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Protective effect of hydroxytyrosol against oxidative stress in kidney cells

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Title
Effetto protettivo dell'idrossitirosolo contro lo stress ossidativo in cellule renali

Key words
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Parole chiave
Idrossitirosolo, perossidazione lipidica, acidi grassi, colesterolo, LLC-PK1

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Summary
Bioavailability studies in animals and humans feeded with extravirgin olive oil have demonstrated that hydroxytyrosol, the major simple phenolic compound present in extravirgin olive oil, is dose-dependently absorbed and excreted. Once absorbed, it undergoes an extensive metabolism and both hydroxytyrosol and metabolites are present in high amount in the kidney, where they may exert an antioxidant action. A dietary intake of antioxidants has an important role in the prevention of oxidative stress induced renal dysfunction. In this study we monitored the ability of hydroxytyrosol to protect renal cells (LLC-PK1) following oxidative damage induced by H_2O_2. Oxidative stress was evaluated by monitoring the changes of the membrane lipid fraction. Hydroxytyrosol exerted a significant antioxidant action, inhibiting the production of fatty acids hydroperoxides and 7-ketocholesterol, major oxidation products of unsaturated fatty acids and cholesterol, and protecting the cells against H_2O_2-induced death.

Riassunto
Diversi studi *in vivo* hanno dimostrato che l'idrossitirosolo, il principale fenolo semplice presente nell'olio extravergine d'oliva, viene assorbito ed escreto in maniera dose dipendente. Una volta assorbito, l'idrossitirosolo viene metabolizzato e si ritrova come tale o come metabolita nel rene, dove potrebbe esercitare un'azione antiossidante. L'apporto di antiossidanti con la dieta è importante nella prevenzione di numerose nefropatie, nelle quali è implicato lo stress ossidativo. In questo lavoro abbiamo valutato la capacità dell'idrossitirosolo di inibire il danno ossidativo in cellule renali (LLC-PK1), trattate con H_2O_2. Il danno ossidativo è stato valutato attraverso il monitoraggio delle modificazioni della frazione lipidica di membrana. L'idrossitirosolo ha mostrato un'efficace azione antiossidante, limitando la produzione di acidi grassi idroperossidi e 7-chetocolesterolo, prodotti di degradazione degli acidi grassi insaturi e del colesterolo, e un'azione protettiva contro la morte cellulare indotta dal H_2O_2.

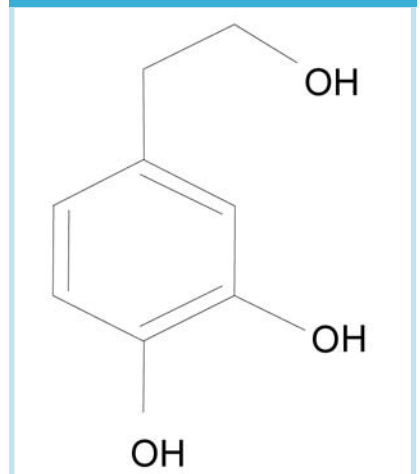
Introduction

Extravirgin olive oil, the principal fat component of the Mediterranean diet, contains a series of phenolic minor components that grant its particular aroma and taste and posses important antioxidant activities (1).
These compounds, together with other components, such as α-tocopherol, contribute to the protection of olive oil against auto-oxidation, and have been demonstrated to exert many potentially beneficial effects both *in vitro* and *in vivo* (2-5)
The major simple phenolic compound in extravirgin olive oil is hydroxytyrosol (HT) (Fig. 1), present as simple phenol or in a conjugated form. Recent *in vitro* studies demonstrate that conjugated forms of olive oil polyphenols are hydrolysed under condition similar to those expected during the transition through the stomach *in vivo*; these results suggest that the amount of HT is increased after ingestion (6, 7).
Bioavailability studies in animals and humans have demonstrated that HT is bioavailable: it is dose-dependently absorbed and excreted (8), and it distributes in few organs with a preferential renal uptake (9).
As HT is present in high amount in the kidney (10) an antioxidant action in this compartment may be supposed.
Oxidative stress mediates a wide range of renal impairment, from acute renal failure (11, 12), to obstructive nephropathy (13), hyperlipidemia (14) and glomerular damage (15).
In the present study, we investigated the protective effect of hydroxytyrosol against H_2O_2-induced injury.
We used the LLC-PK1 cell line, which maintains the characteristics of renal proximal tubular cells (16) and has been used extensively *in vitro* studies to examine both physiological and pathophysiological mechanisms in renal tubules (16, 17).
We measured the changes of the membrane lipid profile induced by the oxidizing action of H_2O_2, and the protective effect of HT; the ability of HT to inhibit H_2O_2 induced death was also evaluated.

Materials and methods

Cell culture. LLC-PK1 cells (a porcine renal epithelial cell line with proximal tubule epithelial characteristics) were obtained from the European Collection of Animals Cell Cultures, ECACC (Salisbury, UK) and grown in Medium 199, containing 10% foetal calf serum and 1% penicillin-streptomycin, at 37°C, under a humidified atmosphere of 5% CO_2. For experimental studies cells were grown to sub-confluence in Petri dishes.
Protective activity. In order to assess the protective effect of hydroxytyrosol (HT) against H_2O_2 induced toxicity, cells were pre-treated with the tested compound (5 and 10 μM; 30 min) prior to H_2O_2 (150 μM; 1h) exposure. The cytotoxic effect was assessed by the MTT colorimetric assay (18).
Antioxidant activity. Cell oxidative stress was induced by H_2O_2 (100 μM). Three sets of cell treatments were performed: a) cells exposed to H_2O_2 in PBS for 60 min; b) cells pre-treated with HT (1÷10 μM) 30 min before H_2O_2 treatment; c) control cells.
After H_2O_2 treatment, the cells were scraped on ice and centrifuged at 1.700 rpm at 4°C for 5 min. After centrifugation pellets were separated from supernatants and the pellet was used for lipid analyses.
Lipid extraction and determination of fatty acids and cholesterol. Lipids were extracted from the cell pellet dissolved in 12 ml of $CHCl_3$/MeOH

Figura 1 - Structure of hydroxytyrosol.



(2/1, v/v) solution as indicated by the Folch et al. procedure (19); an aliquot of 1.5 ml was used to quantify the lipid fraction as previously described (20). Separation of cholesterol and free fatty acids was obtained by mild saponification (21).
Separation of UFA and cholesterol was carried out with a HPLC-DAD system, as detailed in a previous paper (22).
Statistical analysis. INSTAT software (GraphPad software, San Diego, CA) was used to calculate the means and standard deviations of three independent experiments (n=9 for each sample/condition). One-way ANOVA was used to test whether the group means differed significantly.

Results

In order to induce oxidative stress, LLC-PK1 cells were treated for 1h with H_2O_2 (100 μM), the highest non toxic concentration able to induce a significant oxidative damage.
To investigate membrane oxidative injury, the lipid fraction was extracted and the modification of the more oxidizable membrane lipids, unsatured fatty acid (UFA) and cholesterol, assessed.
The treatment with H_2O_2 induced a significant decrease of the concentration of the major UFA (expressed as total UFA) around 20-30% of the initial level as shown in figure 2. Pretreatment with the HT (5 μM) preserved the concentration of all the measured UFA. The depletion of the UFA concentration was inversely correlated with the increase of their major oxidation products, the fatty acids hydroperoxides (HP). In the H_2O_2 treated cells the amount of HP was doubled with respect to the controls, while in the samples pretreated with HT, at all the tested concentrations, HP value remained at the control level.

Figure 3 shows the concentration of cholesterol and its major oxidation product 7-ketocolesterol (7-keto), measured in the control and treated cells. Cholesterol level was significantly decreased after H_2O_2 treatment, with a reduction of 15%, and a related increase of 7-keto, three times the initial value, was also observed. Pretreatment with the phenolic compound significantly protected cholesterol from oxidation: there was no loss of choleste-

Figura 2 - Values of total unsaturated fatty acids (UFA) and fatty acids hydroperoxides (HP) measured in LLC-PK1 cells after 1h incubation with 100 μM H_2O_2 and treated with HT (1÷10 μM), expressed as % of the control values (UFA tot 198,89±34,55 μg/mg protein; HP 0,35±0,06 nmol/ng protein).
*** = p<0.001 versus controls, °°° = p<0.001; °° = p<0.01 versus H_2O_2 treated

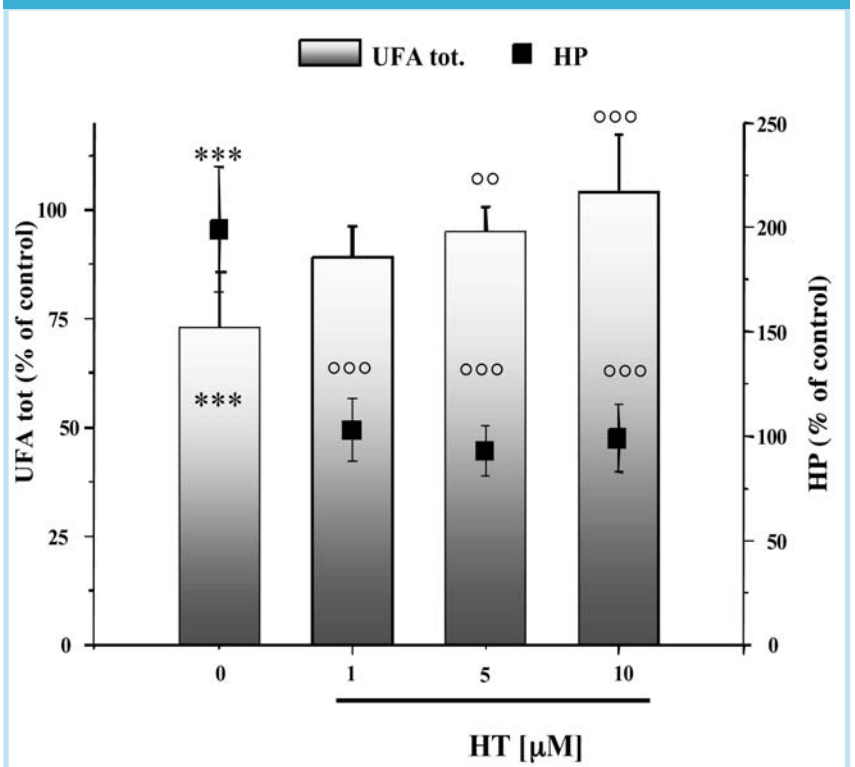
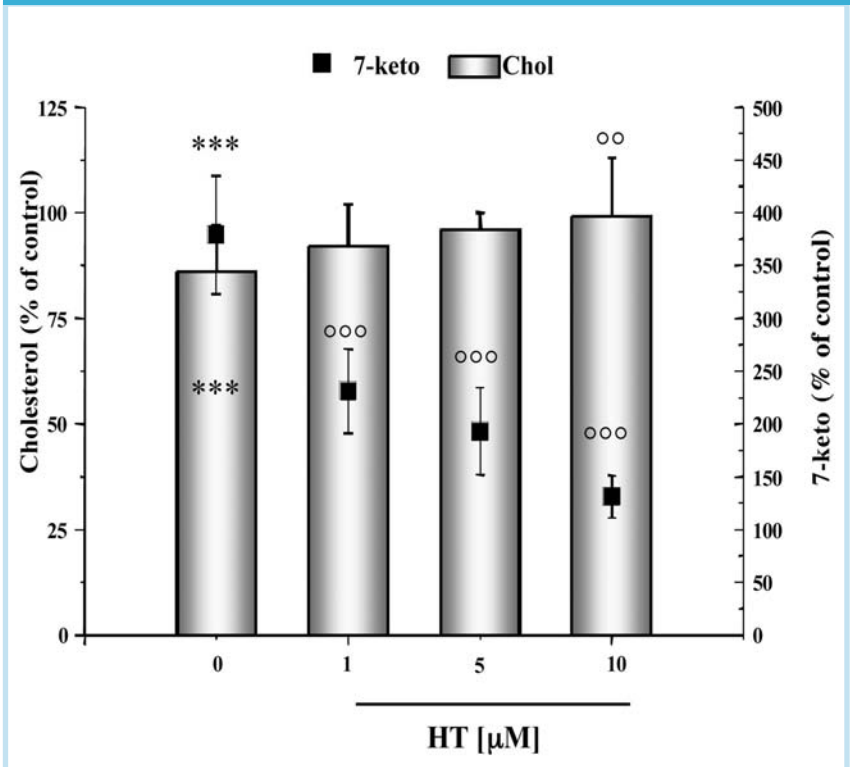


Figura 3 - Values of cholesterol and 7-ketocholesterol (7-keto) measured in LLC-PK1 cells after 1h incubation with 100 μM H_2O_2 and treated with HT (1÷10 μM), expressed as % of the control values (chol 70,89±9,21 μg/mg protein, 7-keto 0,11±0,02 μg/mg protein).
*** = p<0.001 versus controls, °°° = p<0.001; °° = p<0.01 versus H_2O_2 treated



rol in the sample treated with 10 μM of HT, and the concentration of 7-keto was significantly lower from 1 μM.

In order to assess the ability of HT to exert a protection against H_2O_2-mediated cytotoxicity, cells were pretreated with HT (5-10 μM for 30 min) prior to the treatment with H_2O_2 150 μM, concentration that induced a 50% of death.

As shown in figure 4 the protection of HT against cell death was significant at all concentrations tested.

Conclusion

Several studies demonstrated that HT possess different biological activities. An antioxidant action on culture cells has also been pointed out: preincubation of intestinal Caco-2 cells with HT prevents H_2O_2-mediate oxidative damage (23); HT exerts a protective effect against the H_2O_2-induced oxidative hemolysis and MDA formation in red blood cells (4).

In order to investigate the ability of HT to protect the kidney cells against H_2O_2-induced oxidative damage, we focused our attention on the modifications of the membrane lipid components more susceptible to oxidation, UFA and cholesterol, measuring the concentration of their major oxidation products HP and 7-keto. The variation of these parameters resulted a sensible and accurate markers of the oxidative damage.

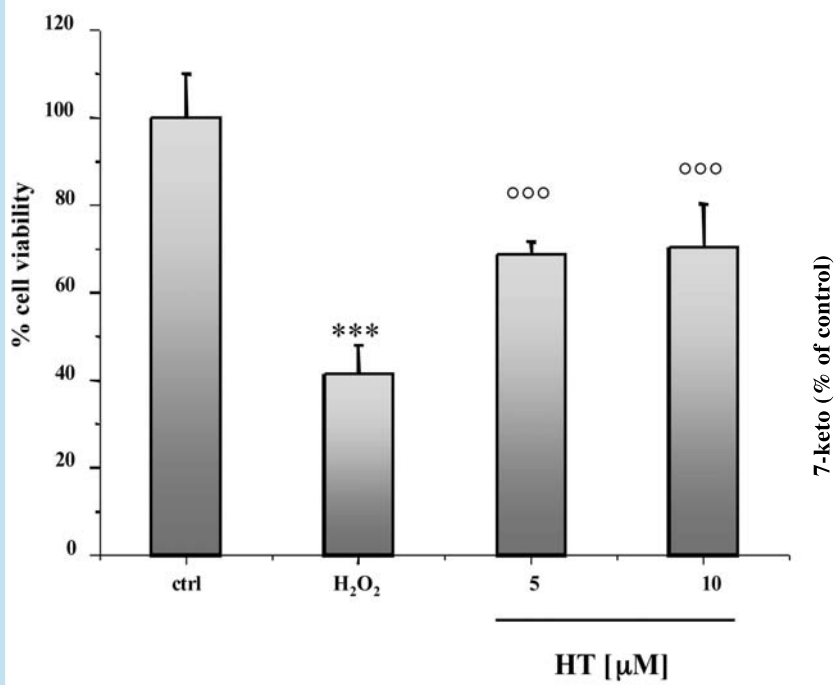
HT protected against oxidative stress at all concentrations tested: the level of membrane lipids was preserved and there was no significant detection of oxidation products. HT protective action may be due to a direct radical scavenging action, as it has been reported that HT can react with H_2O_2 and other aqueous radical near the cell membrane.

HT was also able to protect LLC-PK1 cells against oxidative stress induced cellular injury, as evaluated by measuring MTT reduction as an index of cell viability.

The cytoprotective effect of HT appears dependent on its direct antioxidant potential (3, 24, 25), but also other mechanism could be involved. In summary our result extend the biological effect attributed to HT.

HT contained in high amount in

Figura 4 - Value of cell viability after 1h of incubation with 150 μM H_2O_2 and treated with HT (5 and 10 μM).
*** = p<0.001 versus controls, °°° = p<0.001 versus H_2O_2 treated



extra virgin olive oil, may be helpful in designing dietary strategies to increase the antioxidant potential.

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