

*Full Length Research Paper*

# Hydroxytyrosol supplementation inhibits oxidative DNA damage, suppresses protein-lipid oxidation and modulates antioxidant enzymes in human peripheral blood lymphocytes

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We investigated the oxidative modifications of DNA, proteins and antioxidant enzymes activities, in peripheral blood lymphocytes (PBL) treated with H<sub>2</sub>O<sub>2</sub> in the presence or absence of hydroxytyrosol. Interestingly, we found a potential protection of DNA from fragmentation after hydroxytyrosol supplementation. In addition, this study showed a prospective protective action of hydroxytyrosol of PBL from the attack of reactive oxygen species. In fact, a considerable increase of superoxide dismutase and catalase activities by 76.92 and 21.67% respectively was showed after 20 µg/ml hydroxytyrosol treatment. Moreover, 20 µg/ml of hydroxytyrosol significantly decreased the lipid peroxidation and protein oxidation rates by 97.89 and 18.5% respectively. In conclusion, the present study reveals the efficacy of hydroxytyrosol in the protection of PBL of DNA fragmentation and oxidative stress induced by H<sub>2</sub>O<sub>2</sub> treatment, which may be attributed to its anti-oxidant potential.

**Key words:** Hydroxytyrosol, thiol, lymphocytes, conjugated dienes, malondialdehyde, superoxide dismutase, catalase.

## INTRODUCTION

The human cells are continuously attacked by reactive oxygen species (ROS), which arise as natural products of normal cellular machinery energy production and by exhaustive exercise or by chemical agents in the environment. In addition, many disorders and diseases such as diabetes, cancer and chronic inflammatory diseases generate ROS as part of the pathophysiologic

mechanism. The dietary intake of antioxidants is thought to play a major role in the prevention and protection from the deleterious effects of ROS and modulation of antioxidant capacity. Among compounds that have antioxidant properties, hydroxytyrosol (HT) present in the phenolic fraction of virgin olive oil, is abundant in naturally fermented table olives and can be also recovered from virgin olive oil induced various beneficial actions and prevented many diseases (Boskou, 1996). In fact, it has reported that HT has been shown to possess anti-inflammatory, bactericidal and bacteriostatic activities (Yang et al., 2007). However, any previous studies concerning the effect of HT on the oxidation of proteins, lipids peroxidation, DNA damage and antioxidant enzymes activities in peripheral blood lymphocytes (PBL) cells are available in the literature. This study examined the hypothesis that HT supplementation decreases DNA damage, protein oxidation, lipid peroxidation, and modulates antioxidant enzymes activities in PBL cells model treated with 0.2 mM H<sub>2</sub>O<sub>2</sub>. The DNA, lipids,

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**Abbreviations:** TBA, Thiobarbituric acid reactivity; MDA, malondialdehyde; SOD, superoxide dismutase; CAT, catalase; PBL, peripheral blood lymphocytes; HT, hydroxytyrosol; SH, thiol; ROS, reactive oxygen species; DNPH, 2,4-dinitrophenylhydrazine; CD, conjugated dienes; OD, optical density; TBA, thiobarbituric acid; NO, nitric oxide; DTPA, tris-cacodylic acid-diethylenetriaminepenta-acetic acid; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide.

proteins damages, and antioxidants enzymes activities were then determined after H<sub>2</sub>O<sub>2</sub> treatment, by measuring the level of malondialdehyde and conjugated dienes as parameters of lipids peroxidation, protein thiol as parameter of proteins oxidation, and superoxide dismutase (SOD) and catalase (CAT) activities determination as markers of antioxidants enzymes.

## MATERIALS AND METHODS

### Lymphocyte preparation

24 normal volunteers donor were recruited into the study after obtaining her informed consent. PBL were isolated from heparinised venous blood by sedimentation in Ficoll-hypaque (Sigma, Germany). Cells were washed three times in PBS (phosphate buffered saline) and immediately used for study.

### Proliferation characteristics of PBL cells after HT treatments

Cell viability was assessed following HT treatments by three Bromure (4.5-diméthylthiazol-2-yl)-2.5-diphényltétrazolium; MTT test; sigma, Germany). Briefly,  $2 \times 10^6$  PBL cells were stimulated with 1, 5, 10, 15 and 20 µg/ml of HT for 30 min, when the cells were in logarithmic phase growth, usually 48 h after placing them in culture. Ten microlitres of MTT (Sigma, Germany; 5 mg/ml stock in PBS) was added to each well for 4 h at 37°C; 180 µl of 50% DMSO/50% methanol was then added, and optical density (OD) at 570 nm was determined. For treated PBL and non-treated PBL, the OD was determined in three experiments.

### *In vitro* treatment with HT

The first group, PBL were incubated with a freshly prepared stock solution of H<sub>2</sub>O<sub>2</sub> (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 µg/ml) for 4 h followed by 0.2 mM H<sub>2</sub>O<sub>2</sub>. After incubation, PBL were washed one time with PBS (phosphate buffer saline), the level of PBL malondialdehyde (MDA) and the activities of SOD and CAT were carried out. One control contained PBL only (without hydroxytyrosol), served as negative controls.

### Lipid peroxidation

MDA determination was performed by the thiobarbituric acid reactive species assay. PBL suspension (30 µl) were diluted in 500 µl distilled water and 2 volumes of thiobarbituric acid (TBA) agent was added (15% trichloroacetic acid, 0.8% TBA, 0.25 N HCl) was added. The mixture was heated at 95°C for 15 min to form MDA-TBA adduct. Optical density (OD) was measured with a spectrophotometer (Biochrom, Libra S32) at 532 nm. Values were reported to a calibration curve of 1, 1, 3, 3-tetraethoxypropane.

### Conjugated dienes

Conjugated diene level was evaluated as described by Kurien and Scofield (2003) with modification. 25 µl PBL suspensions were extracted with 3 ml chloroform/methanol (2:1, v/v). After centrifugation at 3.000 rpm 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 thiol levels determination

Protein thiols were quantified spectrophotometrically using 5, 5-dithionitrobenzoic acid (DTNB); 250 µl of freshly prepared 10 mM DTNB in 0.05 M phosphate buffer pH 8, were added to 50 µl of cell lysat in 1200 µl of 0.05 M phosphate buffer. After incubation in the dark for 15 min at room temperature, the release of 5-thiobenzoic acid was quantified by measuring the absorbance at 412 nm and converted to absolute values using N-acetyl cysteine as standard (0 to 0.1 mM). A correlation coefficient of  $r^2 = 0.999$  was obtained. The absorbance of samples lacking DTNB was subtracted to account for the background absorbance at 412 nm. Samples were analysed in duplicate.

### Catalase activity determination

Catalase activity was measured as described previously by Aebi (1984). This method is based on the principle that the absorbance at 240 nm decreases because of H<sub>2</sub>O<sub>2</sub> dismutation. The extinction coefficient of 43.6 L mol<sup>-1</sup> cm<sup>-1</sup> for H<sub>2</sub>O<sub>2</sub> was used for calculation. One unit is defined as the amount of H<sub>2</sub>O<sub>2</sub> converted into H<sub>2</sub>O and ½O<sub>2</sub> 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 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 to 8.2) was determined (A1). Pyrogallol autoxidation was evaluated under the same conditions after addition of 25 µl of supernatant (A2). The inhibition percentage of pyrogallol oxidation was determined using the following formula:

$$\% \text{ Inhibition} = ((A1 - A2)/A1) \times 100.$$

### DNA fragmentation assay

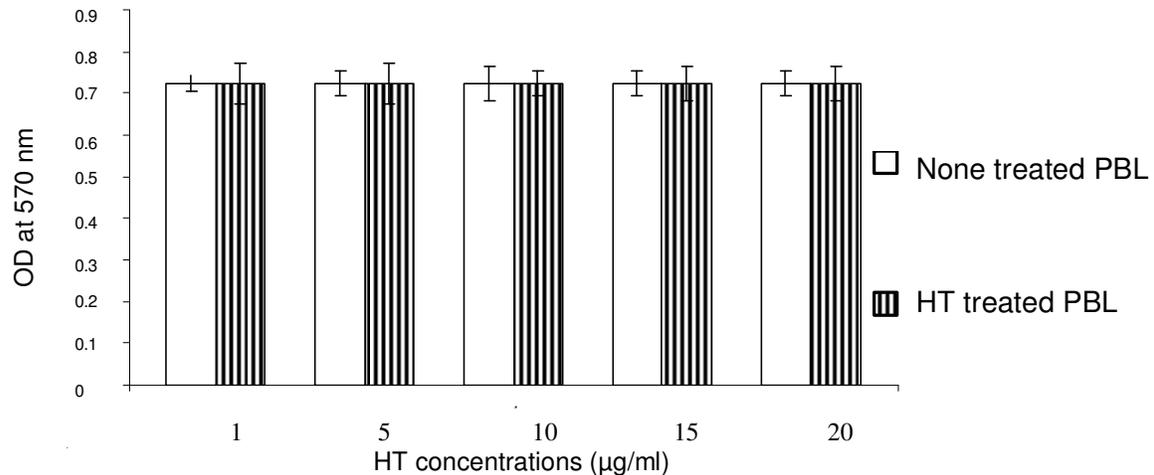
After treatment with H<sub>2</sub>O<sub>2</sub> and or HT, PBL cells were lysed in a buffer containing 10 mM Tris (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 30 min on ice. Lysates were vortexed and cleared by centrifugation at 10 000 g for 20 min. Fragmented DNA in the supernatant was extracted with an equal volume of neutral phenol chloroform isoamyl alcohol mixture (25:24:1) and analyzed electrophoretically on 2% agarose gels containing 0.1 mg/ml of ethidium bromide (Ju-Hyung et al., 2003).

### Protein quantification

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

### Statistical analysis

The statistical analysis was carried out using the student's t-test to assess the statistical significance of the differences between the control group and all concentrations (The statistical analyses were performed using IBM SPSS Statistics 19). All data are expressed as



**Figure 1.** Proliferation of HT-treated and non-treated PBL cells. The growth rate of PBL cells was determined following HT treatment and was compared with that of non-treated PBL cells. No differences in cell viability were observed for 1, 5, 10, 15 and 20 µg/ml HT concentrations.

means  $\pm$  standard error. The results among the different concentrations were evaluated by one-way ANOVA variance analysis. When a significant F ratio was found, the Turkey multi-comparison post-hoc test was used to determine the statistical significance between the means. The level of significance was taken as  $P < 0.05$ .

## RESULTS

### Effect of HT treatment on lymphocytes proliferation

The proliferation rate of lymphocytes was determined following 1, 5, 10, 15 and 20 µg/ml HT treatments using MTT test. The results were compared with those of untreated cells. No cytotoxic effect of all concentrations was observed during incubation times (Figure 1).

### Effect of HT on DNA fragmentation

To investigate the DNA fragmentation, lymphocytes were treated by 0.2 mM  $H_2O_2$  and/or 1; 5; 10; 15 and 20 µg/ml HT. Non treated lymphocyte was used as controls. DNA was isolated and analyzed by agarose gel electrophoresis. Figure 2 showed that the percentage intact DNA was potentially reduced in PBL exposed to 0.2 mM  $H_2O_2$ . However, supplementation of PBL cells with HT at 1, 5, 10, 15 and 20 µg/ml protected efficacy the cells DNA integrity and protected against  $H_2O_2$ -induced DNA damage and fragmentation.

### Evaluation of lipid peroxidation

To determine lipid peroxidation, MDA and CD levels were

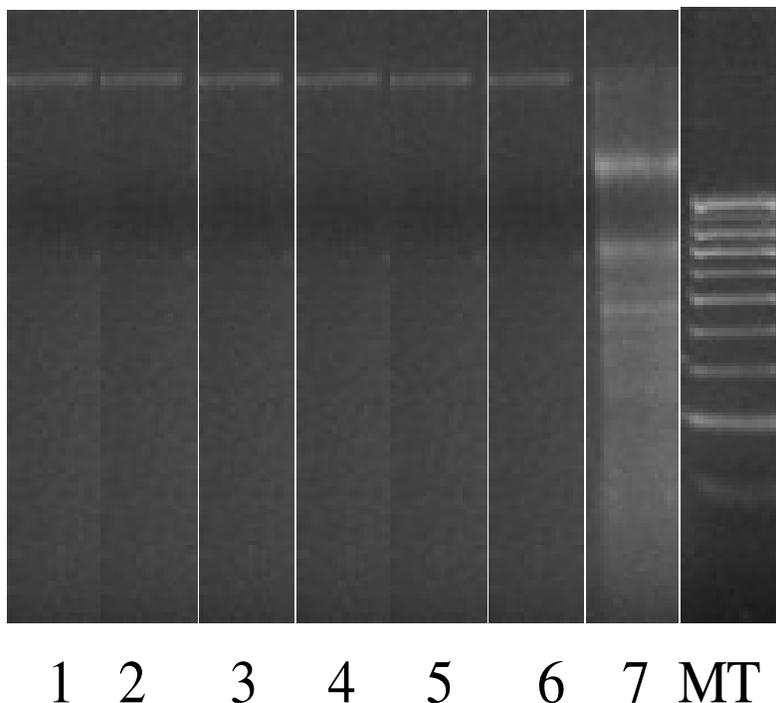
assessed in PBL treated by 0.2 mM  $H_2O_2$  and/or 1; 5; 10; 15 and 20 µg/ml HT, as well as in non treated PBL as controls. 0.2 mM  $H_2O_2$  caused an increase in MDA and CD levels ( $p = 0.001$ ;  $p = 0.001$  respectively) (Table 1 and Figure 3). This increase was significantly inhibited by supplementation of 1; 5; 10; 15 and 20 µg/ml HT (MDA:  $p = 0.001$ ; CD:  $p = 0.001$ ) (Table 1). The inhibitory effect of HT on lipid peroxidation was greater with increasing HT concentrations (Table 1). 20 µg/ml of HT significantly decreased the lipid peroxidation by 97.89%, as compared to PBL incubated only with  $H_2O_2$ .

### Evaluation of protein oxidation

0.2 mM  $H_2O_2$  caused a decrease in SH levels ( $p = 0.001$ ) (Table 2). Protein oxidation was significantly inhibited by supplementation of 1; 5; 10; 15 and 20 µg/ml HT ( $p = 0.002$ ; 0.001) (Table 2). Our data shows that the inhibitory effect of HT on protein oxidation was greater with increasing HT concentrations (Table 2). 20 µg/ml of HT significantly increased protein oxidation rates by 18.5%, as compared to PBL incubated only with  $H_2O_2$ .

### Evaluation of SOD and CAT activities

SOD and CAT activities in PBL treated by 0.2 mM  $H_2O_2$  and/or 1; 5; 10; 15 and 20 µg/ml HT, as well as in non treated PBL as controls are shown in Tables 3 and 4. 0.2 mM  $H_2O_2$  caused a significant decrease of SOD and CAT activities ( $p = 0.001$ ; 0.001). HT at concentrations as high as 1 µg/ml significantly increased SOD and CAT activities. The effect of HT on SOD and CAT activities was greater with increasing concentrations of HT (Tables 3



**Figure 2.** Illustrates the effect of HT on DNA fragmentation.  $H_2O_2$  treatment induces DNA fragmentation in PBL (Lane 7), untreated lymphocytes used as control (Lane 6). Supplementation of PBL with HT at 1, 5, 10, 15 and 20  $\mu\text{g/ml}$  protect efficacy the cells DNA integrity and protects against  $H_2O_2$ -induced DNA damage and fragmentation (Lanes 1 to 5).

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

$H_2O_2$ concentrations (mM)	HT concentrations ( $\mu\text{g/ml}$ )	[MDA] nmole/mg proteins	(HT) inhibition (%)
0.2	0	0.95801359***	
0	0	0.28141683***	
0.2	1	0.28972653***	69.833
0.2	5	0.15853659***	84.21
0.2	10	0.10506567***	89.94
0.2	15	0.04828776***	94.94
0.2	20	0.02542813***	97.89

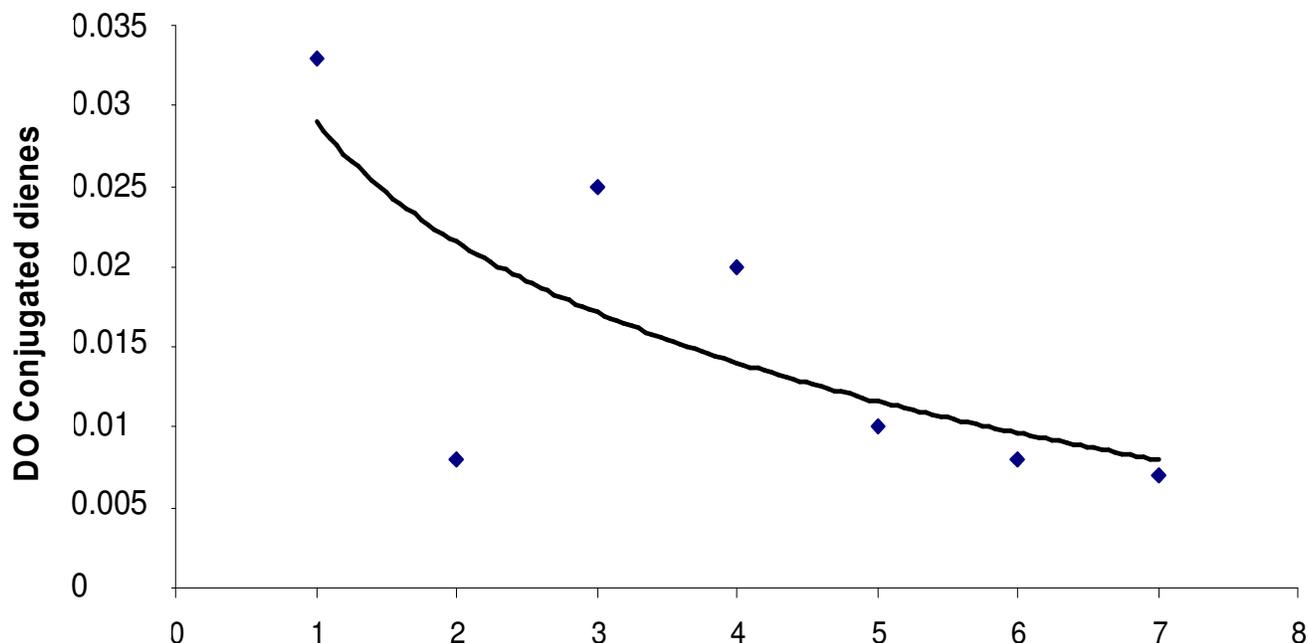
0.2 mM  $H_2O_2$  caused a significant increase in MDA level ( $p = 0.001$ ). This increase was inhibited by supplementation with hydroxytyrosol. The effect of hydroxytyrosol on inhibition of lipid peroxidation was seen at 1  $\mu\text{g/ml}$ . The inhibitory effect of hydroxytyrosol on lipid peroxidation was greater with increasing concentrations of hydroxytyrosol. 20  $\mu\text{g/ml}$  of HT significantly decreased the lipid peroxidation by 97.89%, as compared to PBL incubated only with  $H_2O_2$ .

and 4). A considerable increased of (SOD) and (CAT) activities by 76.92 and 21.67% respectively was showed after incubation of PBL with HT at dose 20  $\mu\text{g/ml}$ .

## DISCUSSION

The results of the present study provide further evidence that dietary antioxidant can protect human blood cells

from death and DNA fragmentation, and increased cells resistance to oxidative stress. In fact, we have shown in this study a decrease in  $H_2O_2$ -induced DNA damage and fragmentation in human peripheral blood mononuclear (PBL) cells after incubation with HT. These results are in accord with others study, which reported the positive effects of antioxidant supplementation on DNA damage in healthy volunteers, with the highest DNA protection within after ingestion (Nousis et al., 2005). The cytotoxicity of



**Figure 3.** The effect of hydroxytyrosol on CD levels. 0.2 mM H<sub>2</sub>O<sub>2</sub> caused an increase in CD levels ( $p = 0.001$ ). Hydroxytyrosol supplementation prevented an increase in CD levels caused by 0.2 mM H<sub>2</sub>O<sub>2</sub>. A significant decrease on CD level was seen at 1  $\mu$ g/ml hydroxytyrosol. The inhibitory effect of hydroxytyrosol on CD was greater with increasing concentrations of hydroxytyrosol.

**Table 2.** The effect of hydroxytyrosol on protein oxidation.

H <sub>2</sub> O <sub>2</sub> concentrations (mM)	HT concentrations ( $\mu$ g/ml)	[SH] $\mu$ mol/mg proteins	(HT) Inhibition (%)
0.2	0	0.00010668***	
0	0	0.00221054	
0.2	1	0.00066118***	5.6
0.2	5	0.00120334***	10.94
0.2	10	0.00153487***	14
0.2	15	0.00177518***	16.7
0.2	20	0.00195527***	18.5

0.2 mM H<sub>2</sub>O<sub>2</sub> caused a significant decrease in SH levels. Our data shows that hydroxytyrosol supplementation prevented protein oxidation caused by 0.2 mM H<sub>2</sub>O<sub>2</sub>. The effect of hydroxytyrosol on inhibition of protein oxidation was seen at 1  $\mu$ g/ml. 20  $\mu$ g/ml of HT significantly inhibited protein oxidation by 18.5%, as compared to PBL incubated only with H<sub>2</sub>O<sub>2</sub>.

**Table 3.** Illustrates the effect of hydroxytyrosol on SOD activity.

H <sub>2</sub> O <sub>2</sub> concentrations ( $\mu$ M)	HT concentrations ( $\mu$ g/ml)	SOD activity (% inhibition)	(HT) Inhibition (%)
0.2	0	10***	
0	0	3.33333333	
0.2	1	23.3333333***	56.52
0.2	5	30***	66.66
0.2	10	33.3333333***	69.69
0.2	15	36.6666667***	72.22
0.2	20	43.3333333***	76.92

0.2 mM H<sub>2</sub>O<sub>2</sub> caused an increase of SOD activity ( $p = 0.001$ ). Hydroxytyrosol at concentrations as high as 1  $\mu$ g/ml significantly increase of SOD activity. The effect of HT on SOD activity was greater with increasing concentrations of HT.

**Table 4.** Illustrates the effect of hydroxytyrosol on CAT activity.

H <sub>2</sub> O <sub>2</sub> concentrations (μM)	HT concentrations (μg/ml)	CAT activity (U/ml)	(HT) Inhibition (%)
0.2	0	452.64***	
0	0	276	
0.2	1	414***	8.40
0.2	5	521.64***	15.26
0.2	10	535.44***	18.29
0.2	15	545.1***	20.42
0.2	20	550.62***	21.67

0.2 mM H<sub>2</sub>O<sub>2</sub> caused an increase of CAT activity ( $p = 0.001$ ). Hydroxytyrosol at concentrations as high as 1 μg/ml significantly increase of CAT activity. The effect of HT on CAT activity was greater with increasing concentrations of HT.

HT at the concentrations of 1, 5, 10, 15 and 20 μg/ml was tested in PBL, to ensure that PBL proliferation is unchanged after HT treatments. No differences in cell viability were observed for 1, 5, 10, 15 and 20 μg/ml HT concentrations. In fact, these concentrations are very low, compared to cytotoxic concentrations (Bouaziz et al., 2008). In addition, this study showed a potential antioxidant effect of HT in human PBL, evidenced by significant increase in SOD and CAT activities after HT supplementation suggesting that HT protected from DNA damage, which contributed to increase in SOD and CAT expressions as activities. SOD and CAT activities have been found to be increased which was therefore interpreted as an adaptative response to oxidative stress (Lassoued et al., 2010). SOD and CAT enzymes usually act in a synergetic manner. SOD, the first line of defense against ROS, catalyzes the dismutation of the superoxide anion into hydrogen peroxide. Hydrogen peroxide can then be transformed into H<sub>2</sub>O and O<sub>2</sub> by CAT, glutathione peroxidase and peroxiredoxins (Michiels, 1994). SOD and CAT activities were increased, suggesting that HT is directly increasing these enzymes or that some endogenous cellular mechanisms that increase these enzymes are activated during HT treatment. Moreover, HT was found to increase SOD and CAT activities, suggesting that scavenging of ROS may be related to the increased antioxidant activity.

This study showed that HT significantly inhibited lipid peroxidation in PBL. This inhibitory effect of HT on lipid peroxidation was greater with increasing HT concentrations. Recently, it is reported that HT inhibited lipid peroxidation and exhibited DNA protective effect in normal human peripheral blood mononuclear cells exposed to H<sub>2</sub>O<sub>2</sub>-induced oxidative stress and intracellular ROS scavenging, and indirect action through induction of anti-oxidative enzymes (Stefania et al., 2005).

Our *in vitro* study show that in PBL model, HT significantly inhibited protein oxidation at 1, 5, 10, 15 and 20 μg/ml concentrations. Our data shows that the inhibitory effect of HT on protein oxidation was greater with increasing HT concentrations. This suggests that the

inhibition of oxidation of proteins may be mediated by an antioxidative effect of HT. This was in agreement with the report of Manna et al. (2005), who found that HT, *in vitro*, prevented protein damage in melanoma cells by ROS induced by long-wave ultraviolet light.

Previous studies have also shown that HT has been shown to have anti-cancer effect on human colon adenocarcinoma HT-29 cells and human promyelocytic leukemia HL-60 cells (Fabiani et al., 2006). HT suppresses nitric oxide (NO), ROS, NF-κ B activation, and increased GSH contents in lipopolysaccharide-stimulated THP-1 cells (Xiaomei et al., 2009).

No clinical trial to determine whether HT supplementation can indeed delay or prevent lipids peroxidation and proteins oxidation has been done. No animal studies indicate a potential benefit of HT supplementation in blood levels of lipids and proteins. The molecular mechanisms by which HT produces its effects in animal models are not known.

## Conclusion

This study has demonstrated for the first time that HT can inhibit lipids peroxidation and proteins oxidation and modulate antioxidants enzymes activities, in PBL 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 and 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 diseases related to oxidative stress.

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