since it is involved in stopping lipid peroxidation. The cutaneous application of vitamin E not only ameliorates photoaging but also can hinder photocarcinogenesis [9]; the activation of antioxidant enzymes and the inhibition of apoptosis play a central role in these protective effects [10, 11]. Also, the antiaging properties of α-tocopherol are related to the inhibition of inflammation and the release of inducible nitric oxide synthase (iNOS)-dependent nitric oxide (NO) [12–14]. This is a Janus-faced molecule, playing either beneficial or harmful effects on the skin. The production of endogenous NO is regulated by 3 isoforms of NOS, namely, (i) constitutively expressed neuronal NOS and (ii) endothelial NOS, which produce small amounts of NO over brief periods, and (iii) iNOS, which is activated in pathological conditions and generates large quantities of NO [15]. Notably, iNOS is overactivated in response to UV irradiation in human endothelial cells [16] and cutaneous fibroblasts [17] and also in response to hydrogen peroxide (H_2O_2) [18]. Its inhibition represents a protective mechanism against senescence exerted by endogenous or exogenous antioxidants [19, 20].

In addition, mitochondria play a central role in skin aging, by acting as a possible source for ROS [21]. Any alteration of the mitochondrial electron transport chain caused by UV radiation and peroxidative stress leads to inadequate energy production in dermal fibroblasts and to functional and structural skin alterations [22]. Herein, we provide a complete study about the role of α-tocopherol on NO release modulation, mitochondrial function, oxidants/antioxidants balance, and inflammation in human skin fibroblasts.

Materials and Methods

Isolation of HPFs

Human primary fibroblasts (HPFs) were isolated from the perilesional skin of nonmelanoma skin cancer (NMSC) patients, after obtaining informed consent. Skin biopsies were washed thrice with ethanol 70% and physiological solution and incubated with Dispase II 2 mg/mL (Merck KGaA, Darmstadt, Germany) overnight at 4°C. The dermis was separated using sterile tweezers, cut in small pieces (2–4 mm), and plated on a 6-well microplate. A squared sterile glass was placed above, and 1 mL of DMEM 20% FBS (Euroclone, Pero, Milano, Italy) was added to each well. After 3 weeks, the dermis and glasses were removed, and fibroblasts were detached with 0.25% trypsin/0.02% EDTA (Euroclone) and plated in 25 cm^2 flasks. Cell media were changed with DMEM 10% FBS every 2 days.

Cell Treatment

HPFs were treated with 1 and 10 μM α-tocopherol given for 24 and 48 h alone (physiological condition) or before 200 μM of H_2O_2 (Merck) for 30 min. The antioxidant N-acetyl-cysteine (NAC; 200 μM; Merck), administrated for 24 and 48 h before 200 μM H_2O_2, was used as a positive control.

Cell Viability (MTT Assay)

0.2 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Merck) solution was added, and cells were incubated for 3 h at 37°C, 5% CO_2, protected from light. Formazan crystals were solubilized with dimethyl sulfoxide (Merck), and absorbance was measured at 570 nm using Victor X multilabel plate readers (PerkinElmer, Milano, Italy).

Intracellular ROS Quantification

The DCFDA-Cellular ROS assay kit (Abcam, Cambridge, UK) was used following the manufacturer's instructions. HPFs (2.5×10^4 cells/well) were seeded into a 96-well microplate and treated as described above. At the end of all treatments, cells were washed twice with PBS and incubated with 1 μM DCFDA solution for 45 min. Cells were washed with fresh PBS, and 100 μL of PBS was added to each well. Fluorescence was read with Victor X multilabel plate readers (PerkinElmer) at 495/529 nm.

Intracellular NO Quantification

The Measure-IT High-Sensitivity Nitrite Assay Kit (Thermo Fisher, Waltham, MA, USA) was used following the manufacturer's instruction. HPFs (4×10^4 cells/well) were seeded into a 24-well microplate and treated as described above. Cells were washed twice and lysated with 200 μL of double-distilled water. Meanwhile, a 96-well microplate was prepared with 100 μL of working solution where 10 μL of the cell supernatant was resuspended and incubated at room temperature (RT) for 10 min. Five microliters of quantification developer was added to each sample, and the plate was read at 365/450 nm using Victor X multilabel plate readers (PerkinElmer).

Quantitative Real-Time RT-PCR

HPFs were treated as described above; then, they were detached and resuspended in 500 μL of Trizol for total RNA isolation. The amount and purity of RNA were quantified at the spectrophotometer (Nanodrop-2000; Thermo Fisher) by measuring the optical

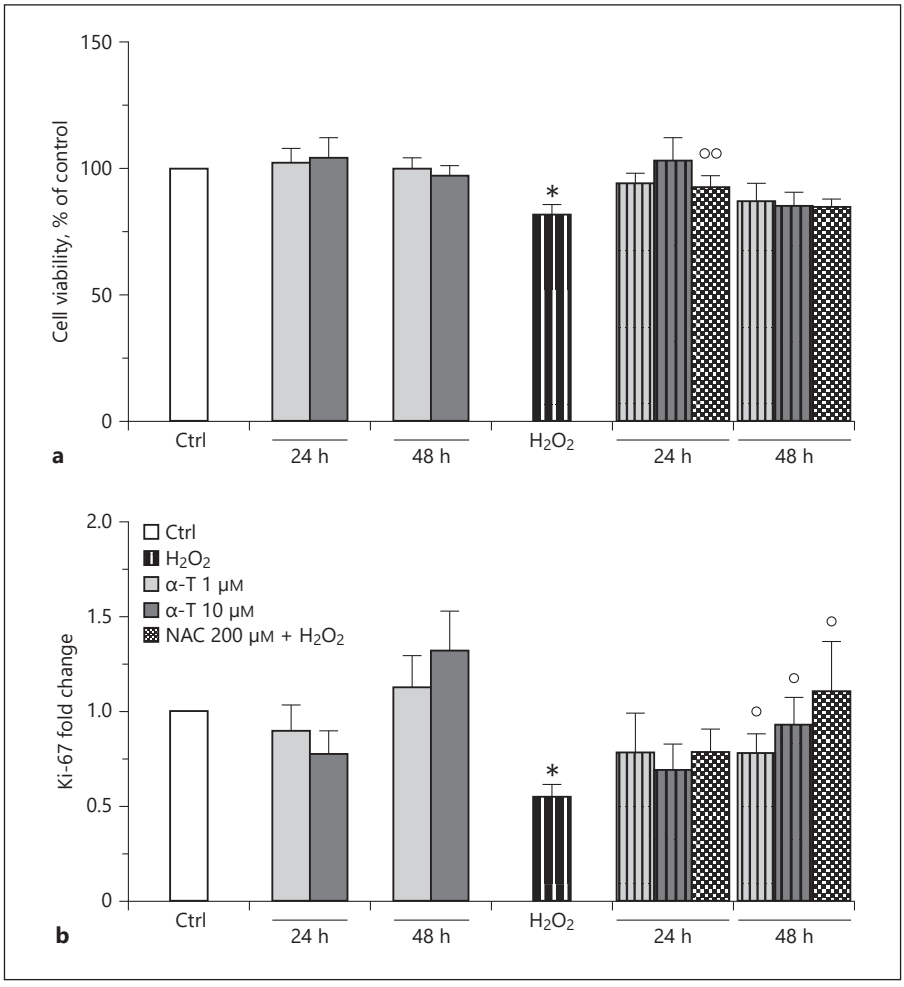

Fig. 1. Effects on cell viability (**a**) and Ki-67 expression (**b**) in HPFs cultured with 1 and 10 μM α-tocopherol for 24 and 48 h in physiological and peroxidative conditions (200 μM H_2O_2). Cell viability was evaluated through MTT assay, while Ki-67 genomic expression was evaluated through qRT-PCR. Reported data are expressed as means ± SD of 7 independent experiments. *p < 0.05 versus Ctrl; $^{○○}p$ < 0.01 versus H_2O_2. HPFs, human primary fibroblasts.

density at 260 and 280 nm. Reverse transcriptase and cDNA synthesis were performed using a high-capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. A 2-step cycling quantitative real-time PCR was performed in a volume of 10 μL per well in a Multiply Optical Strip (Sarstedt, Nümbrecht, Germany) containing SensiFast SYBR No-ROX kit (Bioline, London, UK), forward and reverse primer 400 nM, and 1 μL of the cDNA template. Primers used are indicated in online suppl. Table 1S (for all online suppl. material, see www.karger.com/doi/10.1159/000517204). GAPDH was used for data normalization, and the relative quantification was determined by the 2ΔCT method.

Indirect Immunofluorescence

HPFs (2×10^4 cells/well) were plated on a 12-mm Ø sterile glass and treated as described above. Cells were fixed with 4% paraformaldehyde for 10 min at 4°C and incubated with a blocking buffer (5% BSA, 0.1% Tritron X-100, and PBS 1X) for 1 h at RT; then, they were incubated with primary antibody anti-iNOS (1:250; Thermo Fisher) for 2 h at RT in 3% BSA, 0.1% Triton X-100, and with secondary antibody (anti-rabbit Alexa Fluor 488 antibody) for 45 min protected from light.

Evaluation of Mitochondrial Membrane Potential

It was measured in HPFs by using 5,51,6,61-tetrachloro-1,11,3,31 tetraethylbenzimidazolyl carbocyanine iodide (JC-1; Cayman, Ann Arbor, MI, USA), as previously performed [23–26]. HPFs (10,000 cells/well in 96 wells) were treated as described above. After stimulations, the medium was removed, and cells were incubated with JC-1 diluted in Assay Buffer 1X for 15 min at 37°C. After incubation, Assay Buffer 1X was used to wash cells twice, and then the mitochondrial membrane potential was determined by measuring the red (535/595 nm excitation/emission) and green (485/535 nm excitation/emission) fluorescence with Victor X multilabel plate readers (PerkinElmer). The data have been normalized versus control cells.

GSH Quantification

The GSH quantification kit (Cayman Chemical) was used as previously described [23, 27, 28]. HPFs (400,000 cells/well in 6 wells) were treated as described above. After treatments, cells were lysed and incubated with an equal volume of metaphosphoric acid (5%; Merck) for 5 min and then centrifuged at 2,000 *g* for at least 2 min. Supernatants were collected, and 50 μL per mL of triethanolamine (Sigma Aldrich) 4 M was added to each sample. Fifty

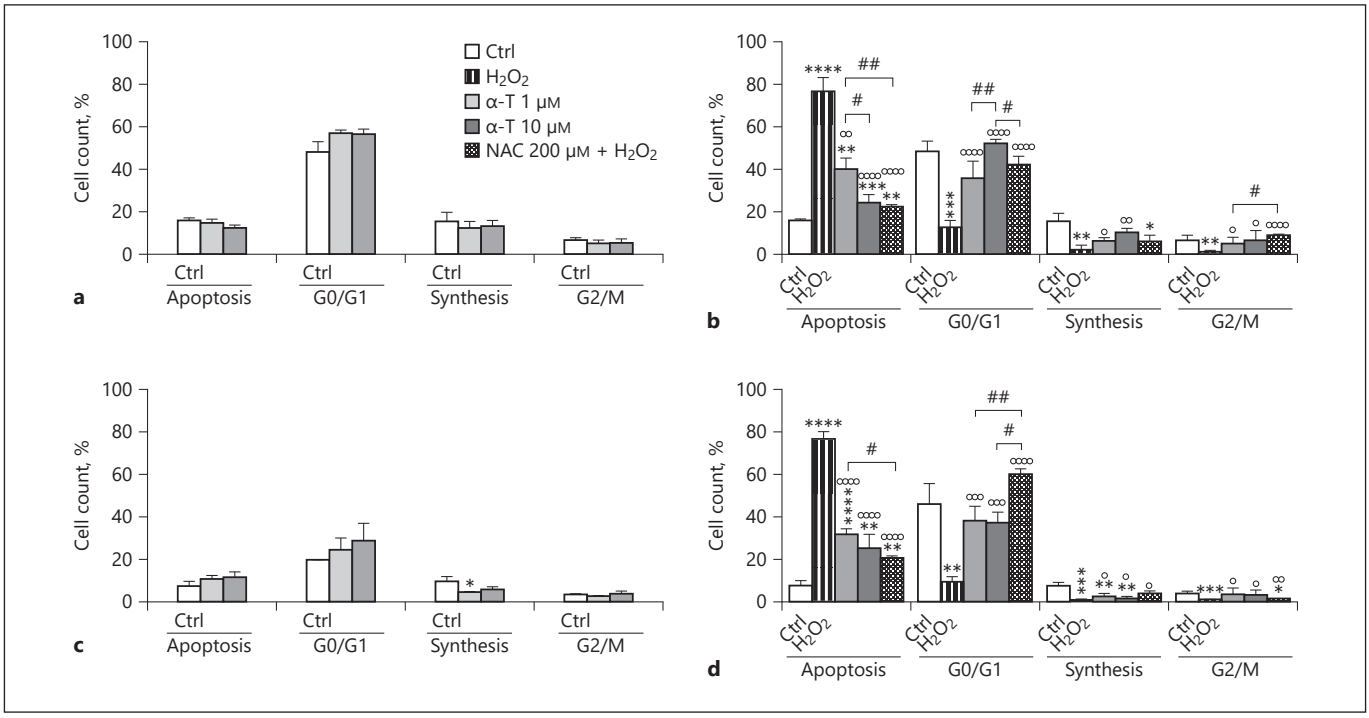

Fig. 2. Effects on the cell cycle in HPFs cultured with 1 and 10 μM α-tocopherol for 24 and 48 h in physiological (**a**, **b**) and peroxidative (200 μM H_2O_2) (**c**, **d**) conditions evaluated using propidium iodide staining through flow cytometry. Histograms represent quantitative analysis of apoptosis, G0/G1, synthesis, and G2/M phase. Reported data are expressed as means ± SD of 5 independent experiments. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, and ****$p < 0.0001$ versus Ctrl; $^{ooo}p < 0.001$ and $^{oooo}p < 0.0001$ versus H_2O_2; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ significance between groups. HPFs, human primary fibroblasts.

microliters of the sample was transferred into a 96-well plate; GSH was detected through Victor X Multilabel Plate Readers (PerkinElmer) at 405/414 nm excitation/emission wavelengths. GSH concentration (μM) was determined using the GSH standard curve.

Cell Cycle Analysis

Flow cytometry was used for cell cycle analysis [29]. HPFs (400,000 cells/well in 6 wells) were treated as described above. Cells were detached from the plate, centrifuged at 900 *g* for 5 min, and fixed in 70% ethanol (Merck) for 1 h at −20°C. Then, ethanol was discarded, and cells were washed with PBS and centrifuged. Cells were resuspended in 200 μL propidium iodide buffer (3.4 mM trisodium citrate, 9.65 mM sodium chloride, and 0.003% tergitol), 25 μL RNasi A (10 ng/mL; Cabru, Arcore, Milan, Italy), and 10 μL propidium iodide (1 mg/mL; Cabru). Fifty microliters of each sample was transferred to a 96-well plate in triplicate and incubated for 15 min at 37°C protected from light. Fluorescence was quantified using Attune NxT (Life Technologies, Monza, Italy) flow cytometry.

Statistical Analysis

All data were recorded using the institution's database. Statistical analysis was performed by using GraphPad Prism 6 (San Diego, USA). Data were checked for normality before statistical analysis, and one-way ANOVA followed by Bonferroni post hoc tests were used. All data are presented as means ± SD of *n* independent experiments for each experimental protocol. A value of $p < 0.05$ was considered statistically significant.

Results

Effects of α-Tocopherol on Cell Viability and Proliferation

The stimulation of HPFs with α-tocopherol alone did not affect cell viability or the gene expression of the cell proliferation marker Ki-67 (shown in Fig. 1a, b). In the presence of peroxidative stimuli, only 10 μM α-tocopherol given for 24 h has improved cell viability. Moreover, 1 and 10 μM α-tocopherol given for 48 h and NAC were able to counteract the reduction of the gene expression of Ki-67 caused by the H_2O_2 stimulation.

Effects of α-Tocopherol on Cell Cycle

In HPFs cultured in physiologic conditions, α-tocopherol did not cause any significant effect on the

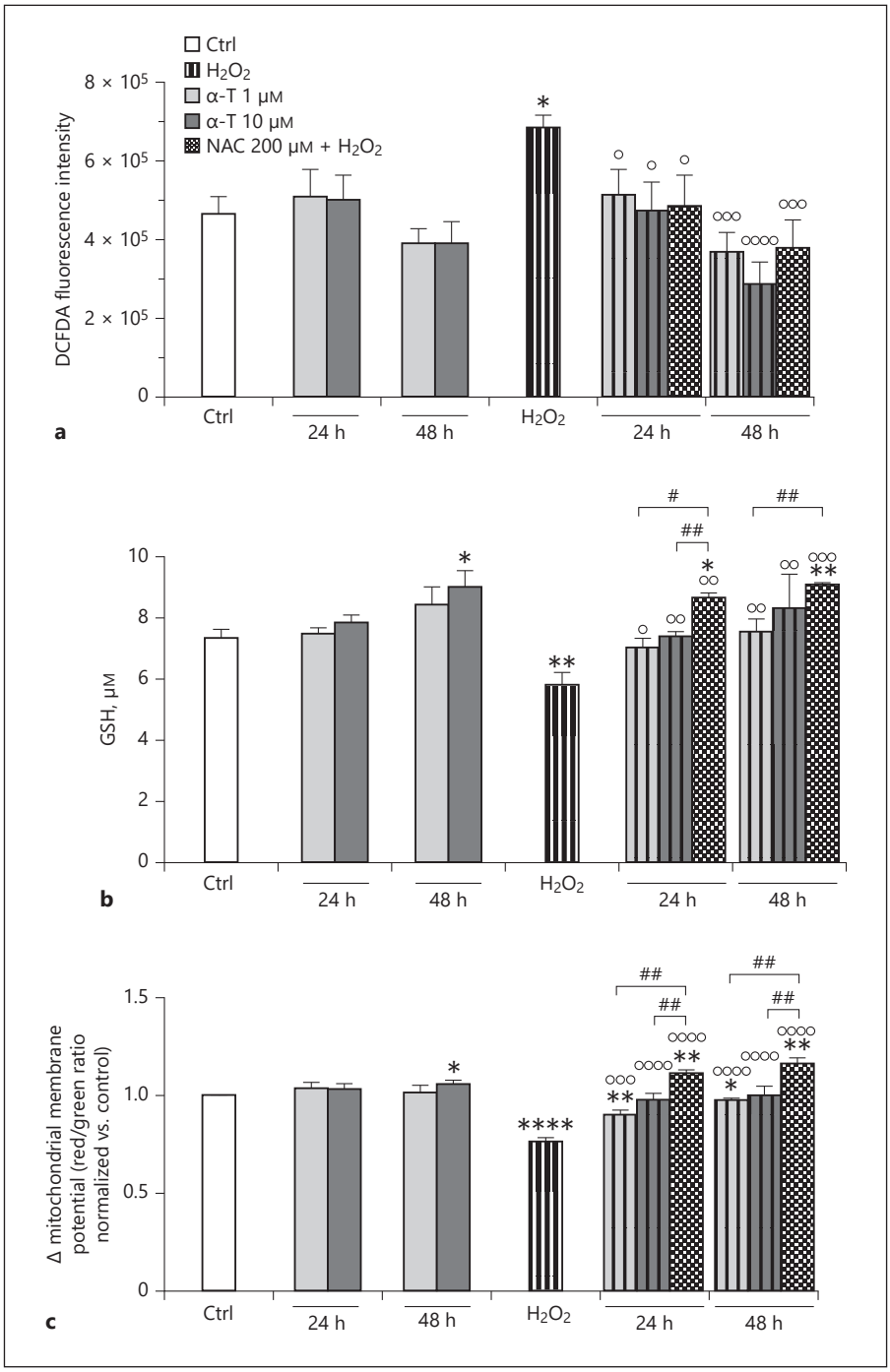

Fig. 3. Effects of α-tocopherol on ROS production (**a**), GSH (**b**), and mitochondrial membrane potential (**c**) in HPFs cultured in physiological and peroxidative conditions (200 μM H_2O_2) with 1 and 10 μM α-tocopherol for 24 and 48 h. Reported data are expressed means ± SD of 5 independent experiments. *$p < 0.05$, ***$p < 0.001$, and ****$p < 0.0001$ versus Ctrl; $^{○}p < 0.05$, $^{○○}p < 0.01$, $^{○○○}p < 0.001$, and $^{○○○○}p < 0.0001$ versus H_2O_2; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ significance between groups. ROS, reactive oxygen species; GSH, glutathione; HPFs, human primary fibroblasts.

cell cycle, except for 48-h stimulation, where a reduction of synthesis was observed with 1 μM α-tocopherol (shown in Fig. 2a, c). In peroxidative conditions, both α-tocopherol doses were able to protect fibroblasts against the H_2O_2-induced oxidative stress. Hence, apoptosis was reduced, whereas G0/G1, synthesis, and G2/M were increased, at both 24- and 48-h stimulation (shown in Fig. 2b, d).

Effects of α-Tocopherol on ROS and GSH Production and Mitochondrial Function

In HPFs cultured in physiological conditions, α-tocopherol did not affect ROS production; however, in the presence of peroxidative stimuli, both concentrations given for 24 and 48 h were able to decrease ROS production, as well as NAC (shown in Fig. 3a). It is to note that

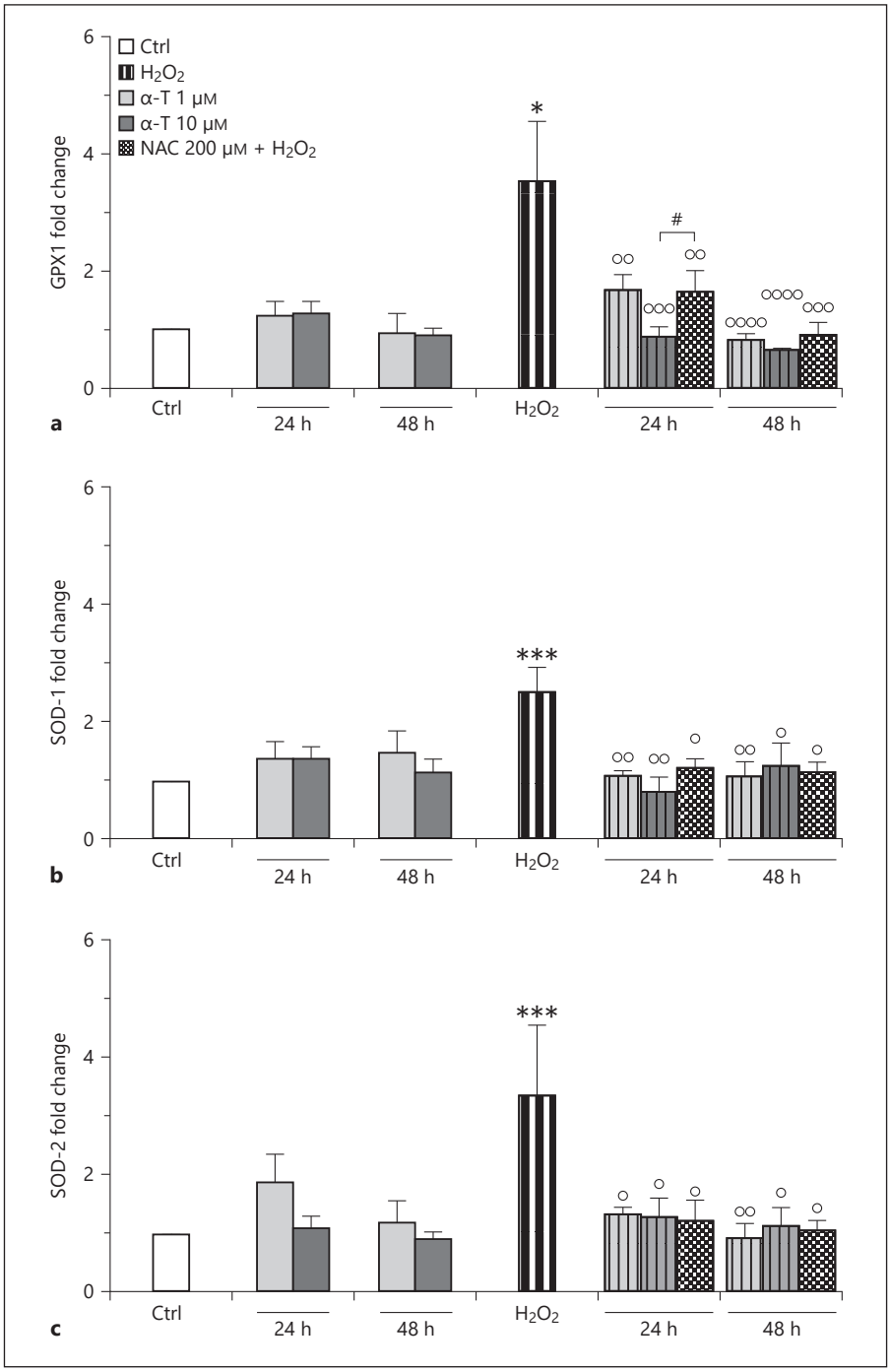

Fig. 4. Effects on GPX-1 (**a**), SOD-1 (**b**), and SOD-2 (**c**) expression in HPFs cultured with 1 and 10 μM α-tocopherol for 24 and 48 h in physiological and peroxidative (200 μM H_2O_2) conditions. Genomic expression was evaluated through qRT-PCR. Reported data are expressed as means ± SD of 7 independent experiments. $^{**}p < 0.01$ and $^{***}p < 0.001$ versus Ctrl; $^{○}p < 0.05$ and $^{○○}p < 0.01$ versus H_2O_2. GPX-1, glutathione peroxidase-1; SOD, superoxide dismutase; HPFs, human primary fibroblasts.

Fig. 5. Effects on intracellular NO production (**a**) and iNOS genomic (**b**) and proteomic (**c**) expression in HPFs cultured with 1 and 10 μM α-tocopherol for 24 and 48 h in physiological and peroxidative (200 μM H_2O_2) conditions. Intracellular NO production was measured by using the Measure-IT high-sensitive kit, iNOS genomic expression through qRT-PCR, and proteomic expression by indirect IF. Reported data are expressed as means ± SD of 7 independent experiments. Indirect IF quantification at 24 h (**d**) and 48 h (**e**) of α-tocopherol stimulation in peroxidative conditions was performed using ImageJ software, and data are expressed as CTCF. Data are expressed as means ± SD of 12 different measured cells of 3 independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$ versus Ctrl; $^{○}p < 0.05$, $^{○○}p < 0.01$, $^{○○○}p < 0.001$, and $^{○○○○}p < 0.0001$ versus H_2O_2; $^{\#}p < 0.05$ and $^{\#\#}p < 0.01$ significance between groups. NO, nitric oxide; iNOS, inducible NO synthase; HPFs, human primary fibroblasts; CTCF, corrected total cell fluorescence.

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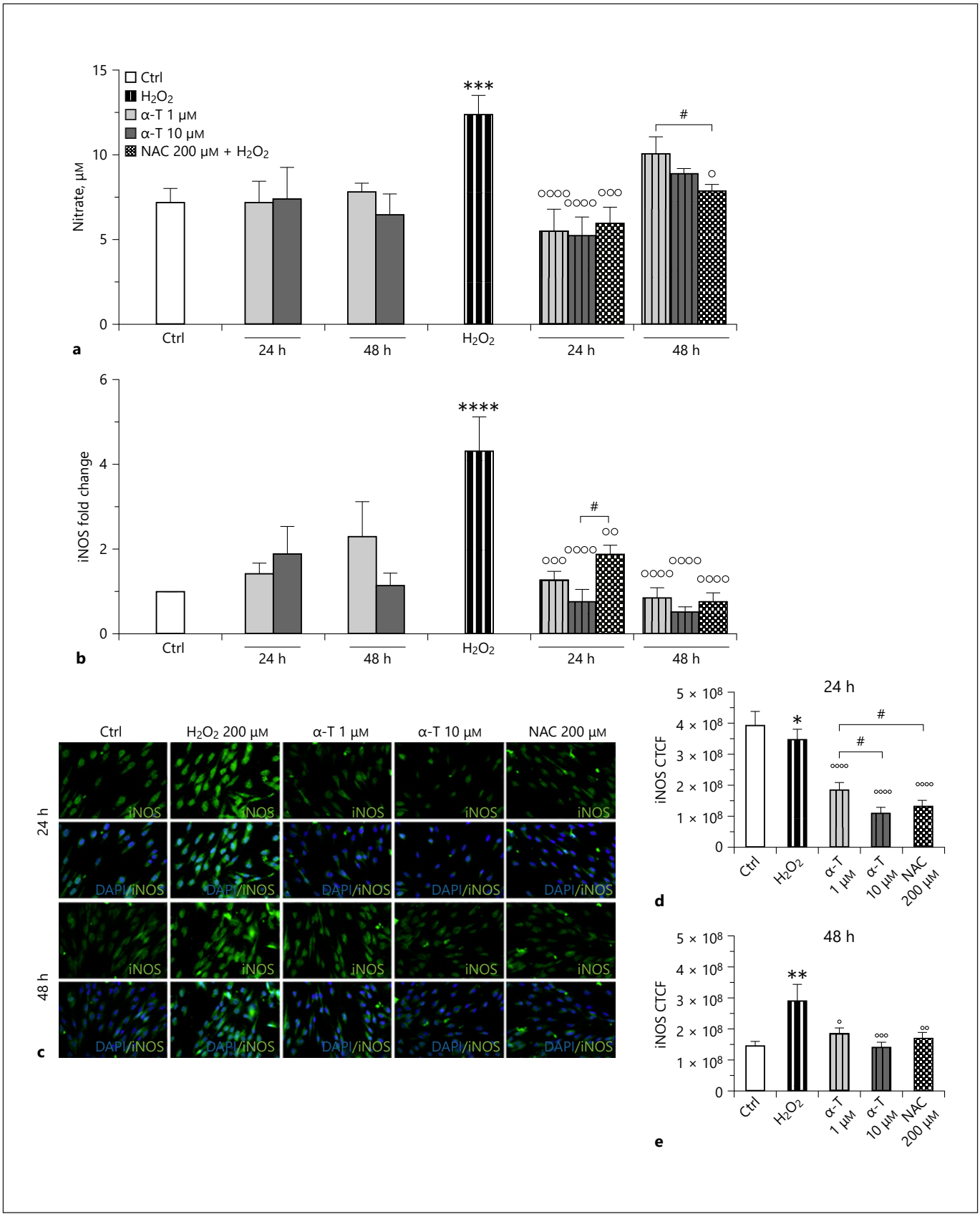

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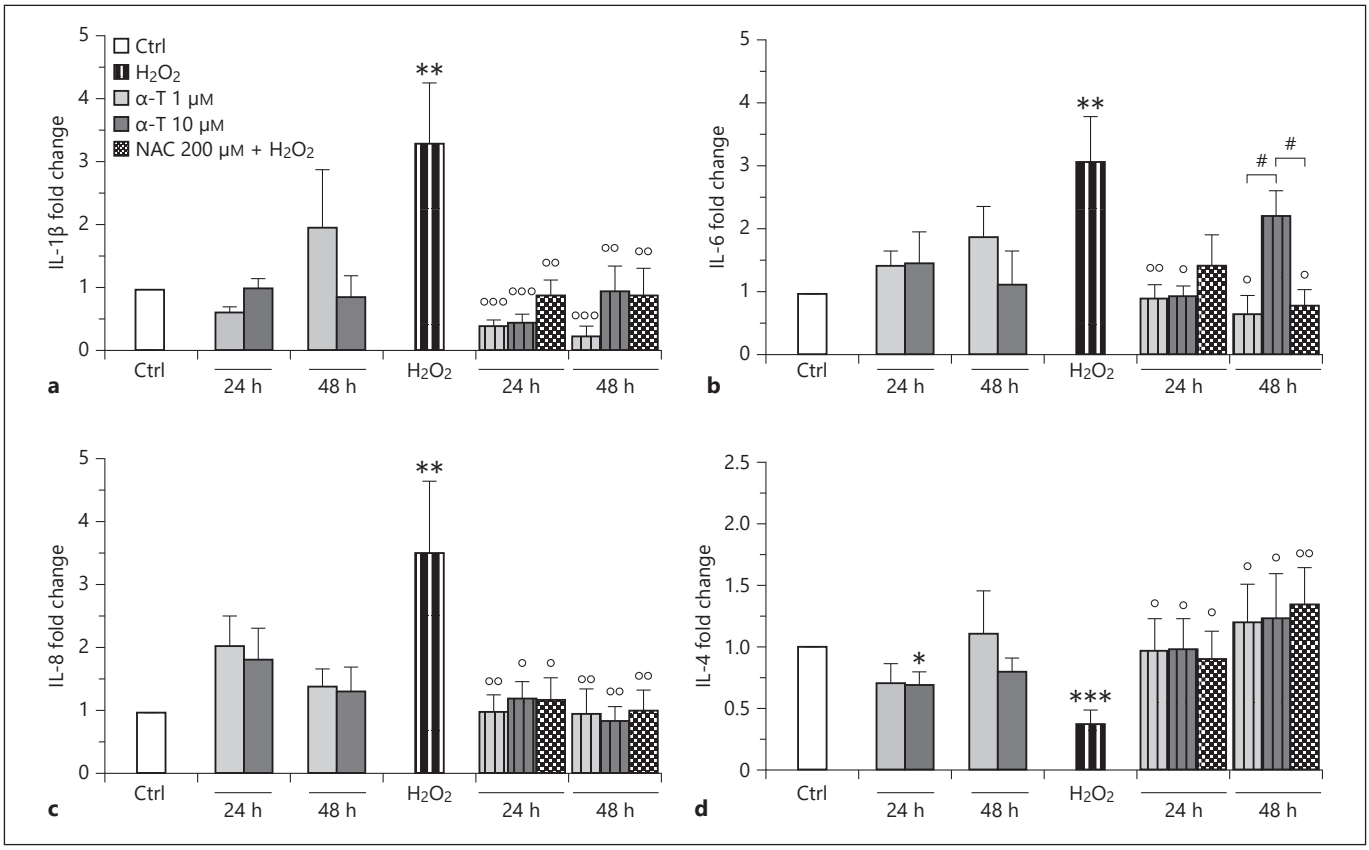

Fig. 6. Effects on pro- and anti-inflammatory cytokine gene expression in HPFs cultured with 1 and 10 μM α-tocopherol for 24 and 48 h in physiological and peroxidative (200 μM H_2O_2) conditions. IL-1β (**a**), IL-6 (**b**), IL-8 (**c**), and IL-4 (**d**) gene expressions were evaluated through qRT-PCR. Data are expressed as means ± SD of 7 independent experiments. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ versus Ctrl; $^{\circ}p < 0.05$, $^{\circ\circ}p < 0.01$, and $^{\circ\circ\circ}p < 0.001$ versus H_2O_2. HPFs, human primary fibroblasts.

the antioxidant GSH was increased by 10 μM α-tocopherol given for 48 h to fibroblasts cultured in physiologic conditions. Also, in the presence of peroxidative stimuli, GSH levels were increased by both 1 and 10 μM α-tocopherol given for 24 or 48 h (shown in Fig. 3b).

The protective effects elicited by α-tocopherol were confirmed by the analysis of mitochondrial membrane potential. This parameter was increased by 10 μM α-tocopherol given for 48 h to fibroblasts cultured in physiologic conditions. Also, the effects of H_2O_2 were counteracted by both concentrations of α-tocopherol given for 24 and 48 h (shown in Fig. 3c).

Effects of α-Tocopherol on Antioxidant Enzyme System Expression

The expression of GPX-1, SOD-1, and SOD-2 was evaluated in HPFs stimulated with α-tocopherol in HPFs cultured in physiological and peroxidative conditions. GPX-1 gene expression (shown in Fig. 4a) was affected by H_2O_2 stimulation; however, both 1 and 10 μM α-tocopherol given for 24 and 48 h in peroxidative condition, as well as NAC, decreased GPX-1 gene expression. Also, SOD-1 (shown in Fig. 4b) and SOD-2 (shown in Fig. 4c) gene expression was increased by peroxidative stimuli, and both α-tocopherol concentrations counteracted H_2O_2 effects, as well as NAC, given for 24 and 48 h.

Effects of α-Tocopherol on Intracellular NO Production and iNOS Expression

As shown in Figure 5a, in peroxidative condition, α-tocopherol given for 24 h to HPFs was able to counteract H_2O_2 effects, as well as NAC. On the contrary, at 48 h of treatment, only NAC was able to reduce the NO production.

iNOS expression was evaluated at both gene (shown in Fig. 5b) and protein (shown in Fig. 5d) levels. H_2O_2 great-

ly increased protein and mRNA expression; conversely, α-tocopherol stimulation counteracted H_2O_2 effects at both gene (shown in Fig. 5b) and protein levels after 24 and 48 h (shown in Fig. 5d, e) of treatment, as well as NAC. Immunofluorescence staining showed a dose-dependent decreased expression of iNOS after 24 h of stimulation (shown in Fig. 5d).

Effects of α-Tocopherol on Inflammation

The gene expression of pro- and anti-inflammatory cytokines was evaluated in HPFs stimulated with 1 and 10 μM of α-tocopherol in physiological and peroxidative (200 μM H_2O_2) conditions. Pro-inflammatory cytokine gene expression of IL-1β, IL-6, and IL-8 (shown in Fig. 6a–c) was enhanced by oxidative stress stimuli, but both α-tocopherol concentrations (1 and 10 μM) given for 24 and 48 h counteracted H_2O_2 effects, as well as NAC. IL-4 cytokine gene expression (shown in Fig. 6d) was decreased by H_2O_2 exposure, while both α-tocopherol concentrations, given for 24 and 48 h, enhanced the anti-inflammatory cytokine expression, as well as NAC.

Discussion

Our results showed for the first time the protective effects elicited by α-tocopherol in HPFs which underwent H_2O_2 exposure; these occurred by the modulation of mitochondrial function and NO release, the keeping of antioxidant systems, and the modulation of inflammation. We focused our attention on fibroblasts since these are the main cell components involved in skin repair and aging processes. While in physiologic conditions, they are almost resting, in the presence of any skin injury, they show abnormalities in the metabolism and proliferation, which can be at the basis of aging skin structure changes [30, 31]. Even more relevant is the involvement of the senescent fibroblasts not only in the early stages of skin carcinogenesis [32] but also in cancer cell migration and metastasis, both in melanoma [33] and in NMSC [34]. Therefore, the preservation of their functionality can be a strategy in the prevention of intrinsic and extrinsic skin aging and even cutaneous carcinogenesis [35, 36].

α-Tocopherol is the most active form of vitamin E [37] and is widely considered as the major membrane-bound antioxidant employed by cells [38], being able to scavenge acylperoxyl radicals, hydroxyl radicals, and O^{2-} generation caused by oxidants like UVA and UVB [39]. It is well known that this molecule has a role not only in the reparation but also in the protection of the skin from exogenous stress factors. The treatment of HaCaT cells before UVA exposure with α-tocopherol showed increased cell viability and decreased intracellular GSH depletion, ROS generation, and lipid peroxidation [40]. In our study, we confirm its potential role in counteracting the oxidative stress induced by H_2O_2 in human fibroblasts too. We have chosen this stimulus for our experimental conditions because it is one of the main ROS released by UVB exposure and may represent one of the possible causes of skin photodamage [41]. In particular, previous studies showed that H_2O_2 can lead to several forms of cellular damages involved in skin aging, including protein oxidation and intrinsic apoptosis, as well as impairment of mitochondrial antioxidant defense [42]. In addition, in fibroblasts, the oxidative potency of H_2O_2 was found to be similar to UVB and significantly stronger than UVA [43]. Furthermore, both UVB and H_2O_2 elicited similar stimulating effects on aging-related biological markers, like β-galactosidase, p53, and p21, in HS68 fibroblasts [44]. However, in our experience, α-tocopherol was able to exert any significant protective effect in human skin fibroblasts only in the presence of harmful conditions; this finding could be explained by the low-grade "reactivity" of fibroblasts in the absence of any stimulation.

Hence, the treatment of fibroblasts with α-tocopherol at concentrations similar to those previously proposed in other human skin models [45] was able to counteract the reduction of mitochondrial membrane potential caused by H_2O_2. Moreover, we demonstrated a reduction in ROS and NO release. The modulation of the fibroblast redox state by α-tocopherol was also confirmed by the analysis of GSH content, which was increased in the presence of the abovementioned agent. These findings are similar to the ones previously observed by our group about the effects elicited by genistein and 17-β estradiol when administrated in the same cellular model cultured in the presence or absence of H_2O_2 [20]. Notably, also the modulation of iNOS expression was similar to those observed in aged fibroblasts and keratinocytes treated with genistein and 17-β estradiol.

The role of mitochondria and NO in skin aging is widely accepted. Mitochondria could both originate ROS or be the target of free radicals. Furthermore, the fall of mitochondrial membrane potential could act as an initiating event leading to the activation of apoptotic cell death by the release of cytochrome C. Thus, in human dermal fibroblasts (HDF), the persistence of mitochondrial DNA deletions caused by UV or other stimuli like

H_2O_2 would alter the electron flow of the respiratory chain leading to inadequate energy production. These events would be followed by oxidative stress and inflammation and altered fibroblast function [22].

NO, as well, can play a key role in regulating the skin's response to external stimuli, like UV. The fact that NO could exert protection or damage strongly depends on its concentration and on the activity of NOS isoforms. While the constitutive NOS may act as a regulator of short-term and physiological phenomena and is involved in a small amount of NO release, iNOS could play a role in longer-lasting cytotoxic and inflammatory immunological functions, by producing NO at higher extent [46–48]. Several studies showed that augmented levels of NO would be pro-inflammatory and could induce skin changes resembling those caused by UV [48].

In our experience, H_2O_2 was able to increase both NO release and iNOS expression by HDF, as previously shown [20]. The fact that α-tocopherol could counteract the increased iNOS-dependent NO and the fall of mitochondria function could, thus, explain the abovementioned protective effects observed as regarding cell viability, cell cycle, and proliferation. The theoretical involvement of other NOS isoforms may explain the absence of reduction observed at 48-h stimulation. In further studies, a deeper analysis of the modulation of the transport across membranes of NO and ROS by α-tocopherol through the involvement of channels like aquaporins (AQPs) could be useful to better define the mechanisms of action. In fact, it is well known that dysregulation of AQPs can lead to oxidative stress and eventually cell death. For those reasons, alterations in AQP-mediated ROS and/or NO transport are assuming an increasing translational value in physiology and pathophysiology with promising nutraceutical and pharmacological implications [49, 50].

Also, the findings about the expression of other antioxidants particularly abundant in the skin, like SOD-1 and -2 and GPX-1, are of particular interest [21, 51]. In fact, SOD can convert superoxide anions into H_2O_2 and O_2, and also GPX-1 plays a crucial antioxidant role and prevents the harmful accumulation of intracellular H_2O_2 by using GSH as a hydrogen donor [52].

In our study, HPFs exposed to H_2O_2 showed increased SOD-1 and 2 and GPX-1 expression, as a putative defensive response toward oxidative stress. About this issue, it was shown that both *GPX-1* and SOD may be transcriptionally upregulated as part of the cellular response to peroxidation [53, 54].

As previously shown [55], H_2O_2 treatment of human fibroblasts led to an accumulation of inflammatory cytokines implicated in the initiation of several age-related inflammatory skin damages. Our results showing an increase of IL-4 and a decrease of IL-1β, IL-6, and IL-8 in response to α-tocopherol could highlight its putative antiaging role through the modulation of inflammation.

Vitamin E has been used for >50 years in experimental and clinical dermatology due to its antioxidant properties [56–58] and photoprotective effects [59]. This potential role is confirmed also by recent clinical findings, reporting lower tissue concentrations of α-tocopherol in NMSC compared with normal skin. Overall, our findings are in line with previously published results and add more information about the mechanisms of action of α-tocopherol in HPFs. Also, our results are of clinical relevance, as the treatment with α-tocopherol could represent useful protection for the skin against intrinsic and extrinsic aging phenomena and a potential therapeutic intervention in NMCS patients.

However, further studies will better examine the relationship between antioxidant expression cross-reaction and free radical release and in the redox state and inflammation cross-reaction. Also, the role of various NOS isoforms in mediating the protective effects of α-tocopherol and the pathways involved should be better analyzed. Finally, it could be also of interest to evaluate the protection exerted on other skin cell lines, such as keratinocytes, and their relationships.

Acknowledgments

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Statement of Ethics

The research was conducted ethically in accordance with the World Medical Association Declaration of Helsinki. All skin donors have given their written informed consent, and the study protocol was approved (No. 620/CE-study: 40/14) by our institute's committee on human research (Comitato Etico interaziendale Novara).

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

L.C., E.G., and P.S. designed the study; L.C., S.F., and P.M. performed the experiments; L.C.G. and E.Z. recruited patients and provided skin biopsies; L.C., E.G., and P.S. analyzed the data; L.C., E.G., S.F., P.M., L.C.G., E.Z., and P.S. wrote and critically reviewed the manuscript.

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