

Full Length Research Paper

## Suppressive effects of Hydroxytyrosol on oxidative stress in erythrocytes

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We investigated the oxidative modifications of lipids, proteins and antioxidants enzymes activities, in erythrocytes treated with H<sub>2</sub>O<sub>2</sub> in the presence or absence of hydroxytyrosol (HT) at 37°C for 30 min. Conjugated dienes level was measured as biomarker of lipid peroxidation. Protein carbonyl level was measured as biomarker of protein oxidation. The antioxidants enzymes activities were evaluated by superoxide dismutase and catalase activities determinations. Conjugated dienes levels decreased in erythrocytes after hydroxytyrosol treatment as compared to erythrocytes treated with 0.2 mM H<sub>2</sub>O<sub>2</sub>. Protein carbonyl levels increased in erythrocytes after hydroxytyrosol treatment as compared to erythrocytes treated with 0.2 mM H<sub>2</sub>O<sub>2</sub>. In addition, this study showed a prospective protective action of HT of human erythrocytes from the attack of reactive oxygen species. In fact, a considerable increase of superoxide dismutase activity by 40% was showed after incubation of erythrocytes with HT at dose 5 µg/ml. Although, there were no significant differences in catalase activity in erythrocytes after hydroxytyrosol treatment as compared to erythrocytes treated with 0.2 mM H<sub>2</sub>O<sub>2</sub>. This study demonstrates that hydroxytyrosol prevents protein oxidation and lipid peroxidation caused by H<sub>2</sub>O<sub>2</sub> treatment using erythrocytes cell model.

**Key words:** Hydroxytyrosol, thiol, erythrocytes, conjugated dienes, malondialdehyde, superoxide dismutase, catalase, peripheral blood mononuclear (PBL), peripheral blood mononuclear.

### INTRODUCTION

The phenolic compounds of olive oil are a complex mixture of compounds that include 3,4-dihydroxyphenylethanol (hydroxytyrosol) (HT), 4-hydroxyphenylethanol (tyrosol), 4-hydroxyphenylacetic acid, protocatechuic acid, caffeic acid and p-coumaric acid, among others (Litridou et al., 1997; Caponio et al., 1999). HT have been shown to possess anti-inflammatory, bactericidal and bacteriostatic activities (Yang et al., 2007). HT possesses marked antioxidant activity and is a good radical scavenger (De La Puerta et al., 1999; Manna et al., 1999). Oxygen free radicals or, more generally, (ROS), are products of normal cellular metabolism. ROS are well recognised for playing a dual

role as both deleterious and beneficial species, since they can be either harmful or beneficial to living systems (Valko et al., 2006). Damage has been reported to occur on all components of biological systems (e.g., DNA, RNA, lipids, proteins, carbohydrates, low-molecular-mass species, antioxidants) due to the high reactivity of many oxidants (Clare et al., 2009). The excess ROS can damage cellular lipids, proteins, or DNA inhibiting their normal function. Because of this, oxidative stress has been implicated in a number of human diseases as well as in the ageing process (Valko et al., 2006). The delicate balance between beneficial and harmful effects of free radicals is a very important aspect of living organisms

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and is achieved by mechanisms called “redox regulation” (Valko et al., 2006). The generation of ROS is controlled by a large number of antioxidant systems which act as protective mechanisms. These antioxidant control systems consist of enzymes and antioxidant substrates. Among antioxidant enzymes, the superoxide dismutase (SOD) catalyzes dismutation of superoxide to hydrogen peroxide ( $H_2O_2$ ) and molecular oxygen. The decomposition of hydrogen peroxide to nontoxic compounds is the main function of catalase (CAT). SOD and CAT usually act in a synergetic manner (Mates et al., 1999). An imbalance between ROS and antioxidant defences creates oxidative stress (Sies, 1991). This disturbance of the oxidative status is the result of either an excessive ROS production or a deficiency in antioxidant systems activities. However, no studies concerning the effect of HT on the oxidation of proteins, conjugated dienes (CD) and antioxidant enzymes activities in erythrocytes are available in the literature.

This study examined the hypothesis that HT supplementation decreases protein oxidation, CD level and modulate antioxidant enzymes activities in erythrocytes model treated with 0.2 mM  $H_2O_2$ . The lipids, proteins damages, and antioxidant enzymes activities were then determined after  $H_2O_2$  treatment, by measuring the level of conjugated dienes as a parameter of lipid peroxidation, protein carbonyl as parameter of protein oxidation, and SOD and CAT activities determination as markers of antioxidant enzymes.

## MATERIALS AND METHODS

Hydroxytyrosol was obtained from sigma. All other chemicals were supplied by Merck (Germany).

### Erythrocytes preparation

Blood was collected into heparinised tubes from 30 normal human volunteers according to a protocol approved by the Institutional Human Review Board for the Protection of Human Subjects. Citrate-blood was centrifuged, the clear plasma and buffy coat layers were discarded. The red cells were washed with cold 0.15 M NaCl solution three times after a 1- to-10 dilution.

### *In vitro* treatment with hydroxytyrosol

The first group, erythrocytes were incubated with a freshly prepared stock solution of  $H_2O_2$  (0.2 mM) at 37°C for 30 min. The second group was preincubated with different concentrations of hydroxytyrosol (1, 5, 10, 15 and 20  $\mu\text{g/ml}$ ) for 4 h followed by 0.2 mM  $H_2O_2$ . After incubation, erythrocytes were washed one time with PBS (Buffer phosphate saline), the level of erythrocytes CD and the activities of SOD and CAT were carried out. One control contained erythrocytes only (without hydroxytyrosol), served as negative controls.

### Conjugated dienes (CD)

In 30 samples, conjugated diene level was evaluated as described by Kurien and Scofield (2003) with modification. 25  $\mu\text{l}$  erythrocytes

suspension were extracted with 3 ml chloroform/methanol (2:1, v/v). After centrifugation at 200 g for 15 min, 2 ml of organic phase was transferred into another tube and dried at 45°C. The dried lipids were dissolved in 2 ml of methanol and absorbance at 233 nm was determined. It corresponds to the maximum absorbance of the extracted compounds.

### Protein carbonyl (PC) levels

PC levels were measured according to the method based on spectrophotometric detection of the reaction of 2,4-dinitrophenylhydrazine (DNPH) with protein carbonyl to form protein hydrazones [30], in 30 samples. Briefly, after precipitation of protein with an equal volume of 1% trichloroacetic acid, the pellet was resuspended in 10 mM DNPH in 2 N HCl or with 2 N HCl as control blank. Next, after the washing procedure with 1:1 ethanol/ethylacetate, the final pellet was dissolved in 6 M guanidine Merck (Germany). The carbonyl group was determined from the absorbance at 370 nm. The results were expressed as nanomoles of carbonyl groups per milligram of protein with an extinction coefficient of 22,000  $M^{-1} \text{cm}^{-1}$ .

### Catalase activity determination

Catalase activity was measured in 30 samples as described previously by Aebi (1984). This method is based on the principle that the absorbance at 240 nm decreases because of  $H_2O_2$  dismutation. The extinction coefficient of 43.6  $L \text{mol}^{-1} \text{cm}^{-1}$  for  $H_2O_2$  was used for calculation. One unit is defined as the amount of  $H_2O_2$  converted into  $H_2O$  and  $\frac{1}{2}O_2$  in 1 min under standard conditions, and the specific activity is reported as units per milligram of protein.

### Superoxide dismutase activity determination

SOD activity was determined in 30 samples by spectrophotometry (420 nm) using pyrogallol assay as described previously by Marklund and Marklund (1974) and modified as follows: the autoxidation rate of pyrogallol in Tris-cacodylic acid-diethylenetriaminepenta-acetic acid (DTPA) buffer (pH 8–8.2) was determined (A1). Pyrogallol autoxidation was evaluated under the same conditions after addition of 25  $\mu\text{l}$  of supernatant (A2). The inhibition percentage of pyrogallol oxidation was determined using the following formula: % Inhibition =  $((A1 - A2)/A1) * 100$ .

### Protein quantification

Protein levels were determined using protein assay kit Biorad and bovine serum albumin served as the standard.

### Statistical analysis

Statistical analysis was carried out by Student's t-test value, to assess the statistical significance of the obtained differences between treated erythrocytes with hydroxytyrosol and  $H_2O_2$  as compared to erythrocytes treated with 0.2 mM  $H_2O_2$  alone. A P value < 0.05 was considered to be statistically significant.

## RESULTS

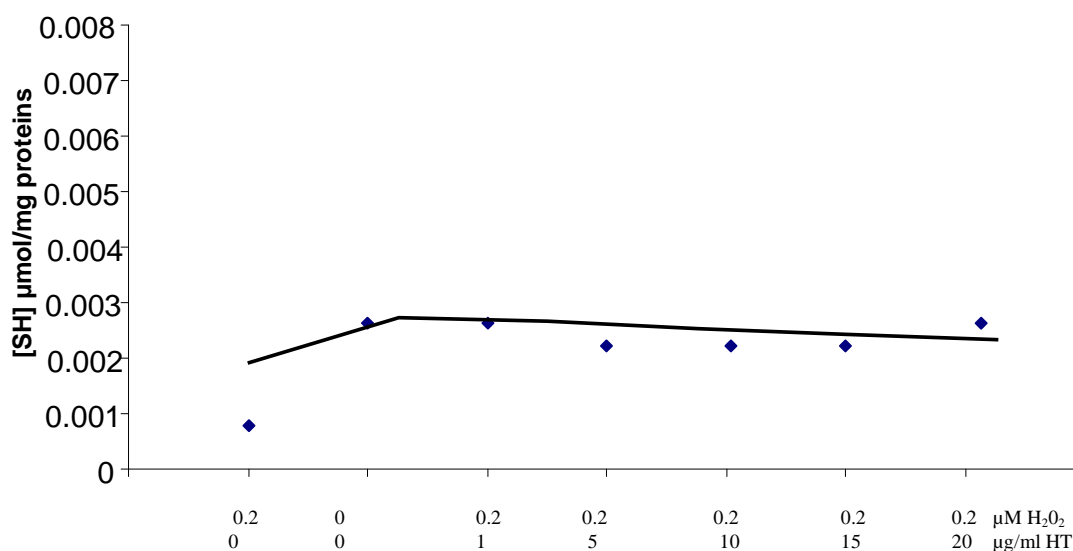
### Evaluation of lipid peroxidation

0.2 mM  $H_2O_2$  caused an increase in CD levels ( $p < 0.001$ )

**Table 1.** The effect of hydroxytyrosol on CD level.

Concentration (H <sub>2</sub> O <sub>2</sub> ) mM	Concentrations (HT) mg/ml	OD
0.02	0	0.03***
0	0	0.006
0.02	1	0.02***
0.02	5	0.018***
0.02	10	0.013***
0.02	15	0.007***
0.02	20	0.007***

0.2 mM H<sub>2</sub>O<sub>2</sub> caused an increase in CD levels ( $p = 0.000$ ). This increase was inhibited by supplementation with HT. The inhibitory effect of HT on CD level was greater with increasing concentrations of HT. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$



**Figure 1.** The effect of HT on protein oxidation. 0.2 mM H<sub>2</sub>O<sub>2</sub> caused a decrease in SH levels ( $p = 0.000$ ). Our data shows that HT supplementation prevented an increase in the protein oxidation caused by 0.2 mM H<sub>2</sub>O<sub>2</sub>. The significant effect of HT on inhibition of proteins oxidation was seen at 1 μg/ml.

(Table 1). This increase was significantly inhibited by supplementation of 1, 5, 10, 15 and 20 μg/ml HT ( $p < 0.001$ ) (Table 1). The inhibitory effect of HT on lipid peroxidation was greater with increasing HT concentrations.

#### Evaluation of protein oxidation

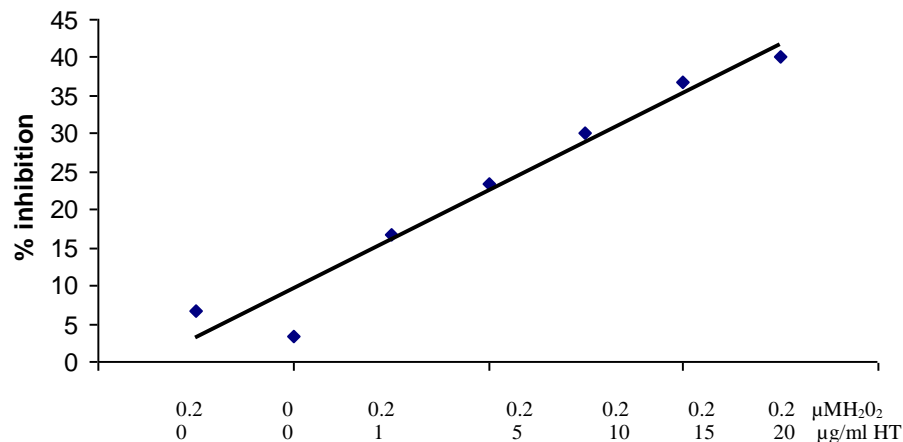
0.2 mM H<sub>2</sub>O<sub>2</sub> caused a decrease in carbonyl levels ( $p < 0.001$ ) (Figure 1). Protein oxidation was significantly inhibited by supplementation of 1, 5, 10, 15 and 20 μg/ml HT ( $p < 0.001$ ). Our data shows that the inhibitory effect of HT on protein oxidation was greater with increasing HT concentrations. 5 μg/ml of HT significantly increased protein oxidation rates by 84.68%, respectively, as compared to erythrocytes incubated only with H<sub>2</sub>O<sub>2</sub>.

#### Evaluation of SOD and CAT activities

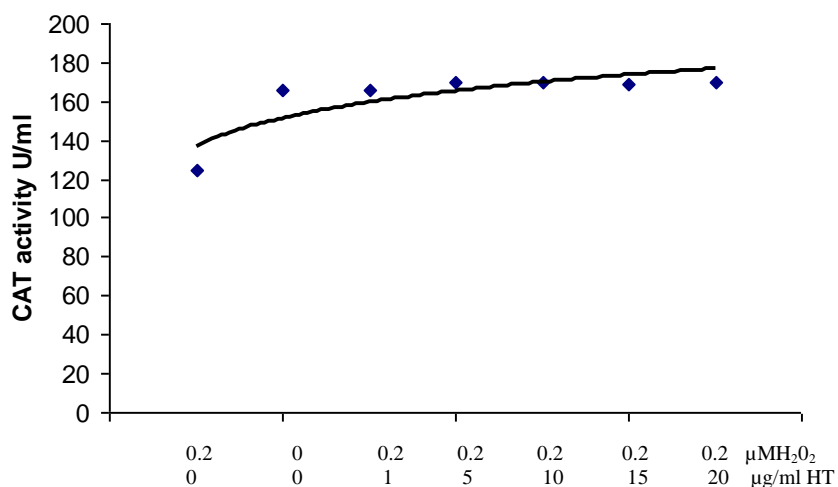
0.2 mM H<sub>2</sub>O<sub>2</sub> caused a significant decrease of SOD activity ( $p < 0.001$ ) (Figure 2). HT at concentrations as high as 1 μg/ml significantly increase SOD activity ( $p < 0.001$ ) (Figure 2). A considerable increase of superoxide dismutase activity by 40% was showed after incubation of erythrocytes with HT at dose 5 μg/ml. Although, there were no significant differences in CAT activity in erythrocytes after HT treatment as compared to erythrocytes treated with 0.2 mM H<sub>2</sub>O<sub>2</sub> (Figure 3).

#### DISCUSSION

Using erythrocytes as a model, this study demonstrates that HT can reduce oxidation of proteins and CD in



**Figure 2.** The effect of HT on SOD activity. 0.2 mM H<sub>2</sub>O<sub>2</sub> caused an increase of SOD activity ( $p = 0.000$ ). HT at concentrations as high as 1  $\mu\text{g/ml}$  significantly increase SOD activity. The effect of HT on SOD activity was greater with increasing concentrations of HT.



**Figure 3.** The effect of HT on CAT activity. No significant differences in CAT activity in erythrocytes after HT treatment as compared to erythrocytes treated with 0.2 mM H<sub>2</sub>O<sub>2</sub>.

erythrocytes exposed to H<sub>2</sub>O<sub>2</sub>. Our *in vitro* study show that in erythrocytes model, HT inhibited protein oxidation at 1, 5, 10, 15 and 20  $\mu\text{g/ml}$  concentrations. This suggests that the inhibition of oxidation of proteins may be mediated by an antioxidative effect of HT. An inhibitory effect of HT on lipid peroxidation at 1  $\mu\text{g/ml}$  was observed. Lipid peroxidation was studied extensively to investigate oxidative stress after HT treatment. HT exerts protective effects against Fe<sup>2+</sup> induced malondialdehyde (MDA) formation in Hela and DG 75 (Bouaziz et al., 2008). HT exerts protective effects against H<sub>2</sub>O<sub>2</sub>-induced malondialdehyde (MDA) formation in red blood cells (Manna et al., 1999).

The complete mechanisms by which HT modulate

antioxidant enzymes activities, in erythrocytes model are not known. The data on the modulation of antioxidants enzymes activities by HT, in erythrocytes model, are novel. Our study demonstrates that HT activates SOD that detoxifies oxygen radicals, thereby reducing the oxidative stress caused by H<sub>2</sub>O<sub>2</sub> treatment. SOD activity has been found to be increased which was therefore interpreted as an adaptative response to oxidative stress. No difference in CAT activity was observed in our study, suggesting that HT is directly increasing SOD or that some endogenous cellular mechanisms that increase SOD is activated during HT treatment. This result also suggests that there may be independent mechanisms for the activation of SOD and CAT, even though these two

enzymes function synergistically.

In conclusion, this study has demonstrated for the first time that HT can inhibit lipids peroxidation and proteins oxidation and modulate antioxidants enzymes activities, in erythrocytes model. Both peroxidation of lipids and antioxidants enzymes perturbation have been related to several types of cancers such as nasopharyngeal carcinoma (Gargouri et al., 2009), and autoimmune diseases such as lupus erythematosus, rheumatoid arthritis (Mansour et al., 2008). The evidence that HT can prevent oxidative stress, lipid peroxidation and protein oxidation needs to be explored at the clinical level to determine whether supplementation can lower levels of lipid peroxidation and protein oxidation and oxidative stress and thereby reduce the incidence of disease related to oxidative stress.

#### ABBREVIATIONS

**SOD**, superoxide dismutase; **CAT**, catalase; **PBL**, Peripheral blood mononuclear; **HT**, Hydroxytyrosol; **SH**, thiol; **ROS**, reactive oxygen species; **DNPH**, 2,4-dinitrophenylhydrazine; **CD**, Conjugated dienes; **OD**, optical density; **DTPA**, Tris-cacodylic acid– diethylenetriaminepenta-acetic acid; **H<sub>2</sub>O<sub>2</sub>**, hydrogen peroxide.

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