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An ascorbic acid-enriched tomato genotype to fight UVA-induced oxidative stress in normal human keratinocytes

Ganna Petruk [a], Assunta Raiola [b], Rita Del Giudice [a], Amalia Barone [b], Luigi Frusciante [b], Maria Manuela Rigano [b,*], Daria Maria Monti [a,c,**]

[a] Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo, via Cinthia 4, 80126 Naples, Italy
[b] Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, 80055 Portici (Naples), Italy
[c] Istituto Nazionale di Biostrutture e Biosistemi (INBB), Rome, Italy

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ABSTRACT

UVA radiations contribute up to 95% of the total UV exposure and are known to induce cell damage, leading to apoptosis. Since the benefic effects of ascorbic acid on human health are well known, a new tomato genotype (named DHO4), highly rich in ascorbic acid, has been recently obtained. Here, we compared the effects of ascorbic acid and hydrophilic DHO4 extracts in protecting human keratinocytes exposed to UVA stress. Keratinocytes were pre-incubated with ascorbic acid or with extracts from the ascorbic acid enriched tomato genotype and irradiated with UVA light. Then, ROS production, intracellular GSH and lipid peroxidation levels were quantified. Western blots were carried out to evaluate mitogen-activated protein kinases cascade, activation of caspase-3 and inflammation levels. We demonstrated that ROS, GSH and lipid peroxidation levels were not altered in cell exposed to UVA stress when cells were pre-treated with ascorbic acid or with tomato extracts. In addition, no evidence of apoptosis and inflammation were observed in irradiated pre-treated cells. Altogether, we demonstrated the ability of an ascorbic acid enriched tomato genotype to counteract UVA-oxidative stress on human keratinocytes. This protective effect is due to the high concentration of vitamin C that acts as free radical scavenger. This novel tomato genotype may be used as genetic material in breeding schemes to produce improved varieties with higher antioxidant levels.

1. Introduction

UV radiations are among the most harmful exogenous factor for the human skin due to ROS production and erythema development. Depending on their UV wavelength, incident photons may determine different damages in the target cells [1]. In particular, in the UVB range (280–295 nm), dimerization reactions between adjacent pyrimidine bases occur by direct light absorption by DNA, whereas UVA radiations (320–400 nm) are less absorbed by DNA, but excite other endogenous molecules, leading to DNA damage [2,3]. Particularly, UVA radiations increase the levels of free radicals/ROS, thus damaging cellular proteins, lipids, DNA, and eventually leading to apoptosis [4–6].

Nevertheless, in the last few years, UVA radiation has been used for cosmetic purposes, for example to cure gel nails, to dry traditional nail polish and for topcoats formulated to protect nails. The effects of UVA nail lamps on human health are still under debate. Some authors reported that UVA wavelengths are responsible for photoaging in human skin and long-term exposure to these lamps can increase skin cancer risk [7,8]. However, the internet market claims that the lamp has no effect on human health [9].

Ascorbic acid (AsA) shows significant ability as electron donor and potent antioxidant in humans; it exerts a relevant role in protecting DNA from damages induced by oxidant species and in the prevention of inflammation [10,11]. Darr and colleagues reported that the topical application of AsA significantly increased cutaneous levels of this vitamin in pigs, and it protected the skin from UVA and UVB damage, probably as a consequence of the reducing properties of this molecule [12].

Abbreviations: AsA, ascorbic acid; DCF, 2′,7′-dichlorofluorescein; DHO4, double homozygote; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; EDTA, 2-({2-[Bis(carboxymethyl)amino]ethyl}(carboxymethyl)amino)acetic acid; EGF, epidermal growth factor; ERKs, extracellular signal–regulated kinases; FW, fresh weight; H_2-DCFDA, 2′,7′-dichlorodihydrofluorescein diacetate; ICAM-1, intercellular adhesion molecule-1; IκB-α, inhibitor of nuclear factor kappa-B kinase subunit alpha; NF-κB, nuclear factor κB; PBS, phosphate buffered saline; P-p38, phosphorylated p38 MAP kinase; P-MAPKAPK-2, phosphorylated MAP kinase-activated protein kinase; ROS, reactive oxygen species; SAPK /JNKs, the stress-activated protein kinase/Jun.-amino-terminal kinase; TBA, thiobarbituric acid; TBARS, TBA reactive substances; TNB, 5-thio-2-nitrobenzoic acid.

* Correspondence to: M.M. Rigano, Department of Agricultural Sciences, University of Naples Federico II, Via Università 100, 80055 Portici (Naples), Italy.
** Correspondence to: D.M. Monti, Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario Monte Sant'Angelo, via Cinthia 4, 80126 Naples, Italy.

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In addition, AsA is known to be a potent anti-inflammatory agent, since it suppresses nuclear factor κB (NF-κB) activation, a key molecule in the inflammatory process [13]. Noteworthy, humans have to introduce AsA with the diet, since they cannot synthesize it, due to mutations in the gene encoding gulonolactone oxidase, which catalyzes the last enzymatic step of the ascorbate biosynthetic pathway ([14] and references therein).

Tomatoes represent one of the most widely used and versatile fruit crop, consumed either fresh or processed (sauce, paste and juice) [15]. It has been reported that intake of tomato and tomato-based products is associated to benefic effects on human health due to the antioxidant compounds present in this fruit [16].

In this work we tested the antioxidant activity of hydrophilic extracts obtained from a novel tomato genotype, named DHO4, recently obtained in our laboratory and characterized by a high amount of AsA in the fruit. Previously, we demonstrated that hydrophilic extracts from the DHO4 genotype showed anti-proliferative activity specific for human cancer cells, as they did not affect the growth of normal cells [17]. Here, we compared the antioxidant activity of DHO4 hydrophilic extracts and of ascorbic acid in human keratinocyte-derived HaCaT cells exposed to UVA stress. We measured oxidative stress cascade, as well as the activation of caspase-3 and inflammation levels. Keratinocytes have been selected since these cells are normally present in the outermost layer of the skin and are more exposed to environmental stress, such as UVA irradiation [18], which contributes up to 95% of the total UV exposure.

2. Methods

2.1. Tomato Extracts and AsA Quantification

Hydrophilic extracts from the DHO4 fruit were obtained according to the procedure reported by Choi et al. [19] with minor changes. Briefly, frozen powder (2 g) was weighed, placed into a 50 mL Falcon tube, and extracted with 25 mL of 70% methanol into an ultrasonic bath (Branson 5200 Ultrasonic Corp.) for 60 min at 30 °C. The mixture was dried by rotovapor (R-210, Buchi), and re-dissolved in dimethyl sulfoxide (DMSO) 5% in PBS (1 mL).

Ascorbic acid quantification was carried out according to the colorimetric method reported by Stevens et al. [20] with modifications reported by Rigano et al. [17]. Briefly, 300 μL of ice cold 6% TCA were added to 500 mg of frozen powder. The mixture was incubated for 15 min on ice and centrifuged at 14,000 g for 20 min at 4 °C. Then, 20 μL of 0.4 M phosphate buffer (pH 7.4), 10 μL of double distilled (dd) H_2O and 80 μL of color reagent solution (prepared by mixing solution A (31% H_3PO_4, 4.6% (w/v) TCA and 0.6% (w/v) $FeCl_3$) with solution B (4% 2,2′-dipyridil (w/v) made up in 70% ethanol) at a proportion of 2.75:1 v/v) were added to 20 μL of supernatant. The mixture was incubated at 37 °C for 40 min prior measurement at 525 nm by a NanoPhotometer™ (Implen) using 6% TCA as reference. Three separated replicates for each sample were carried out, each one with three determinations. The concentration was expressed in nmol AsA according to the standard curve, designed over a range of 0–70 nmol; then the values were expressed in milligrams per 100 g FW.

2.2. Cell Culture

HaCaT cells (ATCC) were cultured in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich), supplemented with 10% foetal bovine serum (HyClone), 2 mM L-glutamine and antibiotics, all from Sigma-Aldrich, in a 5% CO_2 humidified atmosphere at 37 °C. For sub-culturing HaCaT cells, the culture medium was removed and cells were rinsed with PBS, detached with trypsin-EDTA and diluted in fresh complete growth medium; sub-culture was done in a ratio of 1:4 every 72 h.

2.3. Oxidative Stress Analyses

To evaluate the protective effect of hydrophilic tomato extracts and of ascorbic acid against oxidative stress, cells were plated at a density of 4×10^4 cells/cm^2. 24 h after seeding, cells were incubated in the presence of 88 μM AsA (Sigma-Aldrich) or 3 mg/mL hydrophilic extracts for 2 h and treated with UVA light for 2 min (20 J/cm^2). In a preliminary experiment we determined that 2 min of UVA treatment were able to induce the highest level of oxidative stress (data not shown). Immediately after UVA treatment, ROS production and GSH levels were determined by DCFDA and DTNB assays, respectively. Lipid peroxidation and western blotting analyses were performed after 90 min incubation. Control experiments were performed by supplementing cell cultures only with identical volumes of DMSO.

2.4. DCFDA Assay

To determine cytosolic ROS levels, cells were incubated with cell permeable, redox-sensitive fluorophore 2′,7′-dichlorodihydrofluorescein diacetate (H_2-DCFDA, Sigma-Aldrich) at the concentration of 25 μM for 30 min at 37 °C. Cells were then washed with warm phosphate buffered saline (PBS) supplemented with 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 30 mM glucose (PBS plus) two times, detached by trypsin, centrifuged at 1000*g* for 10 min and resuspended in PBS plus at a cell density of 1×10^5 cells/mL. H_2-DCFDA is non-fluorescent until it is hydrolysed by intracellular esterases and readily oxidized to the highly fluorescent 2′,7′-dichlorofluorescein (DCF) in the presence of ROS. The DCF fluorescence intensity was measured at an emission wavelength of 525 nm and an excitation wavelength of 488 nm using a Perkin-Elmer LS50 spectrofluorimeter. Emission spectra were acquired at a scanning speed of 300 nm/min, with 5 and 5 slit widths for excitation and emission, respectively. ROS production was expressed as percentage of DCF fluorescence intensity of the sample under test, compared to the untreated sample. Three independent experiments were carried out, each one with three determinations.

2.5. DTNB Assay

To estimate glutathione levels, cells were detached by trypsin, centrifuged at 1000*g* for 10 min and resuspendend in lysis buffer (0.3 M NaCl, 0.5% NP-40 in 0.1 M TrisHCl, pH 7.4) containing protease inhibitors. After 30 min incubation on ice, lysates were centrifuged at 14,000*g* for 30 min at 4 °C. Supernatant protein concentration was determined by Bradford assay. Then, 50 μg of proteins were mixed with 3 mM EDTA, 144 μM 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) in 30 mM TrisHCl pH 8.2, centrifuged at 14,000*g* for 5 min at room temperature and the absorbance of the supernatant was measured at 412 nm using a multiplate reader (Biorad).

The interaction of the sulfhydryl group of GSH and DTNB produced a yellow-coloured compound, 5-thio-2-nitrobenzoic acid (TNB). The rate of TNB production is directly proportional to the rate of this recycling reaction, which is in turn directly proportional to the concentration of GSH in the sample. Thus, the net absorbance at 412 nm yields an accurate estimation of the amount of GSH in the sample. GSH levels were expressed as percentage of TNB absorbance of the sample under test, compared to the untreated sample. Three independent experiments were carried out, each one with three determinations.

2.6. Measurement of Lipid Peroxidation

The levels of lipid peroxidation were determined by using the thiobarbituric acid reactive substances (TBARS) assay [21]. Briefly, cells were detached by trypsin, centrifuged at 1000*g* for 10 min and 5×10^5 cells were resuspendend in 0.67% thiobarbituric acid (TBA) and an equal volume of 20% trichloroacetic acid was added. The samples were then heated at 95 °C for 30 min, incubated on ice for 10 min and

centrifuged at 3000*g* for 5 min, at 4 °C. TBA reacts with the oxidative degradation products of lipids in samples, yielding red complexes that absorb at 532 nm. Lipid peroxidation levels were expressed as percentage of absorbance at 532 nm of the sample under test, compared to the untreated sample. Three independent experiments were carried out, each one with three determinations.

2.7. Western Blot Analyses

Cells were plated at a density of 2×10^4 cells/cm^2 in complete medium for 24 h and then treated as described above. Both untreated and treated cells were incubated for 30 or 90 min at 37 °C to detect IκB-α or ICAM-1 and p38 or MAPKAPK-2 phosphorylation levels, respectively. To prepare cell lysates, cells were scraped off in phosphate buffer, centrifuged at 1000*g* for 10 min and resuspended in lysis buffer (1% NP-40 in PBS, pH 7.4) containing protease and phosphatase inhibitors. After 30 min incubation on ice, lysates were centrifuged at 14,000*g* for 30 min at 4 °C. Upon determination of total protein concentration in the supernatant by the Bradford assay, samples were analyzed by SDS-PAGE and Western blotting using specific antibodies (Cell Signal Technology). To normalize to internal standard signals, antibodies against β-actin and GAPDH were used. The chemiluminescence detection system (SuperSignal® West Pico) was from Pierce.

2.8. Statistical Analyses

The biological replicates of samples were analyzed in triplicate. Quantitative parameters were expressed as the mean value ± SD. Significance was determined by Student's *t*-test at a significance level of 0.01.

3. Results and Discussion

3.1. Pre-treatment with AsA or Hydrophilic DHO4 Extracts Inhibits UVA-induced Damage in HaCaT Cells

A novel tomato genotype, DHO4, was previously produced in our laboratory, which contained a high content of ascorbic acid in the fruit. We demonstrated that hydrophilic extracts from the DHO4 genotype have anti-proliferative activity on human cancer cells whereas they don't affect the growth of normal cells [17].

To analyze the effects of hydrophilic DHO4 extracts on UVA stress-induced cells, we selected a concentration of the extract at which, in previous work, no cytotoxicity was observed (3 mg/mL), which corresponded to an AsA content of 88 μM [17]s.

For all the experiments, we used immortalized human keratinocyte cells (HaCaT) and UVA radiation as a source of stress. Cells were pretreated with 88 μM AsA or 3 mg/mL hydrophilic tomato extracts for 2 h and then oxidative stress was induced by UVA irradiation (20 J/cm^2). Immediately after UVA irradiation, ROS production and intracellular GSH levels were determined (Fig. 1A–B).

Incubation of cells with hydrophilic DHO4 extracts or with AsA did not alter ROS production (Fig. 1A, grey and white bar, on the left). We found that HaCaT cells exposed to UVA radiation showed a significant increase of fluorescence intensity levels (about 2-fold increase), which is related to ROS generation (Fig. 1A, black bars). Interestingly, when cells were pretreated with DHO4 extracts prior to UVA irradiation, no increase in ROS levels was observed after UVA exposure (Fig. 1A, grey bar, on the right). The same results obtained with DHO4 extracts were obtained by incubating HaCaT cells with AsA (Fig. 1A, white bars), thus suggesting that AsA contained in the DHO4 extract was responsible for the inhibition of ROS generation after UVA exposure.

Since the protective role of GSH from oxidative skin damage has been well documented, we measured intracellular GSH levels in both treated and untreated cells. When AsA or hydrophilic DHO4 extracts were added to the cells, no significant alteration in the GSH level was observed compared to untreated control cells (Fig. 1B, on the left). In UVA irradiated HaCaT cells we found a significant decrease (30%) in GSH level compared to untreated cells (Fig. 1B, black bars). Interestingly, when cells were treated with AsA or hydrophilic DHO4 extracts and then irradiated by UVA, the intracellular GSH level was similar to that observed in the absence of UVA treatment (Fig. 1B, grey and white bars, respectively). It has been reported that free radicals can cause irreversible damage to lipids and DNA, and resulted byproducts can be used as biomarkers of oxidative stress [22,23]. Therefore, we analyzed lipid peroxidation levels in HaCaT cells by measuring thiobarbituric acid reactive substances (TBARS), 90 min after UVA irradiation. We found that cells treated with AsA or hydrophilic tomato extracts did not show significant alteration in lipid peroxidation levels (Fig. 1C, on the left). A high increase (4-fold increase) in lipid peroxidation levels was measured in HaCaT cells upon UVA irradiation, compared to untreated control cells (Fig. 1C, black bars). Noteworthy, when cells were pretreated with AsA or DHO4 extracts and then irradiated by UVA, lipid peroxidation levels were found to be similar to those observed in not-irradiated cells (Fig. 1C, grey and white bars, respectively). A growing body of evidence suggests that UVA radiation generates ROS in the form of free radicals, such as superoxide anion and hydroxyl radical, as well as non-radical intermediates such as hydrogen peroxide [24,25]. Moreover, oxidative stress decrease intracellular levels of anti-oxidant molecules, including GSH, which participates in eliminating lipid

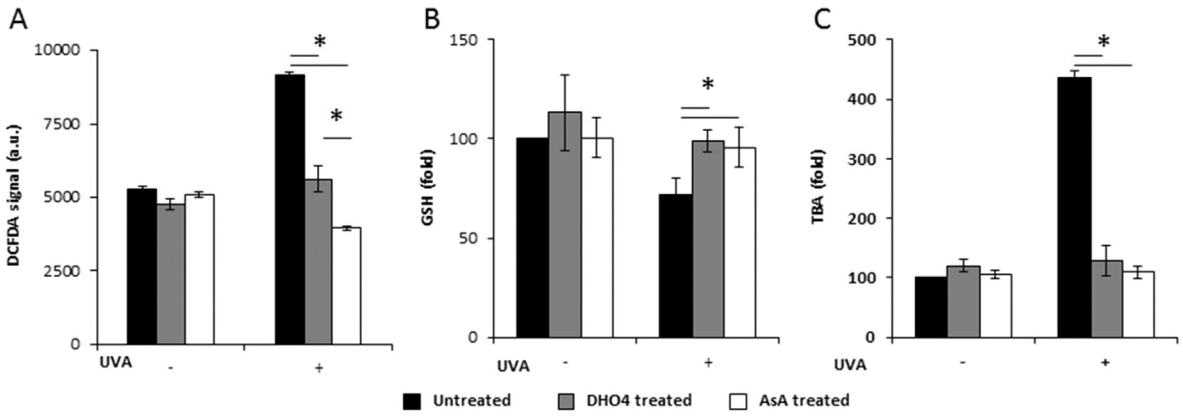

Fig. 1. AsA and DHO4 extracts suppress UVA-induced ROS production, GSH oxidation and lipid peroxidation in HaCaT cells. Cells were pre-incubated in the presence of 88 μM AsA (white bars) or 3 mg/mL DHO4 extracts (grey bars) for 2 h and then irradiated by UVA (20 J/cm^2). A, intracellular ROS levels were evaluated by DCFDA assay after UVA irradiation; B, intracellular GSH levels were determined immediately by DTNB assay; C, lipid peroxidation levels were evaluated after 90 min incubation, by TBARS assay. In B and C, values are expressed as fold increase with respect to control (i.e. untreated) cells. Data shown are the means ± S.D. of three independent experiments. Asterisks (*) indicate values that are significantly different ($p < 0.01$).

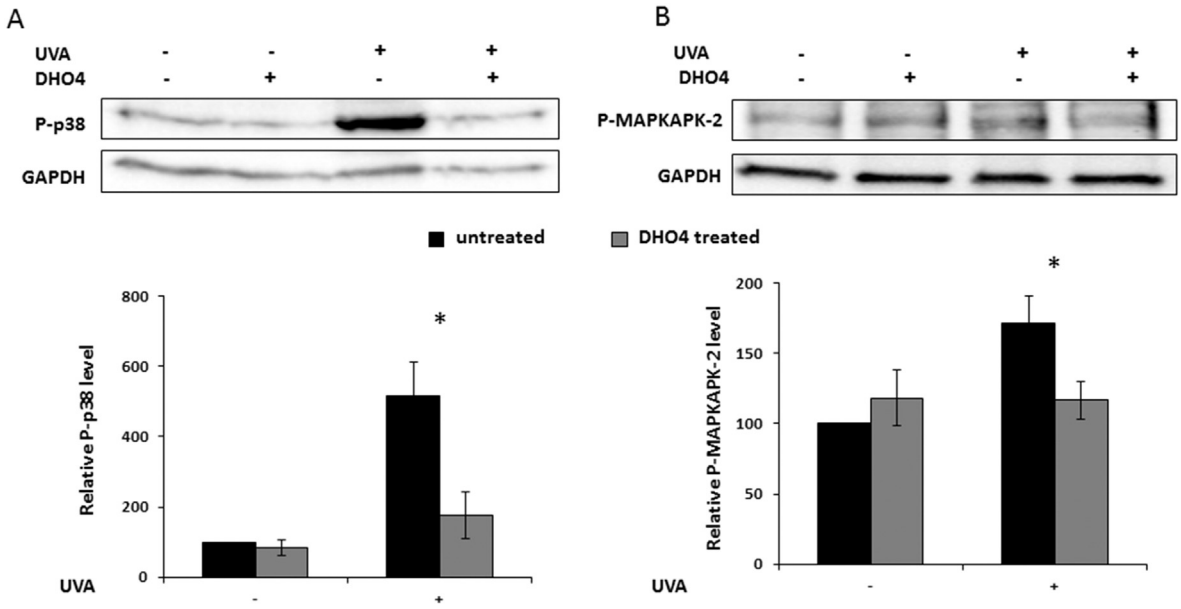

Fig. 2. Effect of DHO4 on UVA-induced oxidative stress markers in HaCaT cells. Cells were incubated with 3 mg/mL DHO4 extract 2 h prior to UVA (20 J/cm^2) irradiation for 2 min and then cells were incubated for 90 min. Western blots show the expression levels of p-p38 (A) and p-MAPKAPK-2 (B), with the relative densitometric analyses in the absence (black bars) or in the presence (grey bars) of DHO4. GAPDH was used as internal standard. Data shown are the means ± S.D. of three independent experiments. Asterisks (*) indicate values that are significantly different ($p < 0.01$).

peroxidation products, including 4-hydroxynonenal, by forming a GSH conjugate [23]. The finding that the AsA present in the tomato extracts is able to prevent alterations in ROS, GSH and lipid peroxidation levels in human keratinocytes irradiated by UVA, can be due to AsA free radical scavenger activity, exerted through its hydroxyl groups.

3.2. Activation of MAPK Cascade

Among the many signaling stress pathways induced by UVA, one involves the mitogen-activated protein kinases (MAPK) cascade. MAPK is a family of evolutionarily highly conserved enzymes that manage the response to growth stimulatory signals, such as insulin or epidermal growth factor (EGF), as well as adverse signals, such as cytotoxic and genotoxic substances or radiations. They include extracellular signal–regulated kinases (ERKs), stress-activated protein kinases, also known as c-jun *N*-terminal kinases (SAPK/JNKs), and the p38-MAPKs [26].

Therefore, we performed Western blot analyses to verify if the protective effect exerted by hydrophilic DHO4 extracts and by AsA from UVA-induced stress was related to MAPKs activation (Figs. 2 and 3). Treatment of cells with hydrophilic DHO4 extracts had no significant effect on p38 and MAPKAPK-2 (p38 target) phosphorylation levels (Fig. 2A and B, second lanes). When cells were irradiated by UVA, instead, we observed a significant increase in the phosphorylation levels of both p38 and MAPKAPK-2 (Fig. 2A and B, third lanes). Interestingly,

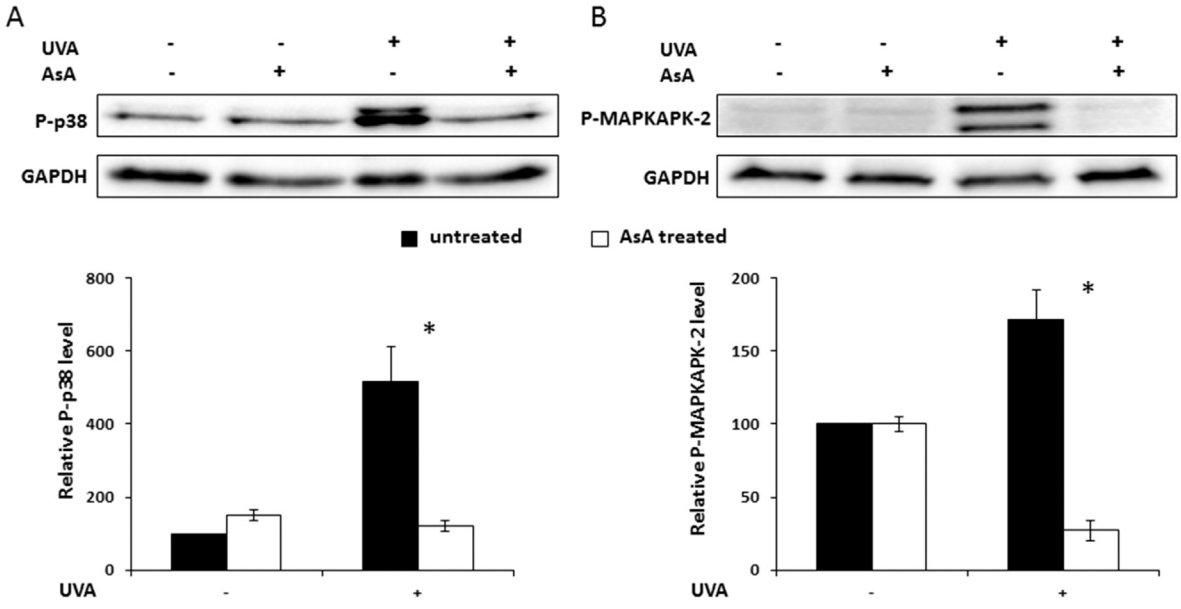

Fig. 3. AsA protects HaCaT cells from UVA-induced oxidative stress. Cells were incubated with 88 μM AsA 2 h prior to UVA (20 J/cm^2) irradiation for 2 min and then cells were incubated for 90 min. Representative western blots show the expression levels of p-p38 (A) and p-MAPKAPK-2 (B), with the relative densitometric analyses in the absence (black bars) or in the presence (white bars) of AsA. GAPDH was used as internal standard. Data shown are the means ± S.D. of three independent experiments. Asterisks (*) indicate values that are significantly different ($p < 0.01$).

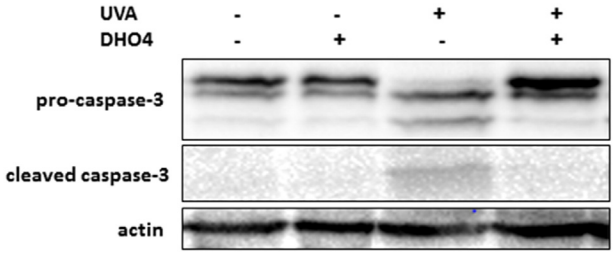

Fig. 4. DHO4 hydrophilic extracts protect HaCaT cells from UVA-induced apoptosis. Cells were pre-incubated with 3 mg/mL DHO4 extracts for 2 h, irradiated with UVA (20 J/cm^2) for 2 min and incubated for 90 min. Western blot was performed using anti-caspase-3, which recognizes both pro-caspase-3 and the activated form. β-actin was used as loading control.

when cells were pre-exposed to hydrophilic DHO4 extracts and then UVA irradiated, phosphorylation levels of both p38 and MAPKAPK-2 were similar to those observed in non-irradiated cells. (Fig. 2A and B, fourth lanes). The same results were obtained when cells were incubated with AsA (Fig. 3A and B), thus confirming that the DHO4 protective effect was due to the ascorbic acid contained in the DHO4 fruit.

ABTS analyses carried out in our laboratory demonstrated that AsA alone has an antioxidant activity comparable to that observed for DHO4 extracts (data not shown). However, treatment of cells with AsA prior to UVA irradiation was found to be more effective than DHO4 extract in preventing ROS production and MAPKAPK-2 phosphorylation levels. We hypothesize that the presence of other structurally different compounds present in DHO4 extracts could mask the AsA antioxidant activity observed on human keratinocytes.

Moreover, since it has been reported that UVA radiation entails, by activation of caspase-3, DNA damage and apoptosis [27,28], we analyzed activated caspase-3 levels in cells after UVA treatment. As shown in Fig. 4, DHO4 treatment had no effect on the activation of caspase-3, since cleaved caspase-3 was not observed. On the contrary, UVA exposure remarkably increased activation of caspase-3, as indicated by the presence of cleaved caspase-3 (Fig. 4, middle panel, third lane). It is worth to notice that when cells were pre-incubated in the presence of DHO4 extracts, no caspase-3 activation was observed, thus suggesting a protective role of DHO4 extracts from UVA irradiation. This result is in agreement with those reported by Ichihashi, who demonstrated that many epidermal skin cells, including keratinocytes, undergo apoptosis following UVA exposure as a result of DNA strand breaks [29].

3.3. DHO4 Protects HaCaT Cells from Inflammation

Finally, since it is known that UVA is also associated with induction of inflammation [30], we analyzed if DHO4 extracts could counteract the signaling pathway that leads to NF-κB activation. Among the signaling molecules that lead to inflammation, we chose IκB-α and ICAM-1, since they participate in the early events of the inflammation process [31]. IκB is the NF-κB inhibitor and its degradation leads to release of NF-κB for subsequent nuclear translocation. ICAM-1, instead, is a pro-inflammatory adhesion molecule whose expression is enhanced by NF-κB activation [31].

Cell lysates were analyzed by Western blotting using specific antibodies against IκB-α and ICAM-1. As shown in Fig. 5A, no differences were observed between DHO4-treated cells and untreated control cells. IκB-α was found to be degraded after UVA treatment. Interestingly, when cells were incubated with tomato extracts and then irradiated, IκB-α levels were similar to those observed in the absence of UVA treatment. ICAM-1 levels were found to be higher in cells exposed to oxidative stress compared to non-irradiated control cells. ICAM-1 levels were similar in cells pre-treated with the hydrophilic extracts and then exposed to oxidative stress and in non-irradiated cells (Fig. 5B). Our results are in line with the literature, since it has been reported that ROS can act as signal transduction molecules that induce pro-inflammatory cytokines and the NF-κB pathway [32]. Moreover, AsA is known to be a potent anti-inflammatory molecule that probably acts by suppressing the activation of transcription factor NF-κB that, in turn, inhibits tumor necrosis factor α_(TNF-α) [33].

Overall, our results suggest that DHO4 extracts are able to protect human normal cells from oxidative stress injury and inflammation process and that the protective effect is due to the high levels of AsA, that acts as an antioxidant molecule by donating its electrons, thus preventing other compounds from being oxidized. We believe that this novel genotype has a potential to be used as genetic material for breeding schemes in order to generate novel varieties with higher antioxidant levels. [17].

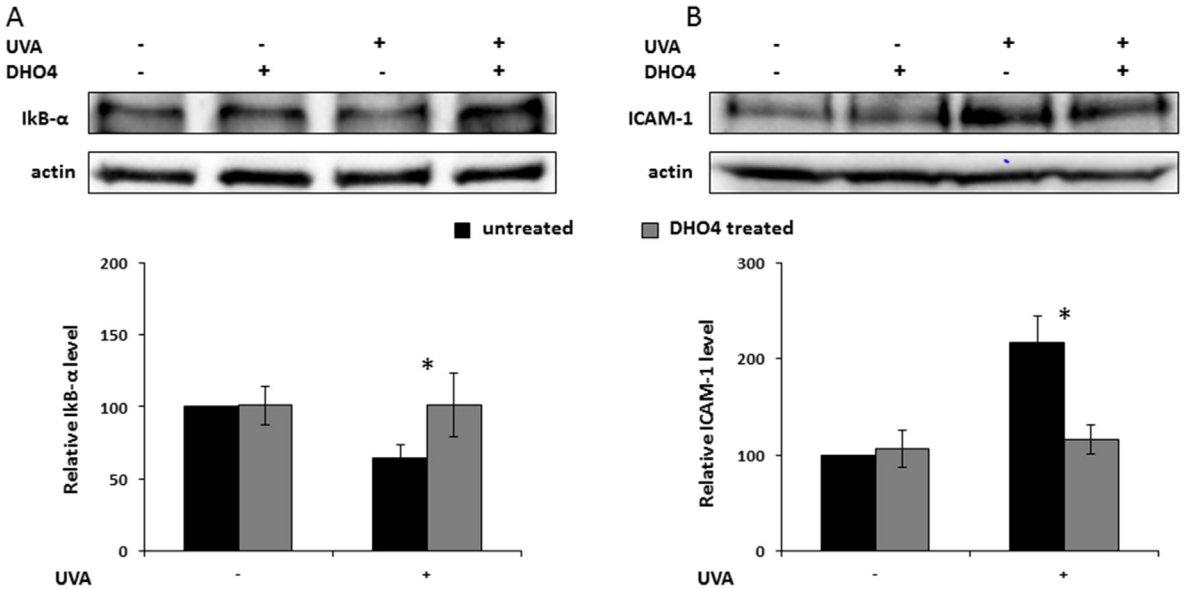

Fig. 5. Effects of DHO4 extracts on NF-kB signaling pathway in HaCaT cells. Cells were pre-treated with DHO4 extracts for 2 h, irradiated with UVA (20 J/cm^2) for 2 min and incubated for 30 min. Cell lysates were prepared and the levels of IκB-α (A) and ICAM-1 (B) were determined by Western blot analyses. β-actin was used as internal standard. The relative densitometric analyses, in the absence (black bars) or in the presence (grey bars) of DHO4, are reported. Data shown are the means ± S.D. of three independent experiments. Asterisks (*) indicate values that are significantly different ($p < 0.01$).

Stahl and Sies demonstrated that skin can be protected against UV-dependent lesions by supplying volunteers with tomato-based products [34]. Therefore, we believe that in the future novel tomato-based skin care products could be developed, including a dermatological lotion or a spray to be used on hands prior to nail care, to support the repair and regeneration of UVA-irradiated skin.

Conflict of Interest

The authors declare no competing financial interest.

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