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# The protective role of olive oil hydroxytyrosol against oxidative alterations induced by mercury in human erythrocytes

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ABSTRACT

Hydroxytyrosol (HT) is a phenolic antioxidant naturally occurring in virgin olive oil. In this study, we investigated the possible protective effects of HT on the oxidative and morphological alterations induced by mercury (Hg) in intact human erythrocytes. These cells preferentially accumulate this toxic heavy metal. More importantly, Hg-induced echinocyte formation correlates with increased coagulability of these cells. Our results indicate that HT treatment (10–50 μM) prevents the increase in hemolysis and Reactive Oxygen Species (ROS) generation induced by exposure of cells to micromolar $HgCl_2$ concentrations as well as the decrease in GSH intracellular levels. Moreover, as indicated by scanning electron microscopy, the morphological alterations are also significantly reduced by HT co-treatment. Taken together our data provide the first experimental evidence that HT has the potential to counteract mercury toxicity. The reported effect may be regarded as an additional mechanism underlying the beneficial cardio-protective effects of this dietary antioxidant, also endowed with significant anti-atherogenic and anti-inflammatory properties.


## 1. Introduction

Hg is a highly toxic heavy metal and is one of the main agents which is responsible for environmental pollution (Rice et al., 2014). In the last century, water and soil contamination by both elementary and organic mercury has increased dramatically through anthropogenic sources, including fuel combustion and incinerators. Human exposure to mercury occurs primarily via medical preparations as well as nutritional sources. In particular, contaminated fish products seem to be the major source of methylmercury in food and represent an increasing public health concern (Booth and Zeller, 2005). The health consequences of human exposure to mercury include immune-toxicity, kidney damage and neuronal disorders (Hong et al., 2012). Moreover, the reported anemia-inducing effect of mercury suggests that erythrocyte (RBC) may be an important target of mercury toxicity (Rooney, 2013). In the last few years, concerns about the negative effects of chronic exposure to mercury on cardiovascular health are rapidly increasing and mercury toxicity is now regarded as a potential new risk factor for cardiovascular diseases (CVD) (Fernandes Azevedo et al., 2012, Houston, 2011; Virtanen et al., 2007). Mechanisms underlying Hg-related endothelial dysfunction include a decrease in nitric oxide bioavailability and Hg-induced increase in oxidative stress (Virtanen et al., 2007). Increased formation of reactive oxygen species (ROS), indeed, is thought to be one of the key mechanisms responsible for Hg-induced toxicity (Ercal et al., 2001). Because mercury is endowed with a high affinity for sulfhydryl groups, it is able to impair the antioxidant defense system by reacting with cellular thiols including glutathione (Rooney, 2007; Velyka et al., 2014). In addition, according to the oxidative stress hypothesis of mercury toxicity, antioxidant compounds are protective (Barcelos et al., 2011; Kaivalya et al., 2011).

The aim of this paper is to further explore the involvement of oxidative stress as an underlying mechanism in metabolic changes related to mercury toxicity and the possible protective role played by dietary antioxidants, using intact human RBC incubated *in vitro* in the presence of mercuric chloride ($HgCl_2$). These cells are a unique cellular model for studies that investigate oxidative stress-related alterations (Manna et al., 1999; Yang et al., 2006) as well as Hg toxicity (Harisa et al., 2012, 2013). This metal, indeed, preferentially accumulates in RBC and induces morphological changes (Pal and Ghosh, 2012) which increase the pro-coagulant activity of these cells (Lim et al., 2010). Among the different antioxidants, our attention has been devoted to hydroxytyrosol (3,4-diidroxyphenylethanol, HT), a simple phenol recalling the structure of cathecol, naturally occurring in olive oil (Napolitano et al., 2010; Zappia et al., 2010). This compound is endowed with a variety of biological activities, including anti-inflammatory and anti-atherogenic properties (Burattini

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et al., 2013; Granados-Principal et al., 2010). Mechanisms underlying the biological effects of HT include both radical scavenging properties and metal chelator activity (Manna et al., 2012).

## 2. Materials and methods

### 2.1. Chemicals

The materials used, 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA), mercuric chloride ($HgCl_2$), and hydroxytyrosol, were from Sigma Chemical Co. All other chemicals used were of the purest grade available.

### 2.2. Preparation of RBC and mercury treatment

The RBC fraction was obtained from whole blood deprived of leukocytes and platelets by filtration on a nylon net, washed twice with isotonic saline solution (NaCl 0.9%) and finally resuspended to 10% hematocrit with Buffer A (5 mM Tris-HCl containing 0.9% NaCl, 1 mM $MgCl_2$ and 2.8 mM glucose, pH 7.4). Mercury treatment was performed by incubation of intact RBC at 37 °C with increasing concentrations of $HgCl_2$.

### 2.3. Assay system for hemolysis

The extent of hemolysis was determined spectophotometrically, according to Manna et al. (1999). At the end of incubation, the reaction mixture was centrifuged at 1100 *g* for 5 min and the absorption (A) of the supernatant at 540 nm was measured. Packed RBC were hemolyzed with 40 volumes of ice-cold distilled water and the lysate centrifuged at 1500 *g* for 10 minutes and the absorption of the supernatant (B) was recorded at 540 nm. The hemolysis percentage was calculated from the ratio of the reading A/B100.

### 2.4. Determination of ROS

The dichlorofluorescein (DCF) assay was performed to quantify ROS generation, according to Manna et al. (2012). Intact RBC were incubated with the non-polar, non-fluorescent 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA), at final concentration of 10 μM for 15 min at 37°. After centrifugation at 1200 *g* for 5 min, the supernatant was removed and the hematocrit value was adjusted to 10% with buffer A and RBC were then treated with $HgCl_2$ in the dark. At the end of incubation, 20 μl of RBC was diluted in 2 mL of water and the fluorescence intensity of the oxidative derivative DCF was recorded ($\lambda_{exc}$ 502; $\lambda_{em}$ 520). The results were expressed as fluorescent intensity/mg hemoglobin (Hb).

### 2.5. Assay for reduced GSH

The intracellular GSH content was determined spectrophotometrically by reaction with 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB) reagent, according to Van den Berg et al. ( 1992). After centrifugation of the samples (0.25 ml) treated as above reported, supernatants were removed and RBC were lysed by addition of 0.6 ml of ice-cold water; proteins were than precipitated by the addition of 0.6 ml ice-cold metaphosphoric acid solution [1.67 g metaphosphoric acid, 0.2 g EDTA and 30 g NaCl in 100 ml of water]. After 10′ incubation at 4 °C, the protein precipitate was removed by centrifugation at 18,000 *g* for 10 min and 0.45 ml of the supernatant was mixed with an equal volume of a 0.3 M $Na_2HPO_4$ . For the reduced GSH determinations, 100 μl of DTNB solution (20 mg DTNB plus 1% of sodium citrate in 100 ml of water) was added to the sample. After 10′ incubation at room temperature, the absorbance of the sample was read against the blank at 412 nm.

### 2.6. Scanning electron microscopy (SEM) analysis

RBC were treated with $HgCl_2$ in the presence or in the absence of HT, as above described. After the treatment, the cells were fixed with a solution of 2.5% glutaraldehyde and 4% paraformaldehyde for 2 h at 4 °C. After fixation, RBC were washed three times with PBS and then post-fixed with 1% osmium tetroxide for 30 min at 4 °C. Cells were washed with PBS several times and then RBC were dehydrated with 50%, 75%, 90%, and 100% ethanol. After drying and coating with gold, the images were observed on a Scanning Electron Microscope (SEM) (FEI QUANTA 200). Echinocytes were quantified by counting ≥200 cells (50 RBC for each different SEM field at a magnification of 1750×) for each experimental condition.

### 2.7. Statistical analysis

Data were expressed as means ± SEM. The significance of differences was determined by one-way ANOVA followed by a post hoc Dunnett's multiple comparisons test with significance set at $p < 0.01$. GraphPad Prism 5 was utilized for statistical analysis.

## 3. Results

In order to evaluate the toxic effects of Hg and the possible protection exerted by HT, intact RBC were exposed *in vitro* to increasing $HgCl_2$ concentrations. As far as the chemical form of mercury utilized is concerned, methylmercury is considered the more toxic form in that, because of its lipophilicity, it is almost completely absorbed and it easily crosses the cellular membrane and the blood–brain barrier. However, methylmercury is rapidly transformed in the body into its mercuric form, the toxic species in human tissue after conversion (Houston, 2011). Accordingly, several papers are present in the literature that utilized $HgCl_2$ to test the toxic effect of this heavy metal in *in vitro* systems (Harisa et al., 2013; Kaivalya et al., 2011; Lim et al., 2010).

### 3.1. HT prevents human RBC from Hg-induced hemolysis

To evaluate the ability of HT to prevent Hg-mediated cytotoxicity, the effect of the phenolic antioxidant on hemolysis was measured. As shown in Fig. 1, exposure of cells to $HgCl_2$ results in a significant cytotoxicity in a dose and time-dependent manner. By comparison of the data at 4 and 24 h, we demonstrated that in our experimental conditions Hg toxicity is a relatively late event: no hemolysis is detectable up to 20 μM upon 4 h treatment. A low value but significant hemolysis (2.6%) is present at 40 μM but only at 80 μM $HgCl_2$ does meaningful hemolysis occur (10.3%). Conversely, prolonged exposure to the heavy metal leads to a dramatic decrease in cell viability: after 24 h the increase in the hemolytic process is significant starting from the 10 μM concentration (2.7%) with about 50% of hemolysis at 80 μM (48.3%).

On the basis of these results, we used 40 and 80 μM $HgCl_2$ to evaluate the effect of HT at the two different time points utilized. The dose-dependency of the protective effect of HT on Hg-induced cytotoxicity is shown in Fig. 2. HT is able to prevent the toxic effect of the metal by reducing cell death. In fact, hemolysis is significantly decreased in the presence of HT concentrations as low as 10 μM at all time points analyzed.

### 3.2. HT prevents Hg-induced ROS generation in human RBC

In order to investigate the role of oxidative stress in Hg-induced cytotoxicity and the possible protective effects of HT, a DCF assay was performed to measure ROS formation. Fig. 3 shows that incu-

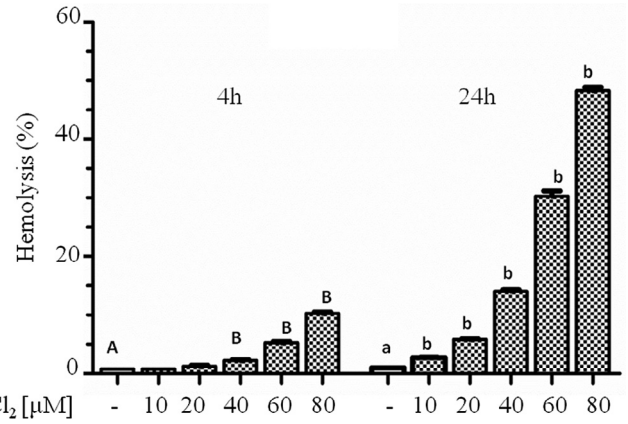


**Fig. 1.** Effect of Hg treatment on hemolysis in RBC. Cells were treated for 4 and 24 h in the presence of increasing concentrations of $HgCl_2$ as described in Materials and methods. Data are the means ± SEM (n = 10). Statistical analysis was performed with one-way ANOVA followed by Dunnett's test ($p < 0.01$). Means with different letters are significantly different.