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ANTIOXIDANT EFFICACY OF NATURAL COMPOUNDS OF VEGETAL ORIGIN IN HUMAN IMMORTALIZED KERATINOCYTES

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Antioxidant efficacy of natural compounds of vegetal origin in human immortalized keratinocytes

Efficacia antiossidante di composti naturali di origine vegetale nei cheratinociti umani immortalizzati

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Riassunto - Abstract

Melanoma and non-melanoma skin cancers are among the most prevalent cancers in the human population and exposure to ultraviolet (UV) light is well-established as the major etiologic factor. Free radicals are generated by normal physiologic processes, including aerobic metabolism and inflammatory response, but may inflict cellular damage when generation is increased and antioxidant defence mechanisms are overwhelmed. The hypothesis of an important role of free radicals in skin carcinogenesis is supported by several evidences including the finding that reactive oxygen species are generated following UV skin irradiation and that the natural antioxidant defences in skin are depleted after UV exposure. Moreover, it has been found that supplementation with antioxidants can inhibit skin carcinogenesis. These findings provide a promising rationale for the development of new antioxidant strategies in the prevention and therapy of skin cancer. In this paper, we evaluated the antioxidant efficacy of different natural compounds of vegetal origin in two different lines of immortalized human keratinocytes both in basal conditions and after exposure to hydrogen peroxide as free radical generator and we found that the compounds, although at different degree, were all able to inhibit reative oxygen species production in this cell model.

KEY WORDS: skin diseases - melanoma - ultraviolet rays.

Il melanoma e il non melanoma skin cancer (NMSC) sono tra i tumori cutanei più diffusi nella popolazione e l'esposizione alla radiazione ultravioletta (UV) ne è sicuramente il principale fattore eziologico. I radicali liberi (RL) sono generati da normali processi fisiologici, incluso il metabolismo aerobio e la risposta infiammatoria, ma i RL possono indurre un danno cellulare quando la loro produzione è sensibilmente aumentata e i meccanismi di difesa antiossidanti risultano insufficienti. L'ipotesi di un ruolo rilevante dei RL nella carcinogenesi della pelle è supportata da diverse evidenze, inclusa la constatazione che le specie reattive dell'ossigeno sono generate in seguito all'irradiazione della pelle con i raggi UV e che i naturali meccanismi di difesa antiossidanti della pelle sono indeboliti dopo l'esposizione agli UV. Inoltre, è stato riscontrato che la supplementazione con antiossidanti può inibire la carcinogenesi cutanea. Questa realtà fornisce un promettente razionale per lo sviluppo di nuove strategie antiossidanti nella prevenzione e nella terapia del cancro alle pelle. In questo lavoro, è stata valutata l'efficacia antiossidante di differenti attivi naturali di origine vegetale in due differenti linee di cheratinociti umani immortalizzati, sia in condizioni basali che dopo esposizione a perossido di idrogeno come fonte di radicali liberi. Si è riscontrato che i composti, sebbene a differente livello, sono tutti capaci di inibire la produzione di specie reattive dell'ossigeno in questo modello cellulare.

PAROLE CHIAVE cute, malattie - melanoma - raggi ultravioletti.

The incidence of both non-melanoma and melanoma skin cancer has rapidly increased during the last decades.[1-3] Ultraviolet radiation is considered the most important causal factor in the development of skin cancer. It has been suggested that an important etiologic role is represented by both the depletion of stratospheric ozone and the recreational intensive sun-exposure.[4-6]

The carcinogenicity of UVB radiation is well established experimentally and involves a direct photochemical damage to DNA from which gene mutations arise. UVA is generally less carcinogenic than UVB radiation, but it is present more abundantly in sunlight than UVB radiation and, therefore, can contribute at a high extent to the carcinogenicity of sunlight. In contrast to UVB, UVA radiation is hardly absorbed by DNA. Its carcinogenic activity is mediated by its absorption by other molecules (endogenous photosensitizers) and reactive oxygen species can be generated that can damage DNA, membranes, and other cellular constituents.[7]

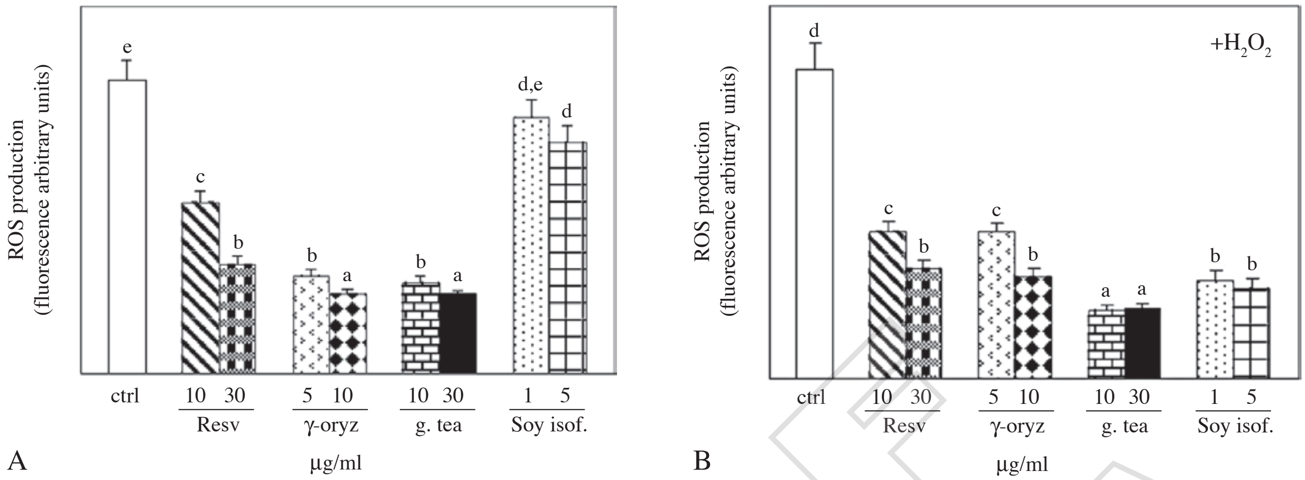

Figure 1.—Antioxidant effect of resveratrol, γ-oryzanol, green tea and soy isoflavones in HaCaT cells. Cells were exposed to two different concentrations of each compound (10 and 30 mg/mL for resveratrol and green tea; 5 and 10 μg/mL for resveratrol; 1 and 5 mg/mL for soy isoflavones) for 24 h and then ROS production was evaluated (see Materials and Methods). Panel A: ROS production in basal conditions; panel B: ROS production after exogenous stimulation with 100 μM H_2O_2. Data are the means ± SE of three different experiments. Values not sharing the same superscript are significantly different ($P<0.05$, One-way ANOVA).

The generation of ROS occurs ubiquitously in the human body during physiological processes, including aerobic metabolism, antimicrobial mechanisms and inflammatory responses. Recently, the involvement of hydrogen peroxide in the response to physiological doses of UVB has been demonstrated.[8] However, ROS may inflict cellular damage when their production increases and antioxidant defence mechanisms are overwhelmed. Such an imbalance between prooxidants and antioxidants is known as oxidative stress.[9] The consequences of an impaired cell oxidative status mainly involve damage to cell membranes through lipid peroxidation, DNA damage and sulfur-containing protein oxidation.[10]

Antioxidants have been shown to effectively reduce cutaneous carcinogenesis by decreasing oxidative stress.

The most convincing approach to confirm the involvement of ROS in carcinogenesis might arise from studies in which antioxidants have been shown to effectively modulate photocarcinogenesis. Among the antioxidant components that have been shown to inhibit UV-induced carcinogenesis there are carotenoids and different polyphenolic antioxidants.

The photoprotective effects of carotenoids were demonstrated in a number of studies in mice and evidenced by both delayed tumor appearance and reduced tumor growth.[11, 12] However, in some human studies beta-carotene not only failed to prove a protective effect but reacted as a pro-carcinogenic agent.[13]

Among the compounds tested for skin carcinogenesis prevention are also green tea polyphenols, resveratrol, curcumarin, sylmarin and ginger. Such compounds have been found to exert antitumor effects.[14] In particular, the main constituent of green tea, polyphenols, epigallocatechin-3-gallate (EGCG) by topical application are able to inhibit UVB-induced inflammatory response in human skin.[15, 16] Similar effects were obtained in different mouse skin models of chemical and UVB-induced skin carcinogenesis by the treatment with green tea polyphenols.[17, 18] Also resveratrol, a polyphenolic antioxidant present in fruits and red wine has been demonstrated to possess antitumor activity in animal models of skin carcinogenesis.[19] Concerning γ-oryzanol, a component of rice bran, an inhibitory effect has not been yet demonstrated in skin carcinogenesis, but an anticancer activity has been found for another component of rice bran, cycloartenol ferulate.[20] Soybeans and their associated food products are a rich source of flavonoids called isoflavones. The most plentiful isoflavones in soy are genistein and daidzein. Soy isoflavones have been reported to exert potent anticarcinogenic effects.[21] In particular, genistein has been reported to act as a strong inhibitor of tyrosine kinases, which are responsible for phosphorylating proteins necessary for regulation of cell division.[22] In animal studies, oral soy or genistein protected against several cancers including bladder, breast, colon, liver, lung, prostate, and skin,[23] but until now there are very few evidences that such compounds may exert their beneficial effect by acting also as antioxidants.[24]

Currently, there is much debate on the mechanisms underlying the beneficial effects exerted by these vegetal compounds in animal models of carcinogenesis and on their effectiveness in human cells.

For these reasons, in the present study, we tested both the cytotoxicity and antioxidant efficacy of some natural compounds of vegetal origin (resveratrol, γ-oryzanol, green tea, soy isoflavones) in two different lines of immoralized human keratinocytes at different degree of differentiation (HaCaT and NCTC 2544) and we also verified if such com-

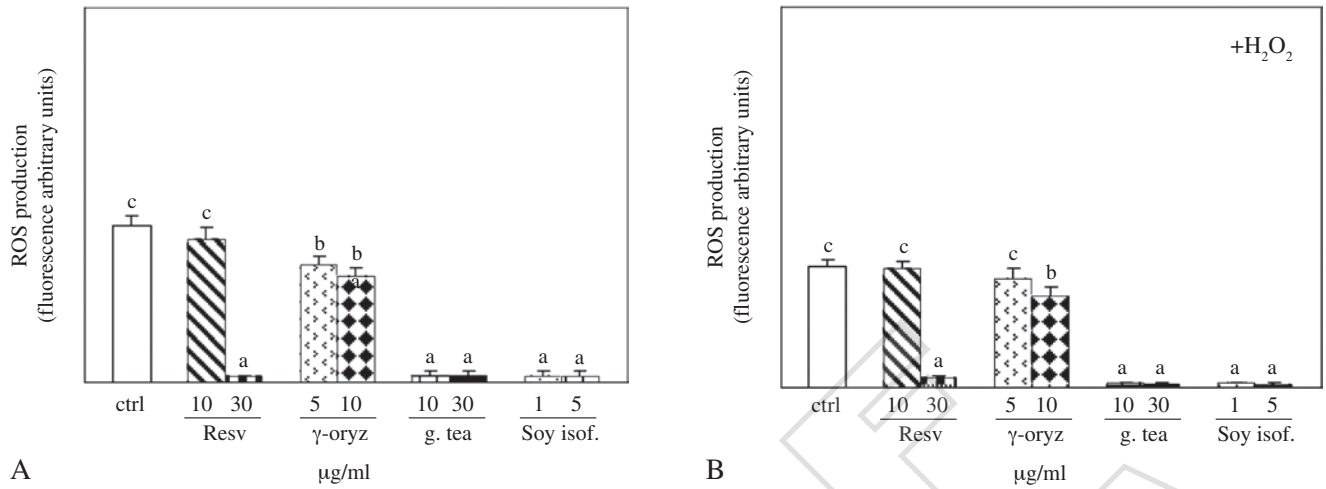

Figure 2.—Antioxidant effect of resveratrol, γ-oryzanol, green tea and soy isoflavones in NCTC 2544 cells. Cells were exposed to two different concentrations of each compound (10 and 30 mg/mL fro resveratrol and green tea; 5 and 10 µg/mL for resveratrol; 1 and 5 mg/mL for soy isoflavones) for 24 h and then ROS production was evaluated (see Materials and Methods). A) ROS production in basal conditions; B) ROS production after exogenous stimulation with 100 µM H_2O_2. Data are the means ± SE of three different experiments. Values not sharing the same superscript are significantly different ($P<0.05$, one-way ANOVA).

pounds may exert an additive antioxidant effect when used in combination.

Materials and methods

Cell lines and treatments

HaCaT human normal immortalized keratinocytes were purchased from ATCC (Rockville, MD, USA). NCTC 2544 human normal immortalized keratinocytes were kindly gifted by Dr. R. De Bellis (University of Urbino, Urbino, Perugia, Italy). Cells were grown in DMEM medium containing 2 mM glutamine and antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin) and supplemented with 10% fetal bovine serum. Cells were maintained at 37 °C in a humidified atmosphere containing 5% CO_2. Cell lines were serially subcultured by treatment with trypsin-EDTA and seeded twice a week at a density of $3x10^5$ cells/mL.

The liophylized resveratrol, green tea and soy isoflavones (all obtained from Biogena, Modena, Italy) were delivered to cell as dimethyl sulfoxide (DMSO) stock solutions (10 mg/mL), while γ-oryzanol (Biogena, Bergamo, Italy) was delivered to cells in acetone (stock solution: 6.7 mg/mL). Control cells were treated with the same amount of vehicle alone. The final DMSO and/or acetone concentration never exceeded 0.5% (v/v).

Measurement of ROS

HaCaT and NCTC 2544 keratinocytes were seeded in six-well plates at the density of 150000 cells/well. After 24 h, cells were exposed to varying concentrations (1-30 µg/mL) of resveratrol, green tea, γ-oryzanol and soy isoflavones. After 24-h treatment, cells culture medium was removed and replaced with phosphate buffer saline (PBS) to evaluate ROS production using the di(acetoxymethyl ester) analog (C-2938) of 6-carboxy-2′,7′-dichlorodihydrofluorescein diacetate (DCF) (Molecular Probes, Inc., Eugene, OR, USA) as previously described.[25] Briefly, cells were incubated at the presence of the fluorescent probe at the concentration of 10 µM for 30 min at 37 °C in the dark. Fluorescent units were measured by use of a CytofluorTM 2300/2350 Fluorescence Measurement System (Millipore Corp., USA) at 502 nm excitation, 504 nm emission. Background fluorescence was subtracted from the measured values. All the compounds tested did not alter the basal fluorescence of DCF. After the first measurement, cells were incubated at the presence of hydrogen peroxide (H_2O_2, 100 µM) for 15 min under the same cell culture conditions and fluorescence after the pro-oxidant addition was measured.

Statistical analysis

Data were analyzed using the one-way analysis of variance (ANOVA), followed by Tukey's test. Values were considered significantly different at $P<0.05$.

Results

Preliminary experiments performed by using the Trypan blue dye exclusion method indicated that the different compounds analyzed did not induce cytotoxic effects in both the cell lines studied when used at concentrations in the range of 1-30 µg/mL. The only exceptions were represented by soy isoflavones and γ-oryzanol which induced cell necrosis at concentrations higher than 5 µg/mL and 10 µg/mL, respectively (data not shown).

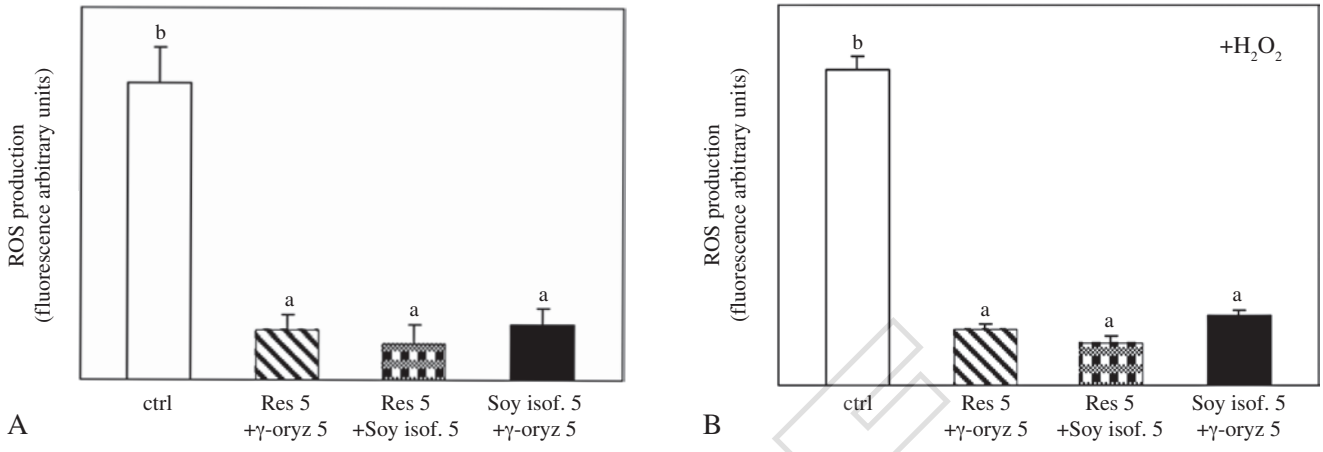

Figure 3.—Antioxidant effect of different combinations of resveratrol, soy isoflavones and γ-oryzanol in HaCaT cells. Cells were exposed to the combinations: resveratrol and γ-oryzanol; resveratrol and soy isoflavones and γ-oryzanol by using all the compounds at the concentration 5 μg/mL for 24 h and then ROS production was evaluated (see Materials and Methods). A) ROS production in basal conditions; B) ROS production after exogenous stimulation with 100 μM H_2O_2.

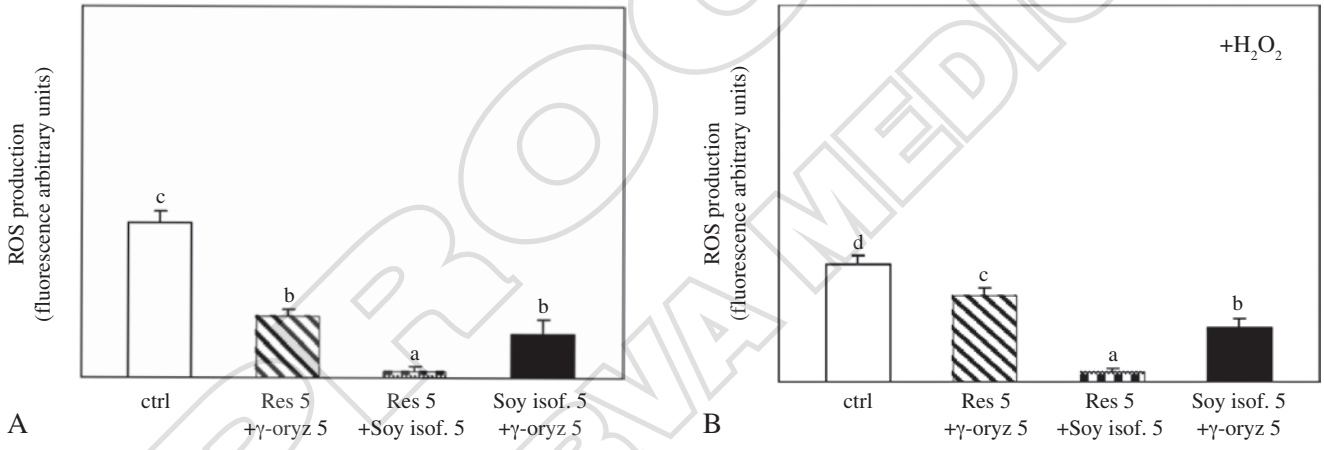

Figure 4.—Antioxidant effect of different combinations of resveratrol, soy isoflavones and γ-oryzanol in NCTC 2544 cells. Cells were exposed to the combinations: resveratroland γ-oryzanol; resveratrol and soy isoflavones and soy isoflavones and γ-oryzanol by using all the compounds at the concentration 5 μg/mL for 24 h and then ROS production was evaluated (see Materials and Methods). A) ROS production in basal conditions; B) ROS production after exogenous stimulation with 100 μM H_2O_2.

We observed that NCTC 2544 cells produce less ROS as compared to HaCaT cells, both in basal conditions and after stimulation with H_2O_2 (Figures 1, 2). All the compounds analyzed demonstrated to possess antioxidant activity. In particular, in HaCaT cells (Figure 1A), the most effective antioxidant was found to be γ-oryzanol, that reduced the basal ROS production by about 75% with respect to control conditions when used at the concentration of 10 μg/mL. Comparable antioxidant efficacy was found for green tea, but such an effect was obtained only with the higher concentration tested (30 μg/mL). The production of ROS was reduced by about 62% with resveratrol (30 μg/mL), whereas soy isoflavones, which were tested only at the doses of 1-5 μg/mL (due to their cytotoxic activity), were able to maximally inhibit ROS production by only 20% with respect to control cells. Slightly different results were obtained in NCTC 2544 cells. In particular, the highest antioxidant efficacy in these cells was observed for soy isoflavones (1 and 5 μg/mL) and for green tea. Both the compounds almost abolished ROS production in basal conditions with respect to control cells (Figure 2A). Also resveratrol at the higher concentration analyzed (30 μg/mL) was found to significantly inhibit ROS production by about 100%, whereas γ-oryzanol showed the lower antioxidant efficacy (28% ROS inhibition with respect to control cells). A similar pattern of antioxidant efficacy was observed after exogenous stimulation with H_2O_2 (Figures 1B and 2B).

In these experiments, we also wished to analyze if combinations of lower concentrations of the compounds studied were able to induce an additive antioxidant effect. In

particular, we analyzed ROS production in the presence of the following combinations of compounds: resveratrol and γ-oryzanol; resveratrol and soy isoflavones and soy isoflavones and γ-oryzanol. All the compounds were used at relatively low concentration (5 μg/mL). We observed that all the combinations analyzed induced a significant inhibition of ROS production in HaCaT cells, both in basal conditions and after exogenous stimulation with H_2O_2 (90% and 85% reduction in basal conditions and after H_2O_2 stimulation, respectively) (Figure 3). As far as NCTC 2544 cells are concerned, although all the combinations showed antioxidant efficacy, we observed that the most powerful was resveratrol and soy isoflavones (92% and 91% ROS inhibition, in basal conditions and after H_2O_2 stimulation, respectively) (Figure 4).

Conclusions

From our results, we can conclude that all the compounds studied are able to induce a significant antioxidant effect in both the cell lines analyzed. Moreover, additive antioxidant effect were observed when such compounds were administered in combinations, even if used at lower concentrations than when were administered singularly. On these bases we suggest the potential efficacy of combinations of these compounds if added in sunscreen formulas.

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