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ORIGINAL ARTICLE

17β-estradiol protects human skin fibroblasts and keratinocytes against oxidative damage

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Abstract

Background Reactive oxygen species (ROS) cause severe damage to extracellular matrix and to molecular structure of DNA, proteins and lipids. Accumulation of these molecular changes apparently constitutes the basis of cell ageing. 17b-estradiol (E2) has a key role in skin ageing homeostasis as evidenced by the accelerated decline in skin appearance seen in the perimenopausal years. Oestrogens improve many aspects of the skin such as skin thickness, vascularization, collagen content and quality. Despite these clinical evidences, the effects of oestrogens on skin at the cellular level need further clarification.

Materials and Methods HaCaT and human fibroblasts were cultured under various conditions with E_2 and H_2O_2; then were subjected to immunofluorescence and western blot analysis. Lipoperoxidation was investigated using BODIPY.

Results In human fibroblasts oxidative stress decreases procollagen-I synthesis, while E_2 significantly increases it. Fibroblasts and HaCaT cells viability in the presence of E_2 demonstrates a notably increased resistance to H_2O_2 effects. Furthermore E_2 is able to counteract H_2O_2-mediated lipoperoxidation and DNA oxidative damage in skin cells.

Discussion In this study we highlight that the menopause-associated oestrogens decline is involved in reduced collagen production and that E_2 could counteract the detrimental effects of oxidative stress on the dermal compartment during skin aging. Furthermore, our data show that physiological concentrations of oestrogens are able to interfere with ROS-mediated cell viability reduction and to protect human skin cells against oxidative damage to cellular membranes and nucleic acids structure.

Conclusion Our experimental data show that the presence of 17β-estradiol may protect skin cells against oxidative damage and that the dramatic lowering of oestrogen levels during menopause, could render skin more susceptible to oxidative damage.

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Conflict of Interest

The authors declare no conflicting or competing commercial interests.

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None.

Introduction

The ageing process involves the whole body and appears visibly in the skin. Skin ageing is influenced by several factors including genetics, environmental exposure, hormonal changes and metabolic processes. These factors together lead to cumulative alterations of skin structure, function and appearance. Clinical signs of natural ageing include fine wrinkles and skin laxity while photoaged skin appears dry, with coarse wrinkles and uneven pigmentation. However, both processes share common features such as reduced collagen levels in the dermis resulting from decreased procollagen synthesis and increased collagen degradation.[1] Collagen-I is the major structural protein of the dermis and provides strength and resiliency to the skin. Collagen-I is primarily produced by dermal fibroblasts and is regulated by a variety of mediators including

growth factors, cytokines, hormones and mechanical tension.[2] In aged skin, a dramatic decrease in procollagen synthesis by fibroblasts is evident.[3] The decrease of oestrogens during menopause is associated with increased skin dryness[4] and decreased skin elasticity,[5] dermal thickness[6] and skin collagen content.[7]

Various studies have shown that oestrogen therapy (ET) improve skin hydration[8] and elasticity,[9] higher density of collagen fibres,[10,11] fewer wrinkles[12,13] and thicker skin.[14] Although many evidences highlighted the beneficial effects of oestrogen on skin, some studies did not show remarkable improvements with ET.[15,16] Thus, the effects of oestrogens on skin are somewhat controversial and still need further evaluations.[17]

Oxidative stress (OS) is considered a primary feature in driving the ageing process. Ageing has been considered the consequence of free radical damage by various endogenous reactive oxygen species (ROS), according to the original free radical theory of ageing.[18,19] ROS form as a natural by-product of the normal cellular metabolism and have important roles in cell signalling and homeostasis. ROS production and release can be influenced by environmental factors such as UV radiation and exogenous toxins (i.e. nitric oxide and cigarette smoke). Key ROS include superoxide ($\cdot O_2^-$), hydroxyl radical ($\cdot OH$) and hydrogen peroxide (H_2O_2). Increased ROS can overwhelm antioxidant defences causing damage to DNA (mutations/breaks), proteins (nitrotyrosinylation), lipids (peroxidation) and sugars.[20–22] Oxidative damage can compromise cell survival,[23,24] proliferation,[24–26] differentiation[24,27,28] and metabolism.[29–31] Long-term effects of oxidative damage are implicated in skin ageing, cancer[32] and inflammation.[33] In particular, in photodamaged and chronologically aged human skin, the increased OS leads to the induction of AP-1 and NF-κB transcription factors,[1,34] which consequently induce collagen degradation by matrix metalloproteinases (MMPs) upregulation.[1,35] These data have been confirmed in different experimental models.[1,34,35]

Many data indicate that, in nervous and cardiovascular systems, oestrogens act as antioxidant,[36,37] providing a protective mechanism against ROS-induced oxidative damage. These data may be extrapolated to the hypothesis that 17β-estradiol (E_2) could exert similar beneficial effects in human skin.

Materials and methods

Materials

Phenol red-free Dulbecco's modified Eagle's medium (PRF-DMEM) and phosphate buffered saline (PBS) were from Gibco (Milan, Italy). Bicinchoninic acid (BCA) protein assay was from Pierce (Rockford, IL, USA). Horseradish peroxidase (HRP)-conjugated anti-mouse antibody was from Millipore (Milan, Italy). Mouse monoclonal antibody against 8-hydroxy-2'-deoxyguanosine (8-OHdG), mouse monoclonal antibody against procollagen-I α2 and mouse monoclonal antibody against β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Alexa Fluor 488-conjugated anti-mouse was from Molecular Probes (Eugene, OR, USA). BODIPY fluorescent probe was from Invitrogen (Milan, Italy). Unless otherwise specified, all other chemicals were from Sigma-Aldrich (Milan, Italy).

Cell culture

Normal human skin fibroblast cultures were established from a punch biopsy obtained from healthy female donors undergoing routine minor elective surgery. Briefly, skin specimens were cut into 1-mm^2 sections and incubated for 1 h in a 5% CO_2 atmosphere at 37 °C in DMEM supplemented with dispase to facilitate separation of the epidermis from the dermis. Human dermal fibroblasts were obtained from explant cultures of the de-epidermized dermis. Fibroblast cultures derived from three healthy donors were polled and maintained in PRF-DMEM supplemented with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 μg/mL streptomycin (P/S). The immortalized human keratinocytes cell line HaCaT was kindly provided by Dr. V. Barygina (Department of Biochemical Sciences, University of Florence, Florence, Italy) and was cultured under the same conditions. For all experiments, cells were grown to 80% confluence and serum starved for 24 h before treatment.

Immunofluorescence analysis

Dermal fibroblasts and HaCaT keratinocytes (1×10^6) were seeded onto glass slides and incubated in a 5% CO_2 atmosphere at 37 °C in PRF-DMEM supplemented with 10% FBS and 1% P/S. After 24 h, the medium was changed to PRF-DMEM without FBS, supplemented with 1% P/S. Cells were treated with E_2 (1 nM) solubilized in absolute ethanol or with vehicle control for 48 h, then washed twice with PBS and fixed for 10 min in 3.7% paraformaldehyde (PFA). After three washes with PBS, non-specific sites were blocked by incubating with 1% bovine serum albumin (BSA) at 37 °C for 15 min. Cells were washed with PBS containing 3% BSA for 30 min at room temperature (RT) and incubated with anti-procollagen-I α2 primary antibody diluted in PBS plus 3% BSA and 0.5% saponin, for 30 min at 37 °C and then for 1 h at 4 °C. The cells were washed three times (15 min each) in PBS plus 0.5% saponin (washing buffer) and incubated with Alexa Fluor 488-conjugated anti-mouse secondary antibody in washing buffer for 30 min at RT. The cells were washed three times with PBS, dried, mounted with Fluoromount and examined with a Zeiss Axiolab microscope (Carl Zeiss, Milan, Italy).

Western blot analysis

Human dermal fibroblasts were treated with vehicle (48 h), E_2 (1 nM; 48 h), H_2O_2 (50 μM; 16 h). The cells were also treated for 16 h with H_2O_2 (50 μM) after a 48-h pretreatment with E_2 (1 nM). The cells were washed three times with ice-cold PBS, scraped off the plates and lysed in 1 mL RIPA lysis buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM Na_2VO_3, 1 mM NaF, 1 mM EGTA, 1 mM EDTA, 1% Nonidet P-40, 0.25% sodium deoxycholatey) containing a protease inhibitor cocktail. The protein concentration

was estimated by BCA assay, and equal amounts of total protein (15 μg) from each sample were separated by SDS–PAGE and transferred to nitrocellulose membranes. Non-specific sites were blocked for 1 hat RT with milk powder. The membranes were incubated overnight at 4 °C with anti-procollagen-I α2 antibody. After washing, blots were incubated with HRP-conjugated anti-mouse antibody for 1 h at RT. After washing, blots were incubated with peroxidase chemiluminescence substrate and signals were detected using ChemiDoc XRS system (Bio-Rad, Milan, Italy). Band intensities were determined by densitometric analysis (ImageJ). Reprobing of the membrane with a β-actin antibody was used to verify equal protein loading.

Mitochondrial activity assay

Mitochondrial activity was assessed by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay in 96-well plates. The cells were treated with increasing concentration of H_2O_2 (50–250 μM), alone or in the presence of E_2 (1 nM) for 24 h. After washing with PBS, 0.5 mg/mL MTT solution in PRF-DMEM was added to the cell cultures and the samples were incubated for 4 h at 37 °C. Finally, MTT formazan crystals were resolubilized by adding dimethylsulfoxide (DMSO) to each well. Plates were agitated on a plate shaker for 5 min and spectrophotometric absorbance at 590 nm was then determined using an ELISA plate reader. Cell viability was expressed as a percentage of MTT reduction.

Evaluation of lipid peroxidation

Lipid peroxidation was investigated in fibroblasts and HaCaT using BODIPY 581/591, a fluorescent probe that is intrinsically lipophilic and thus mimics the properties of natural lipids. BODIPY acts as a fluorescent lipid peroxidation reporter that shifts its fluorescence from red to green in the presence of oxidizing agents. Briefly, cells were cultured on glass slides and treated with vehicle (24 h), E_2 (1 nM; 24 h) and H_2O_2 (70 μM; 16 h). The cells were also treated with H_2O_2 (70 μM) for 16 h after a 24-h pretreatment with E_2 (1 nM). The cells were then loaded with dye by adding BODIPY, dissolved in 0.1% DMSO (2.5 μM final concentration), to the cell culture media for 30 min at 37 °C. The cells were fixed in 3.7% PFA for 10 min at RT and the BODIPY fluorescence was analysed (at an excitation wavelength of 581 nm) using a Zeiss Axiolab microscope. Moreover, lipid peroxidation was quantified by flow cytometry. Cell suspensions were washed twice with PBS and incubated in the dark for 30 min at 37 °C with BODIPY. Cells were washed and resuspended in PBS. Green fluorescence and red fluorescence were revealed, respectively, by the FL-1 (515–555 nm wavelength band) and the FL-2 (563–607 nm wavelength band) detectors of a FACScan flow

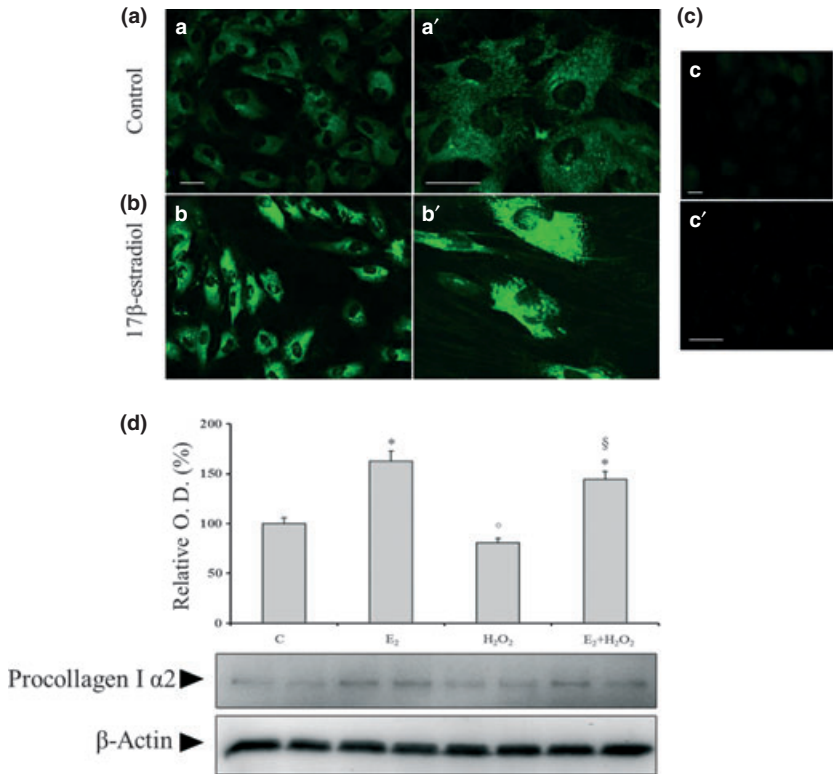

Figure 1 Effects of 17β-estradiol and oxidative stress on procollagen-I synthesis. (a–c) Immunofluorescence microscopy of human dermal fibroblasts and HaCaT keratinocytes immunolabeled using anti-procollagen-I α2 antibody. (a, a') control fibroblasts; (b, b') fibroblasts treated with E_2 (1 nM; 48 h); (c, c') HaCaT. (a–c magnification 40×; a', b', c' magnification 100×). Scale bar: 10 μM. (d) Western blot analysis. The samples were normalized for protein loading (15 μg) by reblotting the membrane with a β-actin antibody. Relative band intensities are depicted in histograms. Data from three independent experiments were combined and analysed by ANOVA. *$P < 0.001$ vs. control, °$P < 0.05$ vs. control, §$P < 0.001$ vs. H_2O_2.

cytometer (Becton Dickinson, San Jose, CA, USA) equipped with a 15-mW argon-ion laser for excitation.

Evaluation of nucleic acids oxidation

Nucleic acid oxidation was investigated using an antibody against 8-OHdG, the most distinctive oxidative biomarker of DNA damage. Human fibroblasts and HaCaT were cultured on glass slides and treated under the same conditions described for lipid peroxidation analysis. Cells were fixed for 10 min in 3.7% PFA and subjected to the immunofluorescence protocol previously described. Hoechst 33342 was used for the nuclear staining. Moreover, nucleic acids oxidation was quantified by flow cytometry. Briefly, the cell suspensions were washed twice with PBS and fixed in 3.7% PFA for 10 min at RT. After three washes with PBS, the samples were incubated with the 8-OHdG antibody diluted in 0.1% Na citrate and 0.1% Triton-X100 for 1 h at 37 °C. The suspensions were washed twice with PBS plus 1% normal goat serum (washing buffer), and then incubated with a FITC-conjugated anti-mouse secondary antibody. After two washes in the washing buffer, the cells were resuspended in PBS. Green fluorescence was revealed by the FL-1 (515–555 nm wavelength band) detectors of a FACScan flow cytometer equipped with a 15-mW argon-ion laser for excitation.

Statistical analysis

All data are reported as mean ± SD of three independent experiments. Statistical analysis was performed using one-way ANOVA. A P value <0.05 was considered significant. The IC_{50} values were calculated using the ALLFIT program.[19]

Results

Human primary dermal fibroblasts were subjected to immunofluorescence and western blot analysis to investigate the role of E_2 and H_2O_2 on procollagen-I synthesis. Figure 1a,b shows the

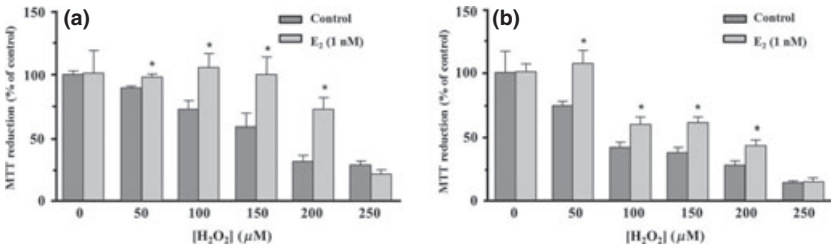

Figure 2 Mitochondrial activity assay performed on human dermal fibroblasts (a) and HaCaT keratinocytes (b) treated with increasing concentrations of H_2O_2 (50–250 μM) in absence or in presence of E_2 (1 nM) for 24 h. *P < 0.001 vs. H_2O_2.

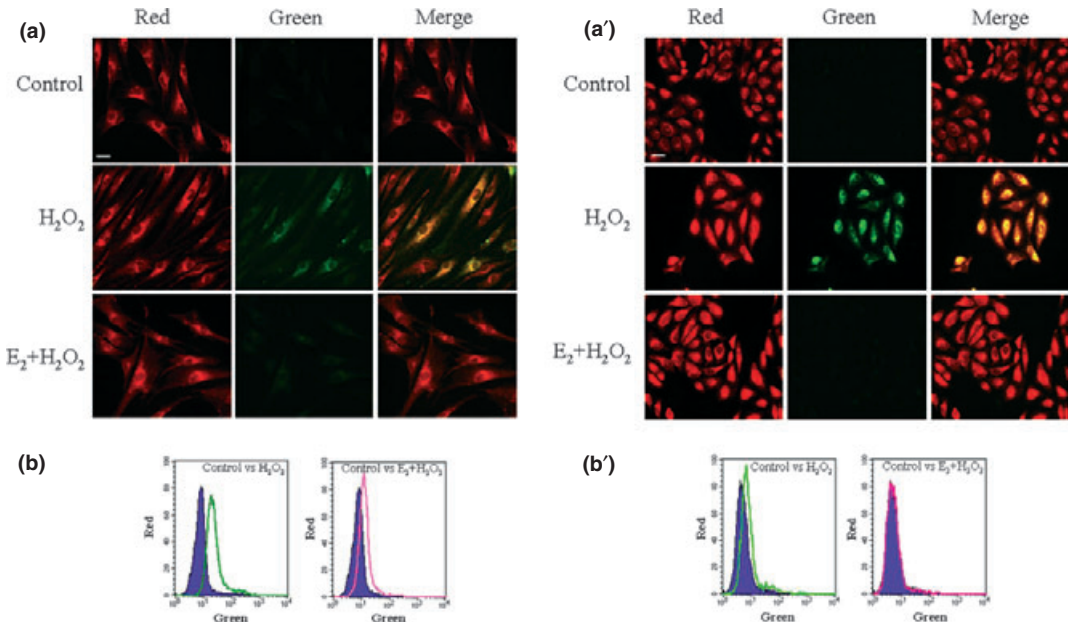

Figure 3 Fluorescence microscopy and flow cytometry analysis of lipid peroxidation. (a, a') Fluorescence microscopy analysis of lipoperoxidation in dermal fibroblasts (a) and HaCaT (a'). BODIPY shifts its fluorescence from red to green in the presence of oxidizing agents. Merged images show an overlap of the red and green fluorescence of BODIPY. Scale bar: 20 μM. (b, b') Quantitative analysis of lipoperoxidation by flow cytometry in dermal fibroblasts (b) and HaCaT (b').

specific immunoreactivity for procollagen-I α2 observed in the pooled cells obtained by skin dermal biopsies of three different patients. A stronger positivity was observed in E_2 (1 nM) treated cells (Fig. 1a; panels b, b') with respect to control cells (Fig. 1a; panels a, a'). The specificity of staining was demonstrated through the complete absence of labelling obtained using an isotype control in the control samples (data not shown) and HaCaT keratinocytes as negative control (Fig. 1c). These results, according to previous data,[7,38,39] highlight that the menopause-associated oestrogens decline could account for the reduced collagen production. To test the hypothesis that E_2 could interfere with OS-mediated collagen decrease, we analysed the procollagen-I expression in control and E_2 (1 nM) 48-h pretreated cells by inducing OS with H_2O_2 (50 μM; 12 h). Western blot analysis shown in Fig. 1d confirmed that E_2 increases procollagen-I α2 expression ($P < 0.001$), while H_2O_2 decreased it ($P < 0.05$). Furthermore, E_2 pretreatment prevented OS-induced collagen decrease ($P < 0.001$) and increased collagen content with respect to control ($P < 0.001$). These results support the previous findings that ROS mediate alterations of skin connective tissue[40] and demonstrate that E_2 could prevent the ROS-induced procollagen-I synthesis decrease in human dermal fibroblasts, suggesting a plausible protective role of E_2 against OS-mediated skin damage during ageing.

To better assess the potential protective effects of E_2 against OS in skin, we performed MTT assay on human dermal fibroblasts and HaCaT keratinocytes (Fig. 2a,b). Fibroblasts and HaCaT were incubated for 24 h with increasing concentrations of H_2O_2 (50–250 μM) in absence or in presence of E_2 (1 nM). The mitochondrial activity measured using MTT test demonstrated a sigmoidal relationship between cell viability and H_2O_2 concentration with an IC_{50} of 158.9 ± 9.3 μM for fibroblasts (Fig. 2a) and of 91 ± 16 μM for HaCaT (Fig. 2b). Fibroblasts and HaCaT viability measured in the presence of E_2 1 nM demonstrated a significantly increased resistance to H_2O_2 treatment, with an IC_{50} of 219.9 ± 3.55 μM for fibroblasts (Fig. 2a) and of 151 ± 18 μM for HaCaT (Fig. 2b). The cells treated with E_2 showed an increased tendency to resist against H_2O_2 adverse effects, giving a further demonstration of the E_2 protective role against OS.

To evaluate a possible interference mechanism of oestrogen with ROS-induced oxidative damage, we analysed the effects of E_2 on H_2O_2-induced lipoperoxidation. Lipid peroxidation was investigated on dermal fibroblasts and HaCaT by fluorescence microscopy analysis using the lipophilic fluorescent probe BODIPY (Fig. 3a,a'). The probe was not oxidized in control cells, whereas the treatment with H_2O_2 (70 μM) for 16 h is capable of considerably increasing the lipid peroxidation (Fig. 3a,a'). Both cellular types were protected against H_2O_2-induced lipoperoxidation by a 24-h pretreatment with E_2 (1 nM), which can bring the lipoperoxidation rate back to comparable levels to control cells (Fig. 3a,a'). These results were confirmed by flow cytometry analysis, as shown

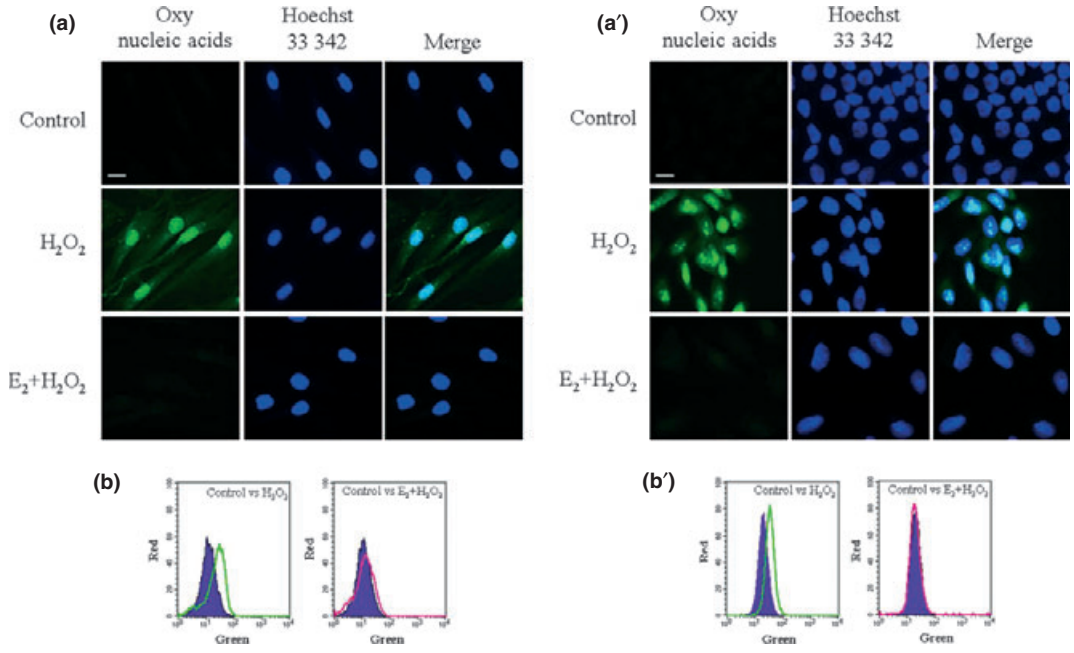

Figure 4 Fluorescence microscopy and flow cytometry analysis of nucleic acids oxidation. (a, a') Fluorescence microscopy analysis of nucleic acids oxidation in human dermal fibroblasts (a) and HaCaT (a'). Oxy nucleic acids staining shows nucleic acids damage (green). Nuclei were stained (Hoechst 33342, blue). Merged images show an overlap of 8-OHdG with Hoechst 33342. Scale bar; 10 μM. (b, b') Quantitative analysis of nucleic acids oxidation by flow cytometry in human dermal fibroblasts (b) and HaCaT (b').

in Fig. 3b,b'. In both cellular types, a significant shift of H_2O_2 treated cells ($P < 0.001$, green curves) with respect to control (purple curves) was observed. This shift is significantly reverted ($P < 0.001$, vs. H_2O_2 treated cells) by the E_2 pretreatment (pink curves).

Nucleic acids oxidation was evaluated by immunofluorescence microscopy analysis on dermal fibroblasts and HaCaT co-stained with a specific anti-8OHdG antibody and Hoechst 33342 (Fig. 4a,a'). The cells treated with H_2O_2 (70 μM) for 16 h showed an increased green fluorescence intensity caused by the rise of nuclear 8-OHdG level, whereas in control cells, no significant fluorescent signal was detected (Fig. 4a,a'). Once more the 24-h pretreatment with E_2 (1 nM) was capable of protecting both cell types against H_2O_2 oxidative effects (Fig. 4a,a'). The quantitative flow cytometry analysis confirms these results (Fig. 4b,b'). The treatment with H_2O_2 induced a considerable shift of oxidized cells ($P < 0.001$, green curves) with respect to control (purple curves), whereas the E_2 pretreatment (pink curves) reverted completely this effect ($P < 0.001$, vs. H_2O_2 treated cells).

Discussion

Human skin is subject to an unavoidable intrinsic ageing process. In addition, skin ageing is strongly influenced by exogenous factors such as UV radiation, which may cause premature skin ageing, also referred to as photoageing or extrinsic skin ageing. However, both intrinsic and extrinsic processes share major biochemical features such as reduced collagen content in the dermis, resulting from increased collagen degradation and decreased procollagen synthesis.[1] Oestrogens exert a profound influence on skin, as highlighted by regressive cutaneous changes that occur after menopause in women.[7,38,41] They not only increase skin thickness and enhance vascularization but also improve collagen content and quality.[39] Our results confirm that E_2 stimulates procollagen-I synthesis in human cultured skin fibroblasts and highlight that the menopause-associated oestrogen decline is involved in reduced collagen production.

OS is considered a primary driving force of the ageing process. The free radical theory of ageing[18,19] describes the progressive accumulation of damage by ROS over a lifetime as a result of aerobic metabolism, combined with a decline in antioxidant defences. ROS such as H_2O_2 were suggested to increase the induction of MMPs in keratinocytes and fibroblasts,[42] resulting in sustained collagen degradation and reduced mechanical tension. Partially degraded collagen and reduced mechanical tension of fibroblasts inhibit new procollagen synthesis and lead to further production of ROS, generating a self-perpetuating cycle, which is a critical mechanism of human skin ageing.[40,43]

We tested the hypothesis that E_2 could exert beneficial effects on OS-mediated collagen decrease by western blot analysis. Our results demonstrate that E_2 could interfere with ROS-induced procollagen-I synthesis decrease in human dermal fibroblasts, counteracting the detrimental effects of OS on the dermal compartment during skin ageing. Furthermore, our results obtained by MTT assay clearly demonstrate that oestrogens are not only involved in the collagen synthesis pathway, but could also interfere with ROS-mediated viability reduction in human dermal fibroblasts and HaCaT keratinocytes, giving a further demonstration of the E_2 protective role against OS in human skin.

Ageing is associated with changes in the molecular structure of DNA, proteins and lipids - all markers of OS. Although transient fluctuations in ROS serve important regulatory functions, when present at high and/or sustained levels, ROS can cause severe damage to these molecules. Considering that an increase in ROS levels has been observed during ageing,[44] the importance of oestrogen-related protective effects need to be considered.

To explore the plausible protective action of E_2 against OS-induced cellular damage, we evaluated two of the major oxidative stress markers within cells, lipoperoxidation and DNA oxidative damage. Lipoperoxidation process proceeds by a free radical chain reaction mechanism when the cellular peroxidized membranes lose their permeability and integrity. DNA oxidative damage can compromise the cellular function, and it is probably the major factor involved in mutagenesis, carcinogenesis and ageing.[45] 8-OHdG induces a guanine to thymine base transversion in DNA structure and thus is considered one of the main biomarkers of oxidative damage. Our data show that physiological concentrations of oestrogens are able to protect human skin cells against oxidative damage to cellular membranes and nucleic acids structure. Considering the high levels of cutaneous ROS generated during UV exposition and also during intrinsic ageing, our data suggested that the dramatic lowering of oestrogen levels during menopause could make skin more sensitive to oxidative damage caused by ROS and that oestrogens could offer a global protection against OS by improving cellular ability to resist against ROS damaging effects.

Although ET has been used for many years to treat the symptoms of menopause and to prevent postmenopausal osteoporosis, recent trials have reported a significant increased risk of breast cancer and other pathologies with this treatment.[46–48] For these reasons, systemic ET cannot be recommended to treat skin ageing. Phytoestrogens, non-steroidal plant compounds with oestrogen-like biological activity, such as Diadzen, Genistein and Resveratrol seem promising alternatives for skin ageing treatment. However, the precise mechanism of action of phytoestrogens in skin is still unknown, and their possible side-effects have not been well investigated.

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