Mini Review

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Nutritional Aspects of Food Toxicology: Mercury Toxicity and Protective Effects of Olive Oil Hydroxytyrosol

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Abstract

Mercury represents one of the main environmental pollutants and human exposure to this heavy metal occurs primarily through nutritional sources, including contaminated fish. This highly toxic compound is known to pose serious threats to human health, including neurological alterations. Moreover, based on its effects on cardiovascular health, mercury exposure is now considered an independent risk factor for cardiovascular diseases. The possibility of reducing heavy metal toxicity through diet has attracted the interest of those responsible for the public health service. In this respect, the use of phytochemicals able to significantly counteract oxidative alterations as an attractive tool for the reduction of mercury toxicity has been proposed. Here we review recent evidence supporting the beneficial role of olive oil hydroxytyrosol in preventing mercury-induced alterations in both human erythrocytes and neuroblastoma cells. This novel biological effect exerted by hydroxytyrosol represents an additional mechanism responsible for the much-claimed health benefits of this dietary phenol. Taken together the reported findings encourage the use of virgin olive oil, characterized by a high hydroxytyrosol content, as an innovative approach in designing combined dietary and/or nutraceutical strategies to contrast mercury toxicity in humans.

Keywords: Food toxicology; Mercury toxicity; Olive oil; Cardiovascular diseases

Introduction

The primary role of nutrition is to provide sufficient quantities of nutrients in order to prevent syndromes of deficiency or excess [1]. Nevertheless, a healthy diet is a vital key in reducing morbidity and mortality from chronic diseases [2]. In recent years nutritional research has focused on studies of dietary components which are able to strengthen biological functions with the aim of preventing and/or reducing the risk of disease [3]. Among these compounds, several secondary plant metabolites are included endowed with important biological activities, in addition to their basic nutritional benefits. Fruit and vegetables, indeed, contain thousands of different biomolecules (phytochemicals), some of which have the potential to promote health and/or retard diseases [4]. In this respect, these bioactive dietary components are believed to play a major role in the positive correlation between adherence to the Mediterranean Diet and a low incidence of several pathologies, including cardiovascular diseases (CVD) and cancer [5]. Moreover, these phytochemicals have been proved to actively counteract the heavy metal-induced body burden and biochemical alterations [6-7].

Mercury Toxicity

Mercury (Hg) is a highly toxic volatile heavy metal, liquid at room temperature [8]. It can exist in three oxidation forms: elementary (Hg^0), mercurous (Hg^+) and mercuric (Hg^{2+}), and it can form both inorganic and organic compounds. Among organic compounds, methylmercury (MeHg) is the most important biologically and ecologically [9]. Mercury is one of the main environmental pollutants. The natural sources of emission of metals are superficial waters, the

soil, volcanic activity and the combustion of vegetation. Among anthropic sources we may consider combustion of fuel and those of incinerators [10]. The biogeochemical cycle of mercury (Figure 1) occurs both in air and in the soil [11]. However, mercury cycling in the aquatic system represents the critical point for human contamination [12-13]. In aquatic sediments, a small fraction of Hg^{2+} is converted to the organic forms. Methylation reaction is mediated by several kinds of bacteria including some strains of sulfate- and iron-reducing anaerobic bacteria [14]. The organic form penetrates inside the aquatic trophic network via plankton (phytoplankton and zooplankton) and invertebrates [15]. Once it is absorbed by living organisms it tends to bioaccumulate in the passage through the aquatic food chain, continuing through small fish and accumulating even further via the process of biomagnification, reaching its greatest concentration in carnivorous fish at the top of the food chain [16]. Thus, sources of Hg exposure to humans are air and water as well as dental amalgam and certain types of vaccines [13]; however, the dominant pathway is through eating contaminated food. In fact, diet plays an important role in exposure to Hg, given that certain foods, especially fish, can contain high concentrations of this contaminant [12,15]. Furthermore, even contaminated soil may represent a risk related to its potential transfer of this metal to crops. Finally, Hg can be transferred into human milk, causing severe damage to infants [17]. The molecular mechanisms underlying Hg toxicity are related to its binding capacity to thiol groups, potentially leading to severe alteration of enzymatic as well as structural proteins [18]. Hg is a well-known inhibitor of glycolytic enzymes; in particular, Ramírez-Bajo et al. report Hg inhibitory activity on both hexokinase and phosphofructokinase in mice, by reacting with crucial cysteine [19]. Furthermore, the human thioredoxin system is reported to be inhibited by Hg [20]. The impact of Hg on the cytoskeleton protein tubulin is well known [21-22]. The metal, binding to SH-groups of the protein, induces depolymerisation of microtubules therefore interfering with cellular processes, including

cell survival, proliferation, migration and differentiation [23]. Besides, sulfur-containing low molecular weight molecules such as glutathione (GSH) can be inactivated, thus reducing the antioxidant endogenous defense system [24]. In this respect, disruption of cellular redox homeostasis, associated with increased levels of reactive oxygen species (ROS), is considered to be one of the main Hg-related toxic mechanisms [25].
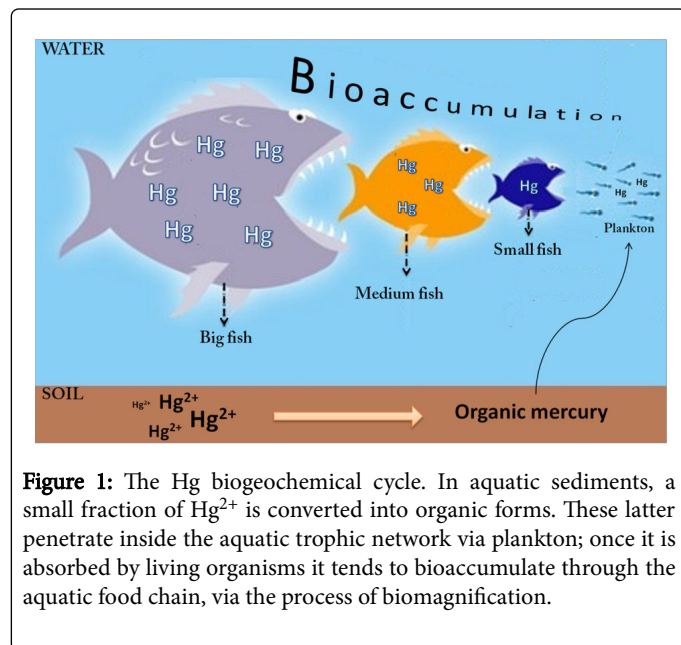

Figure 1: The Hg biogeochemical cycle. In aquatic sediments, a small fraction of Hg^{2+} is converted into organic forms. These latter penetrate inside the aquatic trophic network via plankton; once it is absorbed by living organisms it tends to bioaccumulate through the aquatic food chain, via the process of biomagnification.

Hg Exposure and Human Health

In the last decade, Hg exposure has increased considerably, especially in relation to anthropic sources, causing serious problems for public health [13]. Health risks for mankind following Hg exposure have been well documented by a long series of epidemiological and experimental studies. Pathologies correlated to mercury include renal damage [26] and neuronal disorders [27]. Hg has also been considered as a contributory factor in Alzheimer's and Parkinson's disease [28] and is able to induce genotoxicity in cultured mammalian cells [29]. A positive correlation between Hg exposure and CVD has also been proposed [30]. Recently, the negative effects of chronic Hg exposure on cardiovascular health have assumed even greater importance and Hg toxicity is now considered by some authors as a new independent cardiovascular risk factor [31]. An increasing number of studies have been undertaken to investigate the possible molecular mechanisms at the basis of Hg-induced damage to the cardiovascular system. Endothelial dysfunction plays a central role in Hg toxicity [32]. Exogenous substances once absorbed come inevitably into contact with endothelial vessels before reaching other organs and tissues, which puts the cardiovascular system at risk of a toxic insult on the part of xenobiotics. Potential mechanisms of the toxic action of Hg on the endothelial cells include a decrease in the bioavailability of nitric oxide, altering the property of dilation of the vessels [33-34]. Interestingly, in a human study aimed to investigate the link between Hg exposure and the metabolic syndrome, Tinkov et al. report a correlation between its concentration in the blood and blood pressure [35]. Moreover, smoking is positively associated with hair Hg accumulation, which in turn results in increased blood pressure [36]. Alteration of coagulation factors, such as Factor V, represents an additional potential molecular

mechanism through which Hg exerts its cytotoxic effects [30]. Finally, Hg exposure enhances pro-coagulant activity of red blood cells (RBC), resulting in a contributing factor for Hg-related thrombotic events [37]. This metal, indeed, preferentially accumulates in RBC and induces morphological changes [38] which are associated with phosphatidylserine (PS) exposure [37]. Ps exposure enables the active participation of RBC to vasocclusion through directly enhancing adhesion PS-expressing RBC to endothelial cells and providing a site for the assembly of the prothrombinase and tenase complex, leading to thrombin generation and clotting (Figure 2).
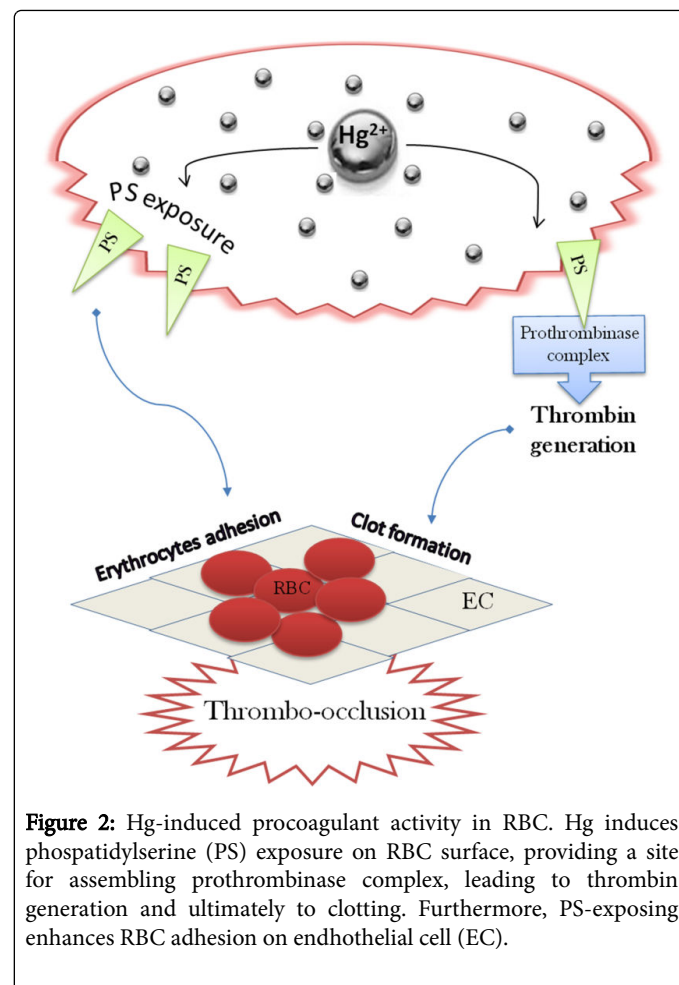

Figure 2: Hg-induced procoagulant activity in RBC. Hg induces phospatidylserine (PS) exposure on RBC surface, providing a site for assembling prothrombinase complex, leading to thrombin generation and ultimately to clotting. Furthermore, PS-exposing enhances RBC adhesion on endhothelial cell (EC).

Hg and Nutrition

Although Hg toxic effects have been well known for a long time, the exposure of humans to this metal still presents a serious health problem, and it is one which is dramatically increasing in certain parts of the world [39]. As previously emphasized, diet represents one of the most important pathways of Hg exposure [40]. However, while there are foods which may favour human exposure to this metal, there are also foods which may reduce its toxicity. Naturally derived products capable of chelating heavy metals, in order to encourage their expulsion, are currently being used and this use is increasing [41,42]. In particular, current research has brought to light the ability of dietary fibers to perform Hg chelation during gastric-intestinal transit [43].

Metal chelation properties have also been found in several compounds of dietary origin including curcumin [44], which is present
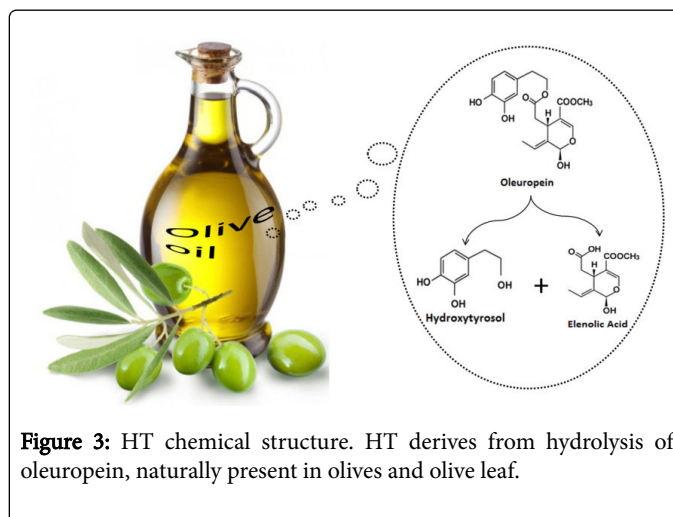
in the rhizome of *Curcuma longa*, a spice widely used in the Indian and Chinese cuisines. Apart from its chelating properties curcumin also exerts a protective action against lipid peroxidation, induced by heavy metals, due to its anti-oxidant activity [45]. In fact, since one mechanism at the basis of Hg toxicity is the deterioration of the antioxidant defence system, molecules with scavenger properties against free radicals have been proposed as potential protective agents [46-50]. Furthermore, there has been a notable increase in the utilization of organoselenium compounds, either for therapy and/or as treatment against Hg-induced toxic effects [51]. Due to the high content of these compounds in the herb garlic [52], it is a dietary component which has an important detoxifying action on heavy metals including Hg [53]. Depending on the conditions of its cultivation, garlic may contain at least 33 different organosulfur compounds, the most abundant being allicin [54]. Garlic is also rich in selenium, an important mineral which hinders Hg toxicity by strengthening the antioxidant defence system, being a co-factor of antioxidant enzymes such as glutathione peroxidase and thioredoxin reductase [55], and directly binds to Hg. Finally, in recent years several studies have revealed the possible protective role of olive oil, against metal toxicity [56-57].

Olive Oil Hydroxytyrosol in the Prevention of Hg Toxicity

Olive oil, the typical lipidic source of the Mediterranean Diet, has been associated with a low incidence of several pathologies [58-59], including CVD [60] and neurological disorders [61]. Olive oil is an excellent source of oleic acid, vitamin E and nonessential nutrients. The olive flesh components are transferred to the oil, which consists of two major fractions, the saponifiable one, made of triglycerides, accounting for 98-99% of the total, and unsaponifiable fraction, containing several liposoluble molecules, including tocopherols, phytosterols, coloring pigments and squalene [25]. Part of the unsaponifiable fraction is several phenolic compounds, plant secondary metabolites. This class includes phenolic acids, phenolic alcohols, hydroxy-isocromans, flavonoids, lignans and secoiridoids such as oleuropein and ligstroside. There is general agreement that the health benefits of olive oil intake result from the combined properties of all its constituents. In particular, converging evidence indicates that the antioxidant fraction, including polyphenols, significantly contributes to its health promoting effect [62-63]. The phenol content is also important for the quality of virgin olive oil, and the contribution of these components to the shelf-life of this food is widely accepted [64].

Hydroxytyrosol (3,4-dihydroxyphenylethanol; HT) is mainly responsible for the antioxidant properties of this food, due to an efficient scavenger activity [65]. This molecule, recalling the structure of the cathecol, is present either simple phenol or esterified with elenolic acid to form oleuropein aglycone (Figure 3). Experiments from our group demonstrated that HT, which effectively permeates cell membranes via passive diffusion [66], counteracts the cytotoxic effects of reactive oxygen species (ROS) in various human systems, including Caco-2 cells [67] and RBC [68-69]. The effects of HT on inflammation/atherogenesis have also been thoroughly investigated. HT inhibits *in vitro* low-density lipoprotein oxidation and modulates the oxidative/antioxidative balance in plasma [70]. Moreover, due to its strong antioxidant activity and presumably counteracting the oxidative stress-induced endothelial dysfunction, HT is able to modulate key mechanisms implicated in the development of atherosclerosis,

including the expression of adhesion molecules [71]. In this respect, it has been demonstrated that this phenol inhibits the expression of adhesion molecules in a human endothelial cell line (HUVEC) exposed to pro-inflammatory cytokines [72]. Even though the majority of HT biological activities can be directly ascribed to its antioxidant activity, emerging evidence [73] supports the view that some effects of this molecule are independent of its scavenging properties. In this respect, several olive oil phenols are able to inhibit homocysteine-induced increased endothelial cell adhesion, regardless of their different antioxidant activity [74]. Additional biological effects include neuroprotection [75] and anti-cancer properties [76]. The interference of polyphenols in the apoptotic model of cell death in nucleated cells is well documented [77] and mainly involves protection against mitochondrial-mediated mechanisms by virtue of their antioxidant capacity. Finally, HT ameliorates acrolein-induced cytotoxicity in retinal pigment epithelial cells, showing a protection from oxidative damage and mitochondrial dysfunction [78] and reduces acrylamide-induced cytotoxicity, preventing DNA damage and intracellular ROS formation in HepG2 cells [79]. In the last few years, several papers report data indicating that this dietary component is able to counteract the toxic effects linked to exposure of heavy metals, including Hg [80-82].

Figure 3: HT chemical structure. HT derives from hydrolysis of oleuropein, naturally present in olives and olive leaf.

HT Prevents ROS Formation and Hg-induced Morphological Alterations in Human RBC

RBC are anucleated cells without organelle cells, thus representing a simplified cellular model of the metabolism. This is particularly advantageous for the study of oxidative stress caused by the high tension of oxygen and the highly toxic free radicals derived from it. In addition, RBC have been utilized as a model for pharmacological and toxicological studies which investigate heavy metal toxicity. Thus, intact human RBC, subjected *in vitro* to treatment with mercury chloride ($HgCl_2$) were utilized to test the potential protective effects of HT. HT has the potential to modulate cytotoxicity and to counteract GSH decrease and the OS induced in RBC by Hg treatment [80]. In this experimental system, Hg-induced ROS generation is a late event (Figure 4) and probably occurs subsequently to a significant decrease of essential antioxidant thiols, which could render cells more susceptible to ROS-mediated OS. Also of great clinical importance is the finding that HT prevents Hg-induced RBC morphological alteration (echinocyte formation), which makes cells more

atherogenic. As pointed out before, Hg exposure enhances pro-coagulant activity of these cells, resulting in a contributing factor for Hg-related thrombotic disease (Figure 5).
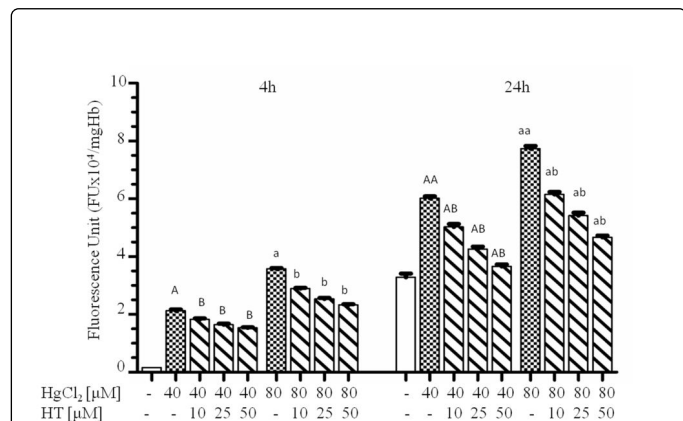

Figure 4: HT prevents Hg-induced ROS formation in human RBC. Cells were subjected *in vitro* to treatment with $HgCl_2$. The 2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA) assay was performed to quantify ROS generation (Courteously granted by Tagliafierro et al. [80]).
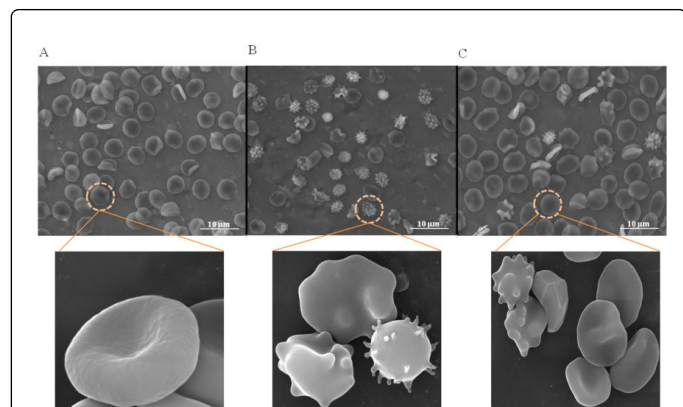

Figure 5: HT prevents morphological alterations in human RBC. Cells were subjected *in vitro* to treatment with 20 μM $HgCl_2$ for 4 hours. After incubation, cells were analyzed by microscopy electronic scan (SEM). (A) Untreated RBC. (B) Hg-treated RBC. (C) HT-pretreated RBC before adding $HgCl_2$ (Courteously granted by by Tagliafierro et al. [80]).

HT Prevents Hg-induced Programmed Cell Death (eryptosis) in Human RBC

Hg-induced programmed cell death has been well documented in both nucleated and anucleated cells [83]. Similarly to apoptosis, RBC may encounter programmed cell death, also called eryptosis [84-85]. This process is characterized by an increase of intracellular calcium and by depletion of ATP and GSH. These biochemical alterations result in RBC morphological changes, associated with a reorganization of the cellular membrane, in which exposure of PS on the cell surface is the major event [37]. Experimental evidence of the efficacy of HT in preventing eryptosis in human RBC exposed *in vitro* to $HgCl_2$

treatment has been recently published by our group [81]. Cell conditioning with HT micro-molar concentrations prior to exposure to Hg causes a decrease in PS-exposing RBC, along with the restoration of ATP and GSH cellular content (Figure 6). Conversely, HT pretreatment shows no effect against influx of extracellular calcium and thus does not interfere with Ca-mediated mechanisms in eryptosis. These data reveal that HT has the potential to modulate suicidal death induced by Hg treatment in anucleated cells, also devoid of mitochondria and thus lacking any mitochondria-mediated apoptotic pathways. Furthermore, no increase in ROS production was observed in the mild experimental conditions utilized, indicating that HT biological activities, which are different from the scavenging potential, are involved in the protective process. In this respect, GSH enhancement may represent a key mechanism, as reported in different cellular systems. In particular, Mohan et al. [82] report that the ability of HT to promote the expression of nuclear factor erythroid 2-related factor 2 (Nrf2), which in turn elevates GSH levels, is crucial in ameliorating the neurotoxic effect of MeHg, as discussed in the next paragraph.
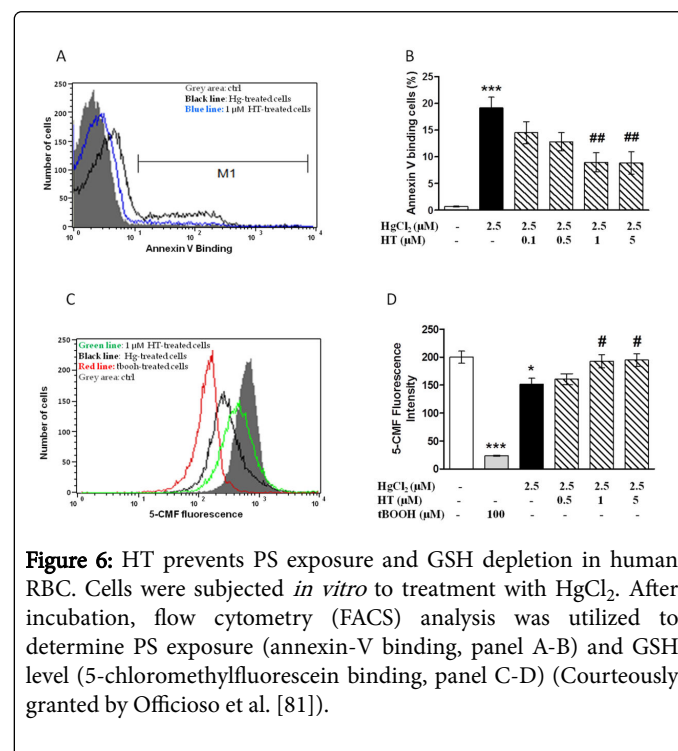

Figure 6: HT prevents PS exposure and GSH depletion in human RBC. Cells were subjected *in vitro* to treatment with $HgCl_2$. After incubation, flow cytometry (FACS) analysis was utilized to determine PS exposure (annexin-V binding, panel A-B) and GSH level (5-chloromethylfluorescein binding, panel C-D) (Courteously granted by Officioso et al. [81]).

HT Prevents Hg-induced Genotoxicity and Apoptosis in Human Neuroblastoma Cells

A recent study highlights the efficacy of HT in preventing MeHg-induced neurotoxicity, using IMR-32 human neuroblastoma cells as a surrogate model for studying the effects of heavy metal on neuronal dysfunction. In this study, Mohan et al. [82] report that cell pre-incubation with HT inhibits MeHg-induced cytotoxicity along with reduction of ROS formation and the maintenance of an efficient endogenous defence system, including GSH levels and superoxide dismutase and catalase activities. Furthermore, HT also prevents genotoxicity and apoptosis, causing downregulation of p53, bax, cytochrome c, and caspase 3 and upregulation of prosurvival proteins including Nrf2 and metallothionein. In particular, the ability of HT to

promote the expression of Nrf2 and, in turn, to modulate GSH levels, appears crucial for the neuroprotective effect of HT. It is well known that ROS accumulation has been implicated as a relevant cofactor contributing to both DNA damage and the cascade of events leading to programmed cell death in nucleated cells. The lowering of oxidative stress, which may be endorsed by its anti-genotoxic and anti-apoptotic properties probably, represents the main molecular mechanism of the observed cytoprotective potential of HT against MeHg-induced toxicity.

Conclusion

Taken together, the data discussed in this review provide experimental evidence that HT, a component normally present in high concentrations in olive oil, has the potential to modulate Hg toxicity, therefore representing an ideal candidate for nutritional/nutraceutical strategies to counteract the adverse effects of Hg exposure in humans. The reported novel biological effect of HT reinforces the nutritional importance of the phenolic fraction which greatly contributes to the beneficial effects of the olive oil on human health. Finally, an interesting observation is that HT protective concentrations utilized in the experimental systems could be approached *in vivo* upon strict adherence to the Mediterranean dietary habit, in the context of a balanced diet. Furthermore, HT has been proved to be devoid of toxicity [86-87], is highly bioavailable [88-89] and potentially able to cross the blood-brain barrier [86].

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