UVC-Protective Effect of Caffeic Acid on Normal and Transformed Human Skin Cells *in Vitro*

(UVC / caffeic acid / UV-protective effect / α-tocopherol)

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Abstract. Possible UVC-protective properties of CA, a plant phenolic compound with antioxidant activity, were investigated on human KF1 diploid fibroblast and A431 epidermoid carcinoma cell lines. Cell populations, untreated and treated by antioxidants (CA and α-tocopherol), were irradiated by UVC at the wavelength of 254 nm and their proliferation activity was determined by the MTT assay. The results show a strong protective effect of CA at both concentrations used (55.5 and 166.5 μM): a significant increase of proliferation activity after UVC irradiation was detected in both cell populations growing in the presence of CA in comparison with cells in DMEM only. The described protective effect of CA was more obvious in transformed cells than in normal diploid cells. This protective ability is probably based on the antioxidant and scavenging activities of CA, which seems to be more efficient than α-tocopherol in protection against the cytotoxic effect caused by UVC irradiation.

In a large number of higher plant species, hydroxycinnamic acids are produced as the secondary metabolites of the shikimate pathway from L-phenylalanine or L-tyrosine (Rice-Evans et al., 1996). Caffeic acid (CA), 3,4-dihydroxycinnamic acid, is a biologically active member of hydroxycinnamates. Esters of CA, above all chlorogenic (5-caffeoylquinic) acid, are widely distributed in tissues of many plant species including fruits and vegetables. The highest concentrations of chlorogenic acid are found in coffee, blueberries, apples, and ciders (Cliford, 1999). After dietary intake of CA esters, CA is released by esterases of gut microflora. Free CA is able to pass through the gut wall and was found in human serum and urine (Cremin et al., 2001).

CA has previously been shown to have a multitude of biological activities. A strong antioxidant action of CA was demonstrated on different *in vitro* models. An increased resistance of cells to the oxidative stress that had been caused by t-butyl hydroxyperoxide was proved in the human U937 monocytic cell line. This effect is ascribed to the power of CA, incorporated into cells without any cytotoxic influence, both to reduce glutathione depletion during the oxidative stress and to inhibit lipid peroxidation (Nardini et al., 1998). At micromolar concentrations, CA effectively protects low-density lipoproteins (LDL) from Cu^{2+}-catalyzed oxidation and forms CA:copper complexes responsible for a transient metal chelating activity. This mechanism accounts for the increase of protective activity of CA (Nardini et al., 1995).

Studies on *in vivo* models also led to similar results. Dietary supplementation in rats resulted in a significant increase of α-tocopherol both in plasma and lipoproteins. Without incorporation into lipoproteins, CA may increase their resistance to oxidation (Nardini et al., 1997). CA as the scavenger of ROS also acts in the protection of some proteins from degradation and fragmentation caused by the increase of oxygen radicals. Facino et al. (1995) demonstrated that CA effectively inhibited collagen (type-III) fragmentation induced by superoxide anions and hydroxyl radicals.

CA is also effective as a selective (non-competitive) inhibitor of 5-lipoxygenase, which catalyzes biosynthesis of leukotrienes from arachidonic acid. Leukotrienes are involved in immunoregulation as well as in a variety of diseases including inflammation, asthma and other allergic conditions. For example, CA in concentration 10^{-4} M completely inhibited leukotriene C4 and D4 biosynthesis in mouse mast tumor cells (Koshihara et al., 1984). The xanthine/xanthine oxidase system represents another pathway strongly inhibited by CA; in this system superoxide anion and hydrogen peroxide are the main reactive agents (Facino et al., 1995). Moreover, changes in the xanthine/xanthine oxidase activity may be associated with certain diseases (gout,

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Abbreviations: CA – caffeic acid, CAPE – caffeic acid phenethylester, CPDs – cyclobutane pyrimidine dimers, 1,5-DCQA – 1,5-dicaffeoylquinic acid, DMEM – Dulbecco's modified Eagle's medium, DMSO – dimethyl sulphoxide, LDL – low-density lipoprotein, MTT – methylthiazolyldiphenyl-tetrazolium bromide, (6-4)PPs – 6-4 photoproducts, ROS – reactive oxygen species, UVA – 400-315 nm, UVB – 315-280 nm, UVC – 280-100 nm.

hepatitis) or with the increased incidence of tumours (Chan et al., 1995).

CA esters also exhibit antioxidant properties. Caffeoylquinic acids, i.e. 5-caffeoylquinic acid (chlorogenic acid), 1,5-dicaffeoylquinic acid (1,5-DCQA), and 1,3-dicaffeoylquinic acid (cynarine), are detected in many plant species, particularly in artichoke (Slanina et al., 1999), and in some foodstuffs: coffee, apples, potatoes (Cliford, 1999). Caffeic acid phenethylester (CAPE) isolated from the apian propolis is another biologically active derivative of CA. This compound is cytotoxic for both tumour and virally transformed cells, but not for normal cells (Frenkel et al., 1993). This finding was confirmed by the inhibition of synthesis of DNA, RNA and proteins in consequence of CAPE application in the HeLa cell line (Huang et al., 1996). Similarly as CA, CAPE also inhibits 5-lipoxygenase and manifests the antioxidant activity (Sud'ina et al., 1993).

The protective effects based on the antioxidant activity of CA against UV irradiation, especially UVA and UVB, were demonstrated both *in vitro* and *in vivo* (Saija et al., 1999, 2000). Similar results were also described using other antioxidants for the cell protection against UVA and/or UVB (Kondo et al., 1990; Malorni et al., 1996; Heo et al., 2001). However, there are no data on the possible protective action of CA against UVC. UVC irradiation preferably causes direct damage of DNA due to cyclobutane pyrimidine dimers (CPDs) and 6-4 photoproducts ((6-4)PPs), and oxidative damage seems to play only a minor role (Kuluncsics et al., 1999; Nishigori, 2000; Yoon et al., 2000).

With regard to the current knowledge on the antioxidant potential of CA and its derivatives after UVA and UVB irradiation, we investigated the possible UVC-protective capacity of CA on two *in vitro* model objects, consisting of normal and transformed human skin cells: KF1 diploid fibroblast and A431 epidermoid carcinoma cell lines. The possible protective effect of CA was assessed by measurement of proliferation activity by means of the MTT assay following UVC irradiation. In comparison with the known antioxidant α-tocopherol, we demonstrated strong UVC-protective properties of CA.

Material and Methods

Cell lines and culture conditions

Human skin fibroblasts (KF1) and human epidermoid carcinoma cells (A431) were chosen for the experiments. The KF1 cell line was diploid, derived in the laboratory using a biopsy sample from a surgically treated donor. The A431 cell line is an established cell line derived from carcinoma epidermis (this cell line was obtained as a gift from the Institute of Molecular Genetics, Prague, Czech Republic). The cells were maintained in DMEM medium supplemented with 10% foetal calf serum, 2 mM glutamine, 100 IU/ml of penicillin and 100 mg/ml of streptomycin at 37˚C in an atmosphere of 95% air : 5% CO_2. All the chemicals used were purchased from PAA Laboratories GmbH (Linz, Austria). The cells were subcultivated twice a week.

Antioxidants

Caffeic acid, CA (Fluka, Buchs, Switzerland) was diluted in DMEM at final concentrations 55.5 and 166.5 μM. For comparison with the effect of a known antioxidant, (±)α-tocopherol (Sigma, St. Louis, MO) at concentrations 100 and 300 μM was used in the experiments. (±)α-tocopherol was dissolved in DMSO (Sigma) and the final concentration of DMSO in DMEM was always less than 0.4% v/v, i.e. non-toxic. The final concentrations of CA and (±)α-tocopherol were chosen on the base of previously published data (Kondo et al., 1990; Facino et al., 1995; Nardini et al., 1995; Malorni et al., 1996). Control cell populations were cultivated in DMEM or in DMEM with 0.4% DMSO, respectively.

UV source and irradiation procedure

A TUV 30 lamp (Philips, Eindhoven, Netherlands) was used as a source of UV radiation. This lamp emits UVC radiation at the wavelength of 254 nm at the dose rate of approximately 920 mW/m^2. The cells growing in 96-well microtitre plates were irradiated in a sterile hood. The uncovered microtitre plates were placed at 50 cm below the center of the lamp tube and were irradiated for 30 min. Immediately after irradiation, the culture medium was removed and replaced with fresh DMEM with/without antioxidants in both irradiated and control microtitre plates.

Measurement of proliferation activity (MTT assay)

The proliferation activity of cell populations – untreated (control) and treated by antioxidants – after UV irradiation was determined by the MTT assay based on the detection of mitochondrial dehydrogenase activity in living cells. For each experiment, cells in suspensions at the concentration of 10^4 cells/ml were seeded into 96-well microtitre plates (Nunc A/S, Roskilde, Denmark) in a volume of 200 μl per well. Antioxidants at given concentrations were added to the cell suspensions before seeding into microtitre plates. The cells were allowed to grow under standard conditions for 24 h and then were irradiated as described above. The MTT assay was performed immediately after irradiation and then after reincubation under standard conditions for the following periods: 24, 48, and 72 h. In order to perform the MTT assay, the culture medium was removed; cells were washed with PBS, and 220 μl of DMEM containing MTT (Sigma) at the final concentration 455 μg of MTT per ml of DMEM were

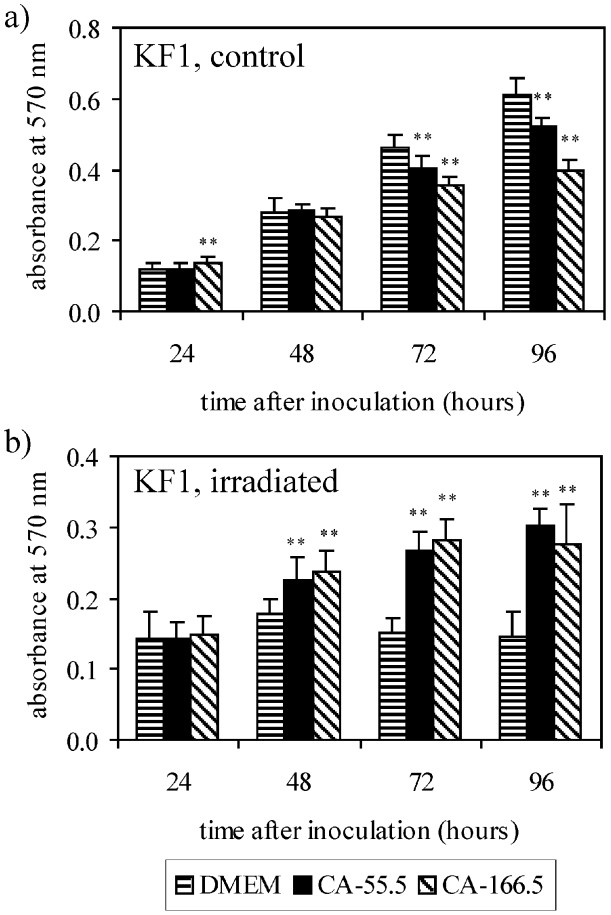

Fig. 1. Proliferating activity (measured using the MTT assay) of control (a) and irradiated (b) KF1 cells growing in DMEM only and in DMEM with CA at concentrations 55.5 μM (CA-55.5) or 166.5 μM (CA-166.5). Mean values ± SD. Statistical significance of difference (Wilcoxon unpaired test): * = $P < 0.05$; ** = $P < 0.01$ as compared with the cell population without CA.

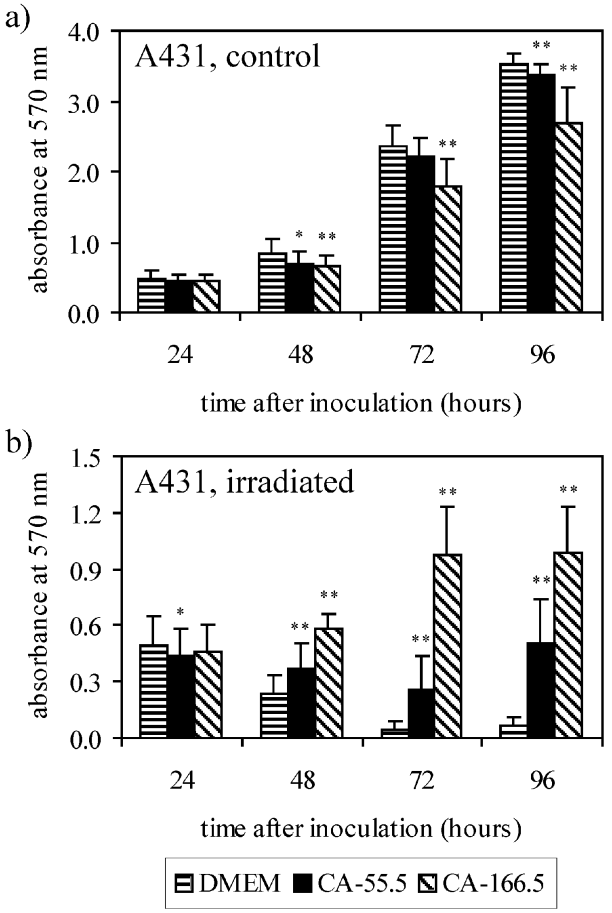

Fig. 2. Proliferating activity (measured using the MTT assay) of control (a) and irradiated (b) A431 cells growing in DMEM only and in DMEM with CA at concentrations 55.5 μM (CA-55.5) or 166.5 μM (CA-166.5). Mean values ± SD. Statistical significance of difference (Wilcoxon unpaired test): * = $P < 0.05$; ** = $P < 0.01$ as compared with the cell population without CA.

added into each well. After 4-hour incubation under standard conditions the medium with MTT was removed and 200 μl of DMSO were added into each well. Absorbance was measured at 570 nm using a Spectra Shell (SLT Laborinstrument GmbH, Salzburg, Austria) microplate reader. At each interval we measured both the irradiated microtitre plates and the control ones, i.e. the non-irradiated ones with the same arrangement of samples.

Statistical analysis

Data were expressed as mean ± S.D. for at least three independent experiments. The significance of differences between mean values was assessed by Wilcoxon unpaired test. The probability of $P < 0.05$ was considered statistically significant. All analyses were carried out using STATISTICA 6 (StatSoft, Inc., Tulsa, OK) and MS-Excel 2000 (Microsoft Corporation).

Results

Our results showed a marked influence of CA on cell proliferation. While in the control non-irradiated cell populations the cell proliferation was significantly reduced by both concentrations of CA used, the same concentrations of CA had a strong protective effect on the cell populations after UVC irradiation.

For the correct interpretation of the potential UVC-protective effect of CA on irradiated cells, we also studied changes in cell proliferation during the cultivation in DMEM containing CA in two different concentrations. Our results proved that the cultivation of cells in DMEM with CA leads to a decrease of proliferating activity (Figs. 1a, 2a) in comparison with cells cultivated in DMEM only, especially after extended reincubation periods, i.e. 48 and 72 h. This effect was similar in both normal (Fig. 1a) and transformed cells (Fig. 2a).

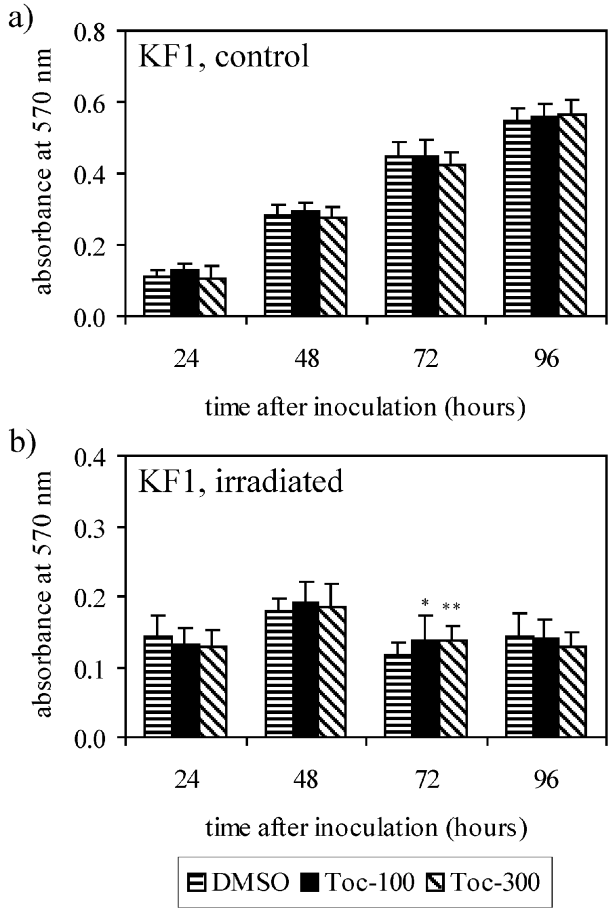

Fig. 3. Proliferating activity (measured using the MTT assay) of control (a) and irradiated (b) KF1 cells growing in DMEM supplemented with 0.4% DMSO and in DMEM with α-tocopherol at concentrations 100 μM (Toc-100) or 300 μM (Toc-300). Mean values ± SD. Statistical significance of difference (Wilcoxon unpaired test): * = P < 0.05; ** = P < 0.01 as compared with the cell population without α-tocopherol.

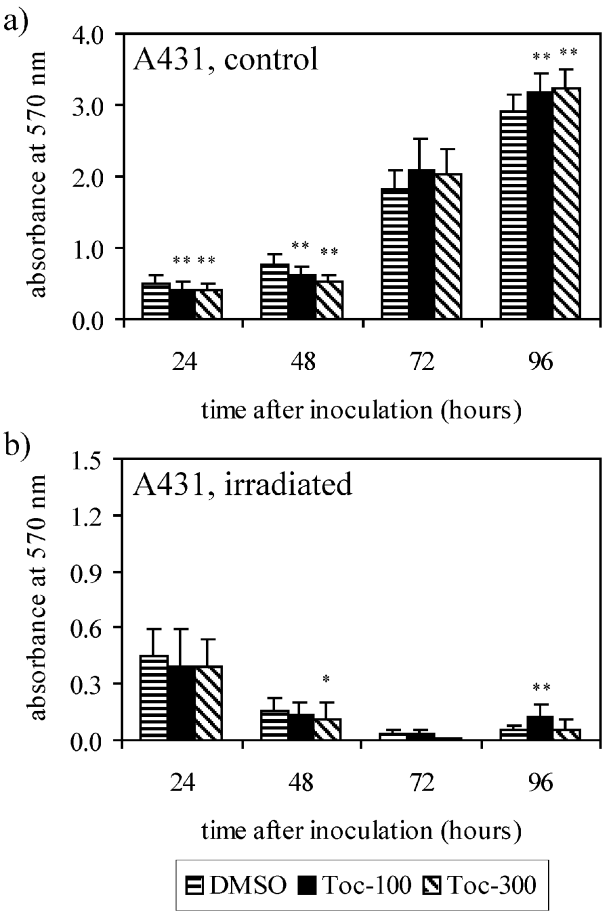

Fig. 4. Proliferating activity (measured using the MTT assay) of control (a) and irradiated (b) A431 cells growing in DMEM supplemented with 0.4% DMSO and in DMEM with α-tocopherol at concentrations 100 μM (Toc-100) or 300 μM (Toc-300). Mean values ± SD. Statistical significance of difference (Wilcoxon unpaired test): * = P < 0.05; ** = P < 0.01 as compared with the cell population without α-tocopherol.

However, the cultivation of KF1 normal skin fibroblasts in DMEM containing α-tocopherol did not significantly affect their proliferation activity (Fig. 3a). The influence of α-tocopherol on non-irradiated A431 cells was partly different: it reduced the proliferation activity during the first 48 h of cultivation, but the cell proliferation was increased after 96 h of incubation in the presence of α-tocopherol (Fig. 4a).

The UVC-protective effect of CA in both concentrations used (55.5 and 166.5 μM) was clearly demonstrated on normal as well as on transformed cells. A significant difference in the proliferation activity after UVC irradiation was detected in both cell populations growing in DMEM containing CA (Figs. 1b, 2b). This protective effect of CA was more obvious in transformed cells (Fig. 2b) than in normal cells (Fig. 1b). Nevertheless, the dissimilarity between the cytotoxic effect of UVC on CA-untreated cell populations and the stimulation of cell proliferation in CA-treated cells after UVC irradiation was distinct and significant. On the other hand, α-tocopherol in concentrations used in our experiments exhibited no or a very low protective effect on cell populations irradiated by the same dose of UV (Figs. 3b and 4b).

Discussion

Demonstration of the protective effect of CA on UVC-irradiated cell populations contributes new information to the current knowledge on antioxidative and protective properties of the phenolic compounds of plant origin.

UV radiation (waveband C) used in our experiments is preferentially absorbed by DNA. Many investigations employing UVC of the 254-nm wavelength have proved that a vast majority of DNA damage is represented by CPDs and (6-4)PPs. Oxidative damage to purines, pyrimidines, and abasic sites was also detected,

although it seems to play a minor role (Kuluncsics et al., 1999; Nishigori, 2000; Yoon et al., 2000).

Generally, the antimutagenic potential of CA was described in a few studies only. In two short-term genotoxicity assays (Ames assay and Drosophila wing spot test), mutations were induced by aflatoxin B_1; CA was effective in reducing these mutational events whereas α-tocopherol did not show any antimutagenic action (Karekar et al., 2000). Similarly, the potential of CA to reduce acridine orange- and ofloxacin-induced genotoxicity was also evaluated by the Ames assay (Belicova et al., 2001). Nevertheless, both studies conclude that the capacity of CA to protect DNA results from the blockage of mutagenic action by means of CA interacting with a genotoxic compound (acridine orange), from the arrest of metabolic activation of a promutagen (aflatoxin B_1) or from scavenging ROS produced by a mutagen (ofloxacin), respectively (Karekar et al., 2000; Belicova et al., 2001). In view of these facts we cannot assume that the primary UVC-protective effect of CA is due to its interaction with damaged or undamaged DNA molecules.

The MTT assay used for the evaluation of cell viability in our experiments yields results at the level of cell populations. We can suppose that the quantity of direct DNA damage, i.e. the number of CPDs and (6-4)PPs induced by the applied dose of UVC is the same in all experimental variants (control, CA-treated, α-tocopherol-treated) for each cell line and that this type of DNA damage is not affected either by CA or by α-tocopherol.

The antioxidant properties of CA were described in many studies as well as its ability to reduce the oxidative damage of DNA (Wells et al., 1997; Li et al., 2000; Yonezawa et al., 2001; Szeto and Benzie, 2002). The photoprotective activity of CA against UVB was reported both *in vitro* and *in vivo;* the former concerned the evaluation of UV radiation-induced peroxidation in phosphatidylcholine liposomal membranes and the scavenging of nitric oxide (Saija et al., 1999), the latter referred to the CA ability to reduce UVB-induced skin erythema in healthy human volunteers (Saija et al., 2000). In view of these facts, the changes in cell viability and proliferation observed in our experiments represent practically the result of antioxidant action of CA via the reduction of indirect DNA damage caused by ROS.

Differences in the proliferation of CA- and α-tocopherol-treated cell populations may be caused by the higher antioxidant potential of CA in comparison with α-tocopherol. This fact is also supported by the results from different antioxidant assays *in vitro* (Maruta et al., 1995; Chen and Ho, 1997). A similar effect was also reported on human lymphocytes using a comet assay for the evaluation of DNA damage (Szeto and Benzie, 2002).

The sole UVC-protective effect of a plant extract was demonstrated in NIH/3T3 mouse fibroblasts using the comet assay. Ku-35 was the ethanol extract of *Prunus persica* flowers and contained also polyphenolic compounds. The possible action mechanism of Ku-35 against UVC- and UVB-induced DNA damage was ascribed to its antioxidant activity and the efficiency of Ku-35 was higher in comparison with α-tocopherol used as a reference compound (Heo et al., 2001).

However, the increased protection of CA-treated cell populations against UVC may not only be due to the antioxidant action of CA that reduces oxidative DNA damage, but also due to the protection of irradiated cells against oxidative damage of lipid-rich membranes. The strong inhibition of lipid peroxidation by caffeic acid was shown with prevention of human LDL oxidation (Nardini et al., 1995). This UV-protective effect of CA was also described on both cellular (Vieira et al., 1998) and cell-free systems (Laranjinha et al., 1994; Laranjinha and Cadenas, 1999; Hung and Yen, 2002).

In conclusion, our data indicate that CA in the concentrations used can protect cell populations against the cytotoxic effect caused by UVC irradiation. This protective effect is probably based on the antioxidant and scavenger activities of CA, which can reduce the oxidative damage both of DNA and lipid-rich membranes. Despite the fact that the primary damage of DNA induced by UVC irradiation, i.e. CPD and (6-4)PP formation, cannot be reduced by CA treatment, the marked protective effect of CA via its antioxidant properties indicates an important role of plant polyphenolic compounds in the protection of skin cells against UV radiation. Further studies aimed at the evaluation of other different parameters of cellular damage after UVC irradiation will be planned to explore the main possible mechanism of the protective action of CA treatment.

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