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**Mechanism of action of Methylmercury on human astrocytes: neuroprotection
of lipoic acid**

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To my brother

**Ognuno sta solo sul cuor della terra
trafitto da un raggio di sole:
ed è subito sera.**

S. Q.

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Abstract

Astrocytes are actively involved in brain development, in mature CNS regulation and brain plasticity. They play a critical role in response to cerebral injuries and toxicants. A large amount of literature highlights the central role of astrocytes in mediating methylmercury (MeHg) neurotoxicity. In fact, mercury is the major neurotoxic pollutant that continues to arouse interest in research because of the severe risk it poses to human health. It has been widely demonstrated that this metal induces acute or chronic neurotoxicity exacerbating an oxidative stress condition and also age-related conditions, generating inflammatory reactions in the brain. These findings support the hypothesis that MeHg and many other environmental pollutants have the potential to cause neurodegeneration, through a variety of pathways similar to those described in human neurodegenerative diseases. However, despite numerous *in vivo* and *in vitro* studies, almost entirely on rat/mouse models, the issue remains controversial and the effects of MeHg in the human population are not completely known. Based on these findings, the aim of this study is to carry out for the first time, according to what has been reported in the literature, an *in vitro* experimental model that was as attributable as possible to humans, precisely human astrocytes (HA), so as to observe the effects of MeHg on some biochemical-molecular pathways. In parallel, I used HA cell cultures, which were treated with Alpha Dihydrolipoic Acid before being subjected to the action of MeHg in order to evaluate the possible protective effect exerted by this compound with antioxidant and chelating properties on cellular metabolism subjected to stress by MeHg.

Abstract

Gli astrociti sono attivamente coinvolti nello sviluppo del cervello, nella regolazione del sistema nervoso centrale maturo e nella plasticità cerebrale. Essi giocano un ruolo critico nella risposta a lesioni cerebrali e alle sostanze tossiche. Una grande quantità di letteratura evidenzia il ruolo centrale degli astrociti nel mediare la neurotossicità indotta dal metilmercurio (MeHg). Infatti, il mercurio è il principale inquinante neurotossico che continua a suscitare interesse nella ricerca a causa del grave rischio che comporta per la salute umana. È stato ampiamente dimostrato che questo metallo induce neurotossicità acuta o cronica esacerbando una condizione di stress ossidativo età correlato e generando reazioni infiammatorie nel cervello. Questi risultati supportano l'ipotesi che MeHg e molti altri inquinanti ambientali hanno il potenziale di causare neurodegenerazione, attraverso una varietà di percorsi simili a quelli descritti nelle malattie neurodegenerative umane. Tuttavia, nonostante numerosi studi in vivo e in vitro, quasi interamente su modelli di ratto/topo, la questione rimane controversa e gli effetti del MeHg nella popolazione umana non sono completamente noti. Sulla base di questi risultati, lo scopo di questo studio è quello di realizzare per la prima volta, secondo quanto riportato in letteratura, un modello sperimentale *in vitro* che sia il più è possibile valido per l'uomo, attraverso l'impiego di astrociti umani (HA), in modo da osservare gli effetti del MeHg su alcune vie biochimico - molecolari. In parallelo, ho usato colture cellulari HA, che sono stati trattati con acido alfa diidrolipoico prima di essere sottoposto all'azione di MeHg, per valutare il possibile effetto protettivo esercitato da questo composto con proprietà antiossidanti e chelanti sul metabolismo cellulare sottoposte all'azione tossica del metallo .

CHAPTER I

Introduction

1.1 Toxic Metals

Metals are substances that account for three quarters of the elements in the periodic table (Fig. 1), but only some of them are essential for life. The majority of known metals are considerably toxic when present in excess, so their concentrations are strictly regulated at the cellular level. Man has been in contact with metals for thousands of years, but only in recent decades has the use of metals, and especially toxic metals, become very intense.

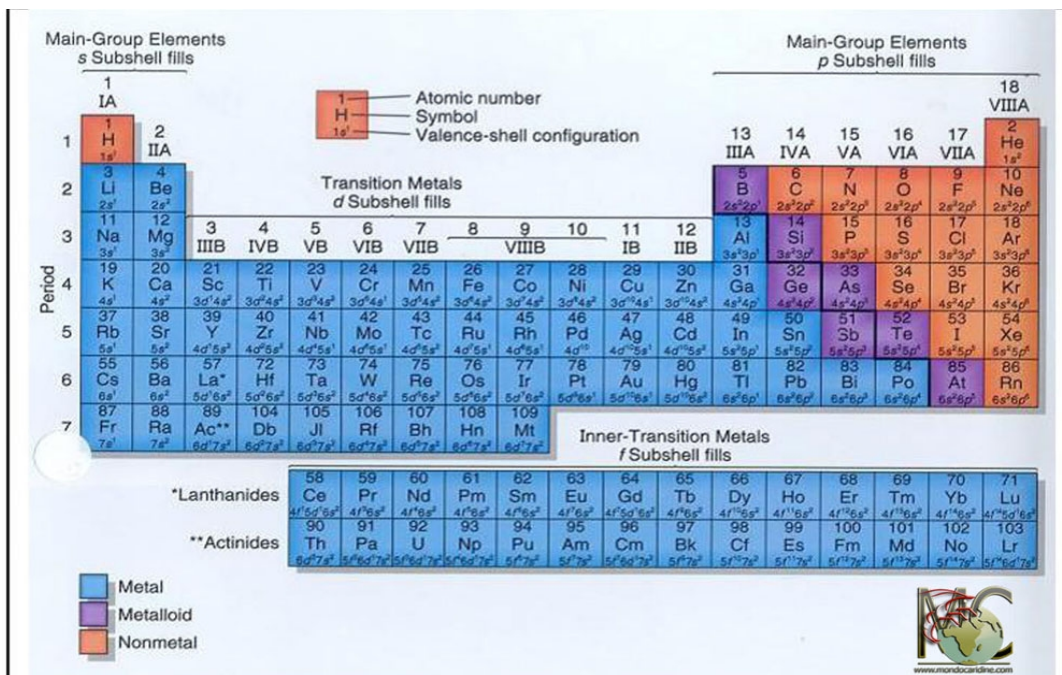


Figure 1. Periodic table of elements, or periodic table of Dmitri Mendeleev (1834 – 1907).

Chapter I: Introduction

The progressive environmental and food pollution that characterises our industrialised society undermines, especially in some subjects, our detoxification system and this causes the spread of various chronic degenerative diseases including cancer, immunodeficiency, autistic spectrum diseases, Alzheimer's disease, fibromyalgia, multiple chemical sensitivity, chronic fatigue, etc. However, whether mercury, lead, aluminium, cadmium and arsenic play a principle role in these diseases is still not well-known.

Heavy metal pollution originates from several sources including coal, natural gas, chlorine-alkali industries and volcanic eruptions (Higuera P. et al. 2006), but the most common are represented by metal purification, copper smelting and from nuclear fuels (Zevenhoven R. et al. 2001). Marine water pollution is mainly due to the development of human activities that introduce, either directly or indirectly, substances capable of causing harmful effects in living organisms and, consequently, in human health into the aquatic environment (Fig 2). In particular, it depends on the contaminants carried into the sea by internal water basins, along which persist many industrial and agro-livestock enterprises, and urbanisation phenomena, while a significant proportion is due to direct input of municipal and industrial waste into coastal waters (Severino L. et al. 2007).

Although the adverse effects of heavy metals on human health have been known for a long time, exposure to such toxic agents continues, which is moreover on the rise in some geographical areas and is very worrying for some amongst them. An example is mercury, which is still used for gold mining in many parts of Latin America (Department of Environment, 2001).

Currently, the thirteen elements of greatest concern in the world are: As, Cd, Co, Cr, Cu, Hg, Mn, Ni, Pb, Sn and Ti (Zevenhoven R. et al. 2001), whose emissions are closely regulated in waste incinerators.

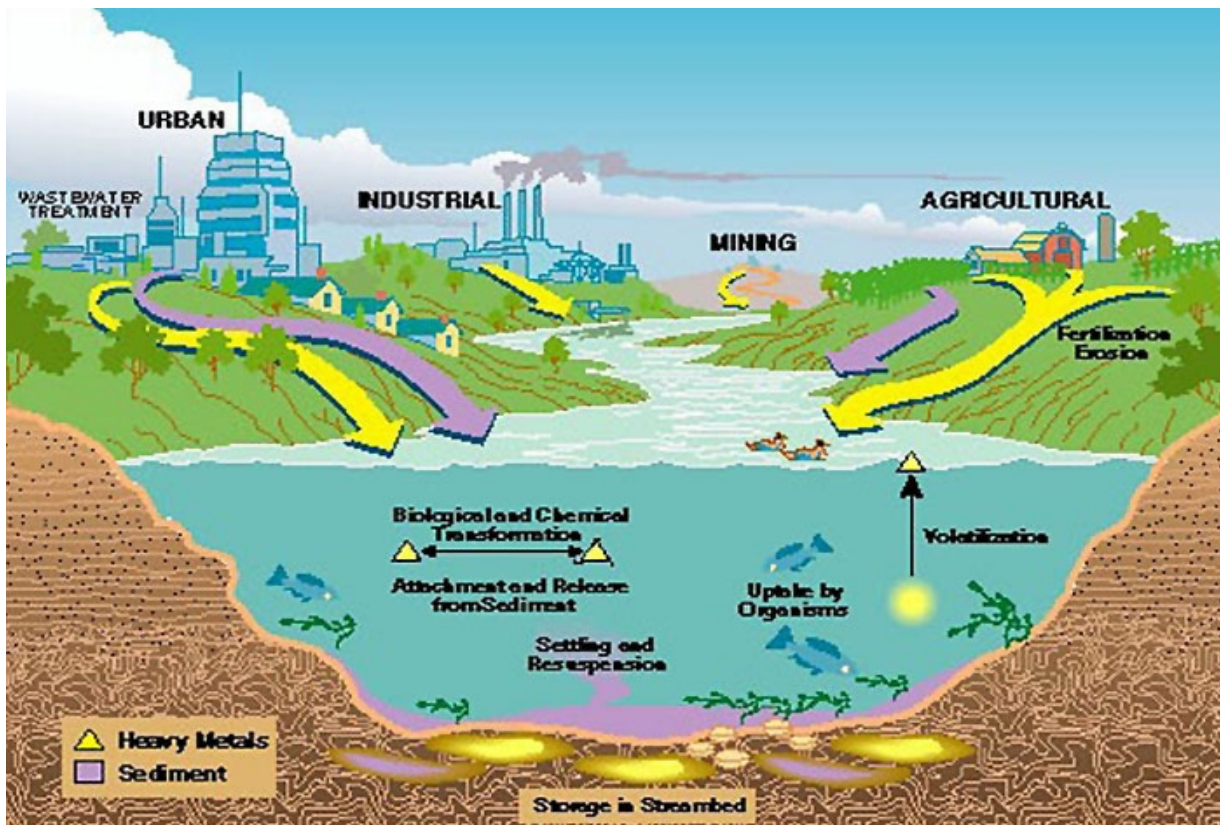


Figure 2. Sources of heavy metal pollution.

Heavy metals such as mercury, cadmium and lead are of particular concern because of their ability to bio-accumulate up the food chain until reaching highest contamination levels in predatory fish, such as tuna and swordfish (Storelli M.M. et al. 2005).

Some of these heavy metals are essential in small amounts (Co, Cu, Cr, Mn and Ni), but others are highly toxic (Hg, Pb and As). In particular, different heavy metals have different target organs. For example, Hg, Mn and Pb cause damage to the CNS, especially during its development stages. The kidneys and liver are susceptible to Cr and Cu, while the hard tissues such as bones and teeth are targets for Ni, Cd and Pb. Once released into the environment, heavy metal pollutants are able to localise and not decay; therefore, their toxicity may persist for generations. Man, therefore, may be exposed to chemical, physical and biological agents that are potentially harmful in

air, food, water or soil (Fig. 3).



Figure 3. Impact of environmental contaminants.

Exposure is a function of concentration and time. "It is an event that occurs for a certain period of time, when there is contact between the human being and the environment in which a contaminant is present at a specific concentration" (National Research Council, 1991). This stresses the importance of environmental factors and increases the possibility of an etiologic role in toxic exposures, which can be pre-natal or post-natal, but also the combination of maternal and gestational effects, and direct exposure of the newborn (Adams J.B. et al. 2007). In the tissues of some people who have been exposed chronically, especially women, there may be (asymptomatically) high levels of one or more heavy metals that can, for the most part, be transmitted to the foetus, or poison infants through lactation (Younes B. et al. 1995). These metals interfere with reproduction and development, causing damage both during the growth of the foetus and at birth. They can also cause neurological damage, including developmental delays, learning disorders and behavioural

abnormalities (Fig. 4). Therefore, a potential diagnostic tool, which allows for the evaluation of previous exposure (Al-Ayadhi L. 2005), is the mineralogram analysis, generally carried out on a sample of hair taken, in appropriate conditions, from the nape. This investigation is backed up by the analysis of the levels of minerals in the urine, a useful method for detecting exposure levels among the various minerals. This is a very useful and often necessary practice to ensure an improvement in metabolism and also the elimination of toxic minerals.



Figure 4. Hg and MeHg toxicity from mother to foetus.

1.2 Mercury and Methylmercury

Mercury is a heavy metal whose presence in the environment is of both natural and anthropogenic origin. During the last century, industrialisation has significantly increased the release of this element into the environment (Mason R.P. et al. 1994). In recent decades, its industrial use has been greatly reduced, but its presence in the

environment appears to be constant, because of its elevated persistence in marine sediments, and because mercury discharged into the environment from natural sources, such as volcanic eruptions, arrives in the aquiferous system (Covelli S. et al. 2009). Once the mercury reaches the water, bacteria present in the oceans convert inorganic mercury into its organic form: methylmercury. This is the most prevalent form of mercury, the most toxic and the form that enters the food chain through plankton and then passes, through invertebrates and fish located at the lower levels of the food chain, to the large predators, where the highest concentrations are found (Fig. 5) (Mason R.P. et al. 1994; Covelli S. et al. 2009).

There is a greater accumulation in muscle tissue than in fat and about 90-99 % of the mercury we find is methylmercury (Ferrara F. et al. 2004).

The result is that the major source of human exposure is represented by consumption of certain types of fish, taking into consideration the type of product and place of capture (Clarkson T.W. 1997; Spry D.J. et al. 1991). The presence of mercury in food from the sea is not uniform. It depends on the concentration of Hg in the water and its pH, the redox potential, the species, age and size of specific fish. The conditions of the microenvironment, especially anoxia, favour the growth of microorganisms and increase the percentage of HG methylation (Boudou A. et al. 2005). But there are also other factors that can significantly increase the toxicity of the fish and / or harm to the individual, including the presence of other metals in the marine environment and, therefore, in fish. In this case, repeated exposure to the metal can lead to the manifestation of symptoms of intoxication. Even though exposure occurs at relatively low levels for each substance, we return to the concept of bioaccumulation (Wecker L. et al. 1985).

1.3 Methylmercury Toxicity

It has been shown that MeHg targets the CNS, and children are most vulnerable to

MeHg toxicity, exhibiting dyskinesia, mental retardation, primitive reflexes, growth disorders, salivation, intestinal dysbiosis and various other cognitive, learning and motility disorders (Tsubaki T. 1967; Harada M. 1995).

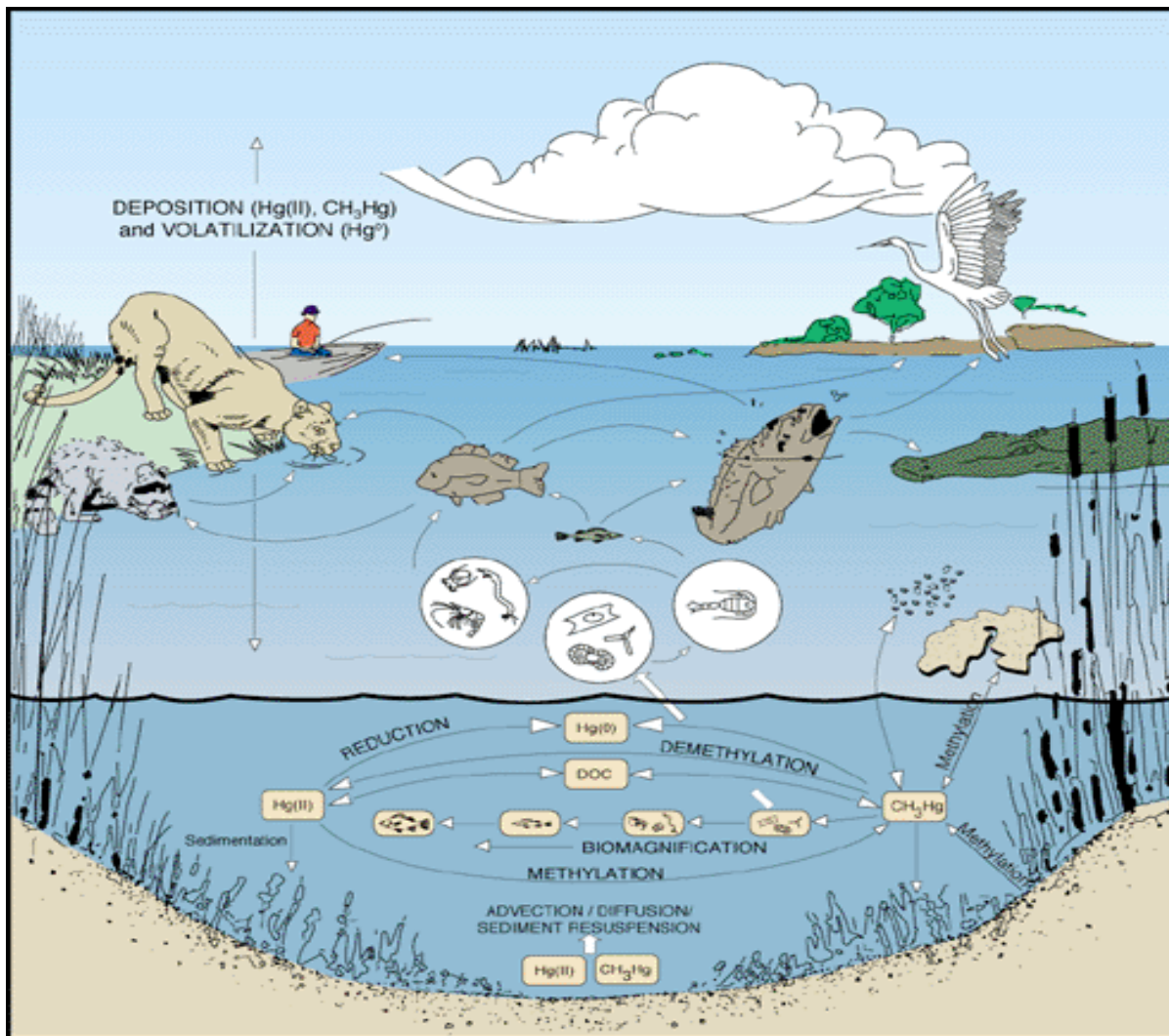


Figure 5. Routes of Transformation of Hg into MeHg.

From a clinical point of view, the symptoms of MeHg poisoning become significant when the levels of exposure to Hg exceed the values of 0.1 mg / Kg per day.

Inside the human body, methylmercury undergoes a redistribution from the gastrointestinal tract to the majority of organs and systems. About 90 % is easily absorbed

and has a long retention time, with a half-life of about 70 days. Distribution in the blood, where it reaches about 7 % of the same ingested dose, occurs within 30 hours following ingestion (Kershaw T.G. et al. 1980). The MeHg present in the systemic circulation accumulates mainly in red blood cells by binding to cysteine residues (-SH) also present in the beta chain of haemoglobin (Doi R. 1991), and is then slowly distributed in other tissues until reaching a balance over a number of days. (Fig. 6) (Kershaw T.G. et al. 1980).

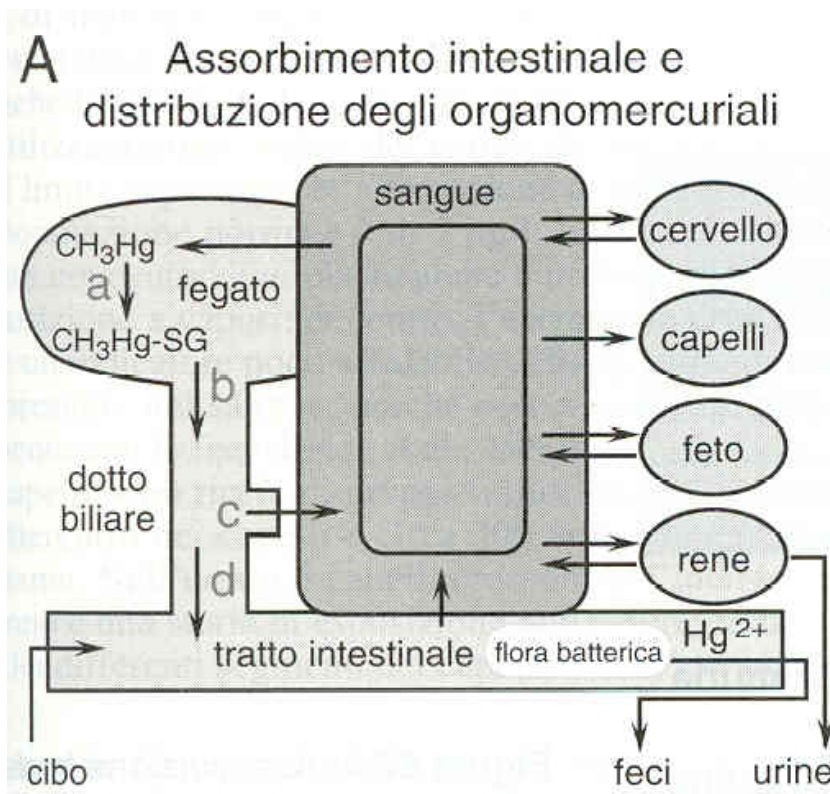


Figure 6. Absorption and elimination of MeHg.

In *steady-state* conditions, the ratio of MeHg concentrations in the blood and hair is approximately 1/250 (Skerfving S. et al. 1974), and in non-symptomatic conditions, the excretion rate of mercury is about 1 % of body weight (Swedish Expert Group 1971).

The elimination of MeHg occurs for the most part from the liver into the bile and

from the kidneys into the urine, but a substantial amount is reabsorbed in the intestine and enters enterohepatic circulation. The rate of excretion may also be affected by diet, as some dietary components may interfere with the absorption of MeHg in the lower intestine by interrupting enterohepatic circulation (Landry T.D. et al. 1979). A characteristic of this organic form of the metal is also its ability to cross the placenta and accumulate in the foetus in a concentration greater than that present in the mother. Therefore, its levels in umbilical cord blood are two times higher than those in the mother's blood at delivery. About 5 % of the MeHg present in maternal blood is found in the breast milk (Bakir F. et al. 1973).

Today it is known that acute intoxication can evolve into pneumonia and pulmonary oedema, neuropathy, gastroenteritis, renal tubular necrosis and glomerular changes, while chronic poisoning leads to gingivostomatitis, pain in the limbs, cardiovascular disorders, tremor, neuropsychiatric disorders and in particular foetal and neonatal neurotoxicity and psychomotor retardation. MeHg, in the CNS, accumulates in particular in the glia, mostly in the astrocytes, which represent up to 50 % of the cell volume (Aschner M. et al. 2000).

1.4 Physiology and Functions of Astrocytes

Astrocytes, also known as astroglia, are a cell type of the CNS, present in the brain and spinal cord, characterised by a typical star shape due to the presence of numerous protrusions in their structure (Fig. 7). They play a fundamental role in maintaining the homeostasis of the CNS, participating in the formation of the blood-brain barrier, providing support and nutrients to neuronal cells and endothelial cells. They also play a major role in the processes of defence and regeneration of the brain and spinal cord following a trauma (Ramon Y. et al. 1909). They are also able to secrete growth factors, release cytokines and modulate synaptic transmission by removing neurotransmitters, such as glutamate, from the synaptic cleft (Schwabe T. et al. 2005;

Allen N.J. et al. 2005).

Astrocytes, considering morphological differences and different anatomical sites, can be classified into two main types (Ramon Y. et al. 1909):

- 1 - fibrous, with long, branched protrusions that wrap around the capillary wall;
- 2 - protoplasmic, present mainly in the grey matter, with short, widely branched protrusions. They are involved in maintaining extracellular ion balance, in the uptake of glutamate (Bechtholt-Gompf A.J. et al. 2010), in controlling GABA receptor function (Beenhakker M.P. et al. 2010), in controlling pH and in regulating the concentration of potassium (K^+). The concentration of that ion in the extracellular fluid tends to increase due to the depolarisation of the neurons; this may cause interference in the normal propagation of an action potential from one neuron to another. Therefore, the presence of a high number of potassium channels in these cells facilitates the removal of the ion from the extracellular compartment, allowing the subsequent transfer to neighbouring cells via gap junctions (Walz W. 2000).

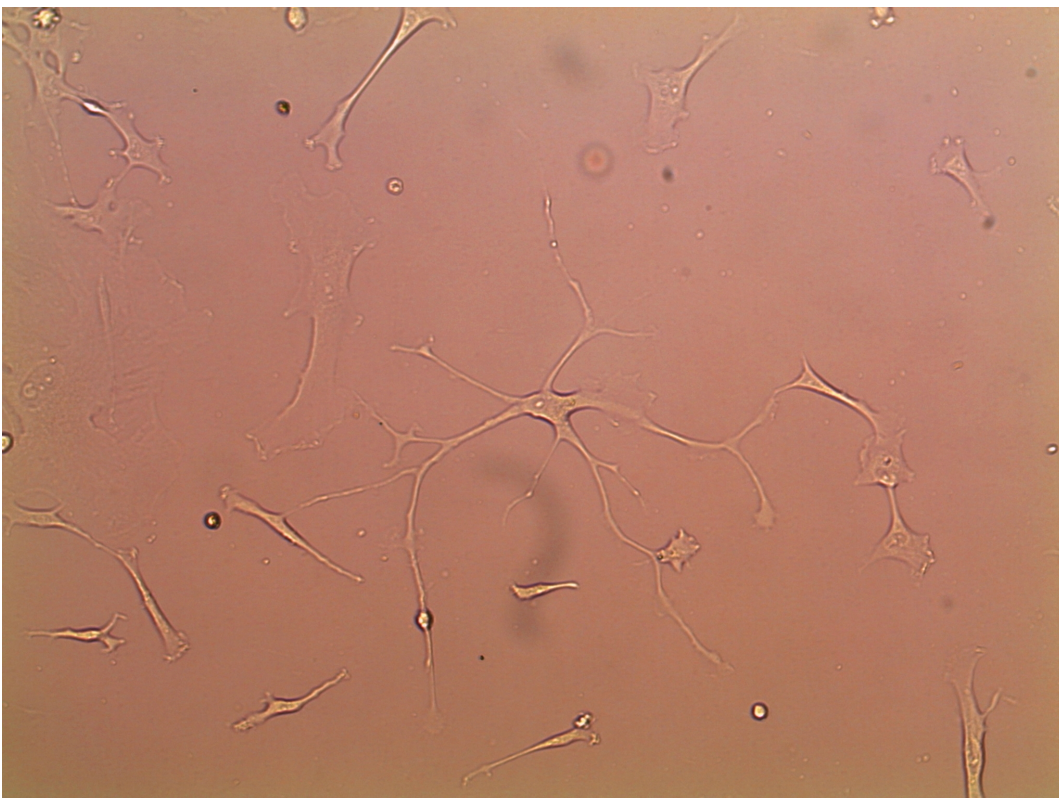


Figure 7. Human astrocytes.

Besides the presence of channels for potassium, astrocytes also present them for sodium; however, unlike neurons, they are not able to trigger or propagate any action potential (Nedergaard M. et al. 2003; Seifert G. et al. 2006). Nevertheless, it does not mean that astrocytes are physiologically silent. They show, in fact, a kind of cell excitability that, depending on the increase in the intracellular concentration of calcium (Ca^{++}), represents the means of intercellular communication between astrocytes and between astrocytes and neurons (Halassa M.M. et al. 2007; Perea G. et al. 2009; Shigetomi E. et al. 2008; Volterra A. et al. 2005).

Recent studies have shown that these cells play a pivotal role in response to cerebral injury and their functional deficiency may contribute to the development of various neurodegenerative diseases, as well as being involved in 'brain ageing' (Araque A. 2006). A brain injury causes a reaction by one of the glial components, defined "reactive gliosis" (Pekny and Nilsson 2005; Norton W.T. et al. 1992). Astrocytes react to the injury by increasing in volume (hypertrophy), proliferating and starting inflammatory pathways, and so isolate the damaged area by forming a glial scar. This phenomenon is very useful in the short and medium term, because it contains the injured area and limits the inflammatory process (Kawano H. et al. 2012). In any event, in an excitotoxic state, the protective functions of reactive astrocytes, such as the uptake of glutamate, the maintenance of electrolyte homeostasis and the elimination of free radicals remain essential for the containment of neuronal damage (Iwata-Ichikawa E. et al. 1999; Chen Y. et al. 2001). It is clear that this kind of glial activation, typical of reactive gliosis, plays a key role in the mediation of the metabolic processes of neurons in physiological, toxic and neurodegenerative processes (Kawano H. et al. 2012).

1.5 Methylmercury Neurotoxicity

Astrocytes, even if not the only cell type to be negatively affected by exposure to MeHg, have been shown to play a key role in mediating neurotoxicity, which is induced by the organic form of this metal for several reasons (Fig. 8):

- Astrocytes are the preferred site of accumulation of MeHg (Garman R.H. et al. 1975; Aschner M. et al. 2000);
- MeHg inhibits the uptake of glutamate (and aspartate) and stimulates efflux. The resulting increased concentration of the two amino acids in the extracellular fluid sensitises neurons to excitotoxic injury (Allen J.W. et al. 2001; Brookes N. et al. 1989; Aschner M. et al. 1993; Dave V. et al. 1994);
- It has been shown that MeHg-induced neuronal dysfunctions are secondary to disorders already present in astrocytes (Brookes N. 1992). In fact, the *in vitro* co-application of non-toxic concentrations of mercury with glutamate generate typical neuronal lesions, typical of excitotoxic stimulation (Matyja E. et al. 1993);
- MeHg also promotes the cytotoxic cycle by activating cytosolic phospholipase A2 with the consequent release of arachidonic acid and inhibition of glutamate transport (Shanker G. et al. 2002; Shanker G. et al. 2003).

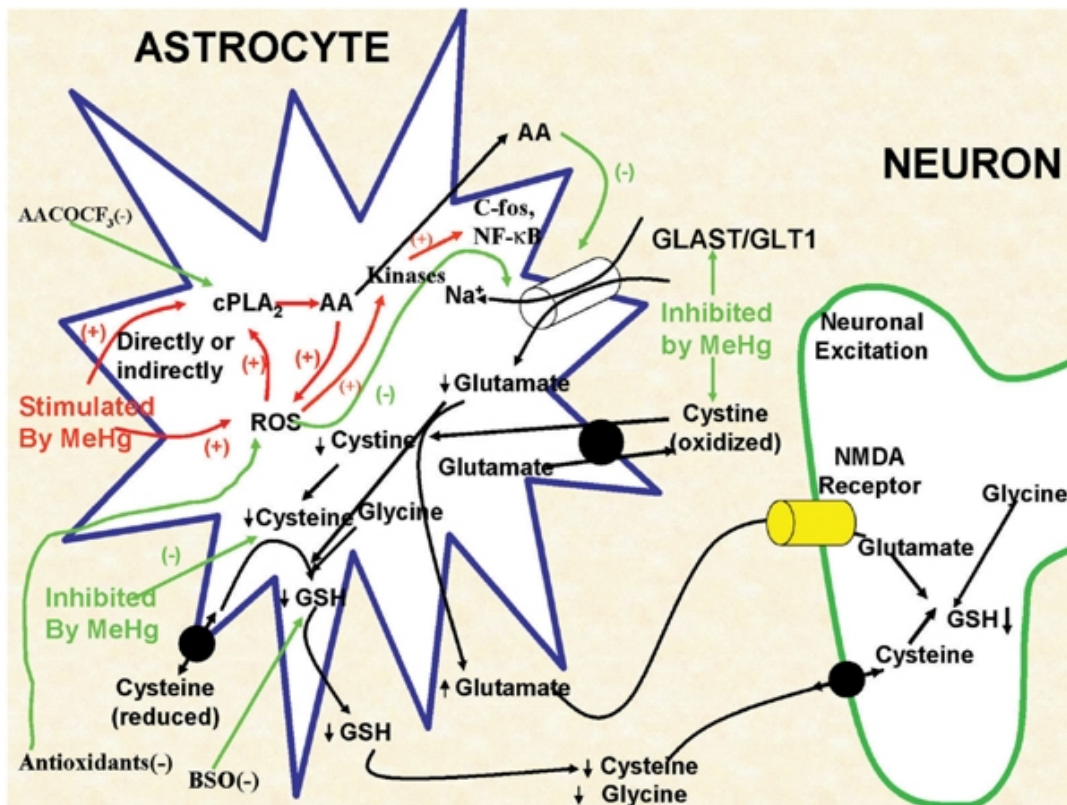


Figure 8. Schematic model proposed by M. Aschner et al. for MeHg neurotoxicity.

It should also be added, as previously mentioned, that as well as damaging the cells in different ways, mercury also binds to sulfhydryl groups, causes microtubules depolymerisation and neurotransmission alteration (Castoldi A.F. et al. 2001).

Since astrocytes are the cell type, which occupies up to 50 % of volume in the CNS, it is clear that one of the main causes of observed neurotoxicity is correlated to their dysfunction, resulting from harmful exposure to mercury (Chen S.H. et al. 2000). It is well known that upon contact with MeHg, about 10 % of this toxic form of the metal is retained in the encephalon, deposited preferentially in certain cell types of the CNS. This can be explained by considering the ease with which this element crosses the blood-brain barrier, accumulating in various brain areas, such as the cerebellum, cerebral cortex and retina (Mottet N.K. et al. 1984; Erie J.C. et al. 2005). The damage induced in the CNS, especially in a fully developed encephalon, is localised in certain areas of the visual cortex and includes the loss of the layer of granules of the

cerebellum. Differently, in a developing CNS, which is extremely sensitive to neurotoxicity induced by the metal, a diffuse cytoarchitecture disorganisation of the cerebral cortex is manifested, accompanied by the disappearance of the granule cells (Vettori M.V. et al. 2003). The onset of these kinds of alterations could also be caused by failure of the process of cell division, mediated by MeHg, in the course of the development phases of the CNS (Vettori M.V. et al. 2003).

Another mechanism of toxicity is the ability of the metal to inhibit the uptake of cysteine, an amino acid precursor in the synthesis of glutathione (GSH), whose levels cause a serious reduction with damage even at the neuronal level as these cells are replenished with GSH from astrocytes (Shanker G. et al. 2002; Allen J.W. et al. 2002). GSH, in fact, plays a key role as a scavenger of reactive oxygen species (ROS), whose concentration is known to increase in response to exposure to MeHg (Yin Z. et al. 2007), resulting in alteration of mitochondrial functions and an increase in oxidative stress, known to be involved in several neurodegenerative disorders, including Alzheimer's, Parkinson's and Amyotrophic Lateral Sclerosis (Kaltschmidt B. et al. 1993; Kitamura Y. et al. 1997). The increased production of MeHg-induced ROS in astrocytes and the consequent alteration of cellular homeostasis are phenomena also related to the increase in glutamate, which cause neurotoxicity. In fact, MeHg also induces inhibition of glutamate uptake in astrocytes with simultaneous stimulation of efflux of this amino acid, causing an excessive concentration at the synaptic level, and so generating neurotoxicity (Aschner M. et al. 1993; Yin Z. et al. 2007). The increase in the concentration of glutamate in the extracellular fluid, causes hyper-activation of the NMDA receptor for glutamate (N-methyl-D-aspartate) with consequent increase in the influence of ions Na^+ and Ca^{++} (Choi D.W. 1992). In a sort of vicious circle, the increase in intracellular levels of Ca^{++} generates an increase in ROS (Lafon-Cazal M. et al. 1993), the production of which inhibits the transport of glutamate by astrocytes.

It is the combination of these effects which provokes a reduction in intracellular GSH

levels, increased levels of synaptic glutamate with increased expression of NMDA receptors on adjacent neurons, and therefore excitotoxicity.

Exposure to MeHg is also able to alter the cell cycle, interfering negatively with the proliferative process during the development phases of the CNS.

Glial cells provide neurotrophic signals to neurons, which are necessary for their survival, proliferation and differentiation. Various proteins are involved in these processes, such as:

- proteins belonging to the cyclins family (cyclin D1);
- the intermediate filament proteins: GFAP (glial fibrillary acidic protein), nestin and vimentin;
- the MAP-kinase (mitogen-activated protein).

1.6 Methylmercury and Oxidative Stress

Although free radicals, reactive oxygen species (ROS) and reactive nitrogen species (RNS) are known to be key molecules in the cell where they act as "molecular beacons". An excess of these, caused by hyper-production or reduced *scavenger* activity of antioxidant systems, i.e. a condition known as oxidative stress is strongly implicated in several neurodegenerative diseases including Alzheimer's disease, Parkinson's disease and amyotrophic lateral sclerosis (Kaltschmidt B. et al. 1993; Hunot S. et al. 1997; Kitamura Y. et al. 1997), but also in other degenerative conditions such as autoimmune diseases and inflammatory diseases (e.g. ischemia and rheumatoid arthritis), cancer, diabetes mellitus and atherosclerosis (Kehrer J.P. et al. 1994; Halliwell B. et al. 1994; Betteridge D.J. 2000), as well as in the toxicity induced by the metal (LeBel C.P. et al. 1992).

ROS are known to mediate neurotoxicity induced by MeHg in different experimental models. For example, MeHg induces the formation of ROS *in vivo* (in the cerebellum of rodents), and *in vitro* (isolated from rat brain synaptosomes) (Ali S.F. et al. 1992),

as well as in neuronal cultures of the cerebellum, in a neuronal hypothalamic cell line and in mixed aggregates cell cultures (Sarafian T.A. et al. 1994; Park S.T. et al. 1996; Sorg O. et al. 1998). Furthermore, an increase in ROS was observed in: a) isolated mitochondria from a rat brain in which MeHg was injected (Yee S. and Choi B.H. 1996), b) isolated mitochondria *in vitro* from a rat brain and subsequently exposed to MeHg (Myhre O. and Fonnum F. 2001), and c) mitochondria of astrocytes and neurons exposed to Hg and glutamate (Dugan L.L. et al. 1995; Brawer J.R. et al. 1998). The production of ROS and glutamate dyshomeostasis induced by MeHg are related phenomena that influence each other.

1.7 Action of Methylmercury on Redox Systems

The interaction of MeHg with nucleophilic groups of biomolecules with both low and high molecular weight and its affinity for -SH groups, support and confirm the observations that oxidative stress is a central event in mediating MeHg-induced neurotoxicity (Aschner M. et al. 2007); in fact, levels of GSH (glutathione) are exhausted after MeHg intoxication.

MeHg targets specific proteins containing thiol groups and selenium that trigger molecular mechanisms involved in secondary MeHg neurotoxicity. In particular, MeHg can interrupt the activity of proteins containing a thiol group or selenium, such as glutathione peroxidase (GPX), thioredoxin (Trx) and thioredoxin reductase (TrxR) (Carvalho C.M. et al. 2008; Farina M. et al. 2009; Franco J.L. et al. 2009; Glaser V. et al. 2010; Branco V. et al. 2011), all major components of the cellular antioxidant system, thus contributing to the rupture of the normal redox balance of brain cells (Farina M. et al. 2011).

However, even if the fundamental role of oxidative stress in MeHg-induced neurotoxicity has been identified, the underlying molecular mechanisms are not yet

fully understood.

In fact, the initial oxidative damage caused by methylmercury in astrocytes, as described above, is also supported by its reaction with endogenous molecules containing thiol (-SH) or selenium (-SEH) proteins at the base of the cellular redox buffer, causing the formation of a very stable complex of the RSHgCH₃ or RSeHgCH₃ type (Aschner M. et al. 2011; Farina M. et al. 2011, Branco V. et al. 2011) and in the case of proteins and enzymes containing these groups, the S-Hg Se-Hg bond formation can affect its function.

The thiol groups are much more abundant than the selenium groups (Nogueira C.W. and Rocha J.B.T. 2010), They are, in fact, found in proteins of low molecular weight (primarily cysteine and reduced glutathione) and high molecular weight, while the selenium groups are found in a small group of selenium-proteins (Araie H. and Shiraiwa Y. 2009; Lobanov A.V. et al. 2009; Lu J. and Holmgren A. 2009), but these groups may be critical for the catalytic activity of several enzymes (such as glutathione reductase etc.), being involved in intermediary metabolism and antioxidant processes. Since MeHg is able to influence enzymatic activity, it is reasonable to hypothesise that this interaction is responsible for imbalances in oxidative metabolism, as well as increasing levels of reactive species. In figure 9, some metabolic pathways in which some reactive species are the mediators of MeHg-induced neurotoxicity are shown:

- Hydrogen peroxide (H₂O₂) is quantitatively the most important peroxide generated by the cells (Dringen R. et al. 2005) and the main sources of H₂O₂ are the disproportionation of mitochondrial superoxide anion (Inoue M. et al. 2003) and reactions catalysed by oxidases, such as monoamine oxidase (Nicotra A. et al. 2004). Although it is known that hydrogen peroxide carries out important physiological functions in the modulation of cellular functions, high levels of H₂O₂ are dangerous for the cells, in large part due to the generation of hydroxyl radicals by the Fenton reaction (McCord J.M.

and Day E.D. 1978).

- MeHg is able to selectively inhibit transport of cysteine and cysteine in astrocytes, thereby negatively influencing their oxidation-reduction state and reducing glutathione content (GSH), an important antioxidant for humans, thus generating oxidative stress (Allen J.W. et al. 2001; Shanker G. et al. 2003);

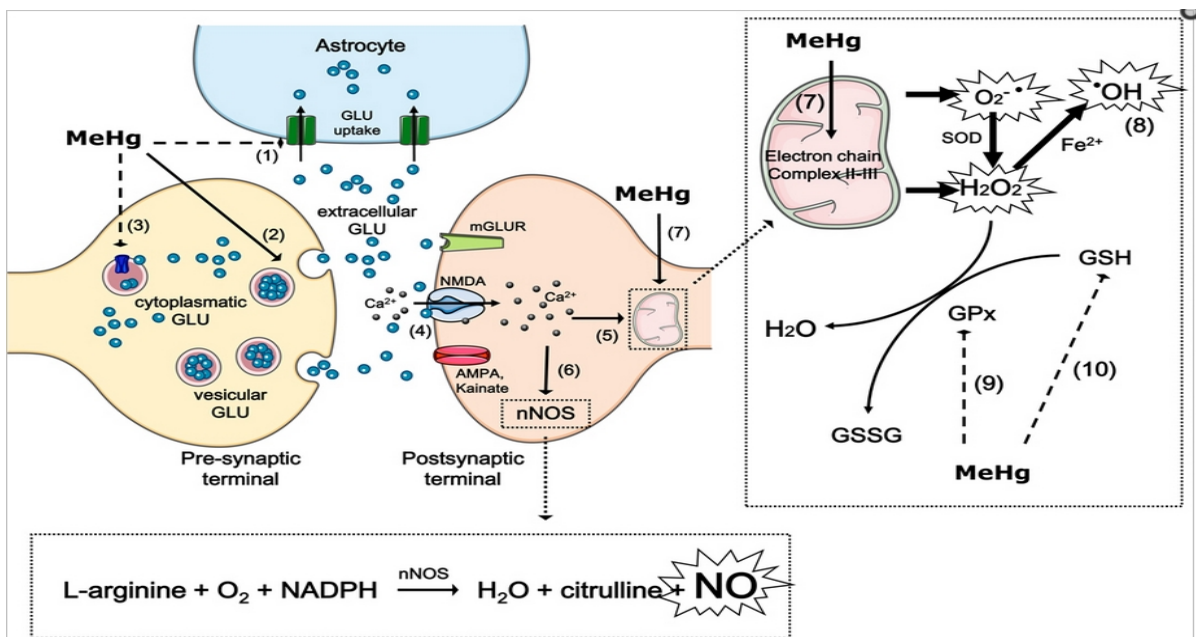


Figure 9. Reactive radical species as mediators of MeHg neurotoxicity.

- The superoxide anion (O₂^{•-}), a by-product of the normal operation of the mitochondrial respiratory chain is produced after the one-electron reduction of molecular oxygen. Even the superoxide anion plays an important role in MeHg-induced oxidative damage. Shanker and collaborators (2004), using a specific probe for the superoxide (hydroethidine), observed an increase in levels of ROS in cultured astrocytes treated with MeHg.

The main pathways for H₂O₂ decomposition involve catalase (CAT),

cytoplasmic glutathione peroxidase (GPx1) and peroxiredoxin (Prx) (Winterbourn C.C. and Hampton M.B. 2008). With particular attention to the pro-oxidant effects of MeHg, the evidence shows that it has the ability to increase levels of H₂O₂ in various experimental conditions (Manfroi C.B. et al. 2004; Shanker G. et al. 2004; Franco J.L. et al. 2007; Mori N. et al. 2007). The increased levels of H₂O₂ observed after exposure to MeHg represent the consequences of various phenomena, such as the inhibitory effects of MeHg on glutathione peroxidase.

GPxs represent a family of selenoproteins whose catalytic activity depends on the reducing power of a selenium group, located in the active site (Brigelius-Flohé R. 2006). Due to the high affinity of MeHg for selenium groups, the decreased activity of GPX after exposure to MeHg has been attributed to direct inhibitory events (Farina m. et al. 2009). Furthermore, a recent study has proposed another molecular mechanism to explain the reduced activity of GPx after exposure to MeHg: MeHg induces a condition called “selenium deficiency,” which affects the synthesis of GPx through a post-transcriptional effect (Usuki F. et al. 2010), thus leading to decreased levels of GPX, reduced peroxidase activity and, consequently, higher levels of hydrogen peroxide.

Another mechanism linked to increased levels of H₂O₂ after exposure to MeHg seems to be the direct effect of this toxic substance on the whole antioxidant system of glutathione.

In addition to direct depletion of reduced glutathione, MeHg also hinders the physiological maturation of many enzymes involved in the metabolism of GSH, thus leading to increased levels of H₂O₂ in the brain (Stringari J. et al. 2008).

While it is known that increased levels of H₂O₂ is a consequence of exposure to MeHg, the precise role of this molecule in mediating MeHg-induced oxidative damage has not yet been fully determined.

- Nitric oxide (NO) is a neurotransmitter widely used as a signalling molecule by all cells of the body. Although NO plays a number of important roles as a regulator of diverse biological processes (vascular tone, platelet adhesion, neurotransmission), it can, however, also cause deleterious effects, including inhibition of some enzymatic functions, the promotion of DNA damage and the activation of inflammatory processes (Hollenberg S.M. and Cinel I. 2009).

Nitric oxide is synthesised from L-arginine and oxygen in a reaction catalysed by nitric oxide synthase (NOS), which has at least three distinct isoforms (inducible and constitutive), (NOS1, NOS2 and NOS3). Indeed, while the activity of NOS2 (inducible isoform) depends on transcription, NOS1 and NOS3 are constitutively expressed and are usually triggered by an increase in intracellular calcium (Alderton W.K. et al. 2001). In particular, an increase in the activity of the NOS enzyme and increased NO levels have been reported in MeHg-mediated neurotoxicity (Yamashita T. et al. 1997; Herculano A.M. et al. 2006), although the mechanisms involved in the interaction between MeHg and NO are not entirely clear.

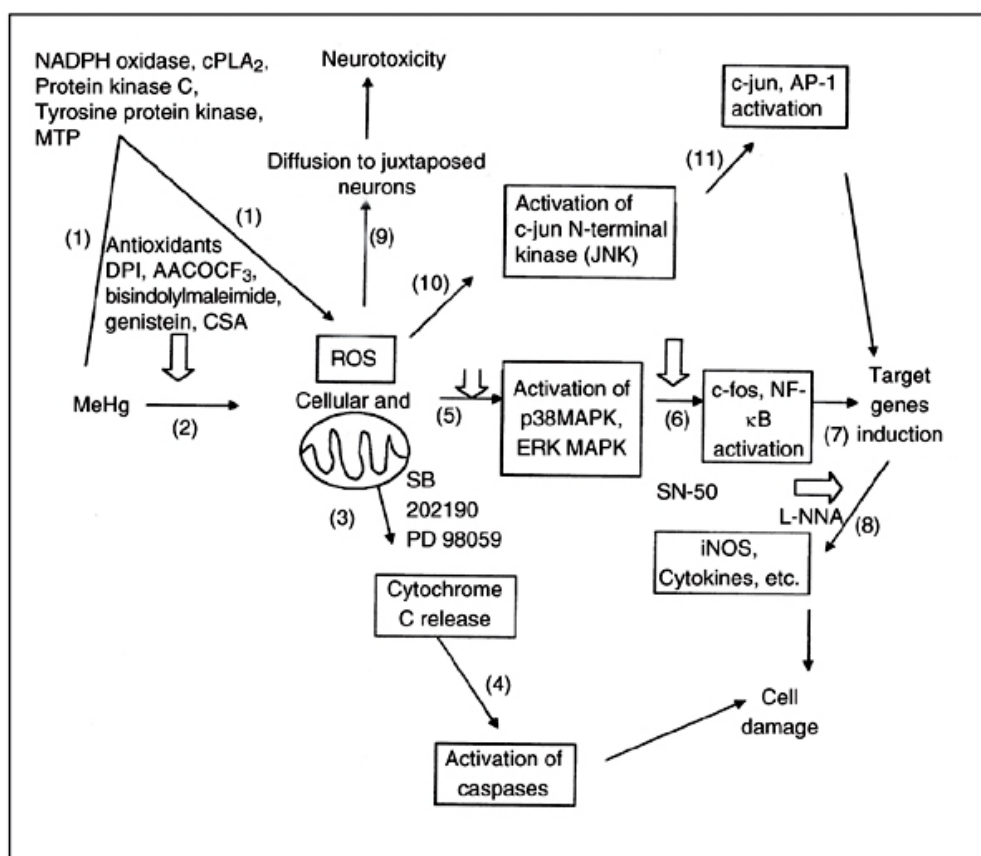


Figure 10. A model that represents the role of oxidative stress in MeHg-induced neurotoxicity.

A number of articles indicate that activation of some signal molecules is induced by oxidative stress (for example, NFκB, protein-1 activator, c-fos, c-jun, etc.). This activation leads to the induction of various target genes (i.e. inducible NOS, COX and SOD, the inducible form (HSPs-72), cytokines, etc.), that contribute to cellular damage (Kumagai Y. et al. 1997; Hsieh H.J. et al. 1998; Goering P.L. et al. 2000). However, to date there is a lack of systematic studies that examine and correlate the formation of ROS in astrocytes with MeHg-induced toxicity, possibly examining the role of different signal pathways involved in the process. Figure 10 shows the objectives that may be affected by MeHg, as proposed by Aschner M. et al. (2007). Considering that hydrogen peroxide is involved in MeHg toxicity, both *in vivo* and *in vitro*, numerous studies have examined the protective effects of treatment with antioxidant molecules (for example, the precursors of GSH, antioxidant and selenium

compounds) (Allen J.W. et al 2001 a, b; Farina M. et al. 2003; Ballatori N. et al. 1998). It has been shown that organoselenium compounds with thiol peroxidase activity exert protective effects, maintaining levels of H₂O₂ low in systems exposed to MeHg (Farina M. et al. 2005; Moretto M.B. et al. 2005).

Although N-acetylcysteine, the precursor of GSH, may contribute to the maintenance of homeostasis of intracellular GSH, which is crucial in the detoxification of hydrogen peroxide by glutathione peroxidase, some of the beneficial effects elicited by N-acetylcysteine in *in vivo* conditions are also linked to its ability to accelerate the urinary excretion of MeHg in poisoned animals (Koh A.S. et al. 2002). To date, the use of molecules with antioxidant activity to prevent/reduce MeHg neurotoxicity continues to have great reticence. Currently, the only way to prevent or ameliorate the effects of MeHg toxicity caused by poisoning is to accelerate the elimination from the body through the following various therapeutic strategies that the medical expert must be able to adapt from individual to individual: hemodialysis, exchange transfusions and chelation therapy performed by intravenous perfusion based on disodium salt of Ethylene-Diamino-Tetraacetic acid (EDTA) (Clarkson T.W. et al. 1981; Lund M.E. et al. 1984).

In addition to traditional chelation therapy, in less serious conditions of intoxication, you can resort to oral "chelates" such as zeolite, chlorella and alpha-lipoic acid.

1.8 Alpha-Lipoic Acid (ALA)

ALA (Figure 11), an essential molecule for cell energy metabolism, a cofactor at entry to Krebs cycle, displays anti-oxidant effects by increasing glutathione peroxidase activity and reducing oxidative stress (Mantovani G. et al. 2003), regulates calcium homeostasis (Sen C.K. et al. 1996), and modulates the activity of the transcription factor NFkB (Packer L. 1998; Packer L. et al. 1997). Indeed, several studies have shown that ALA exerts multiple pharmacological actions that can

prevent nerve degeneration in experimental *in vitro* models of diabetes (Vincent A.M. et al. 2005), Parkinson's disease (Bharat S. et al. 2002), and Alzheimer's diseases (Abdul H.M. and Butterfield D.A. 2007). It also inhibits oxidative stress in HIV infection in experimental studies (Packer L. et al. 1995) and reduces damage from ischemia-reperfusion in the central nervous system and cardio-vascular system in animal studies (Cao X. and Phillis J.W. 1995; Freisleben H.J. 2000).

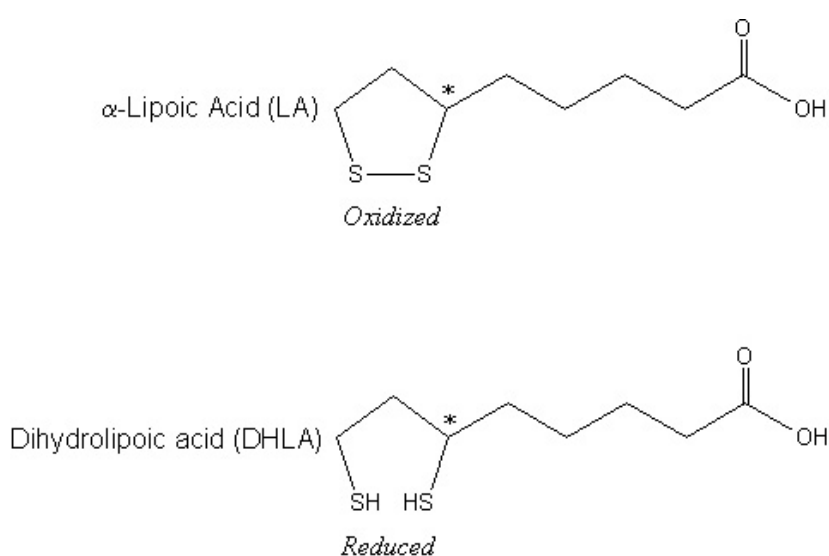


Figure 11. Alpha-lipoic acid

Alpha-lipoic acid, also known as thioctic acid, was first isolated from bovine liver in 1950 (Reed L.J. 2001). Lipoic acid contains two thiol groups, which may be oxidised or reduced. Like the thiol antioxidant glutathione, ALA is part of a redox pair, being the oxidised partner of the reduced form dihydrolipoic acid (DHLA). Unlike glutathione, of which only the reduced form is an antioxidant, both the oxidised and reduced forms of lipoic acid are antioxidants. ALA is reduced *in vivo* to its dithiol form, DHLA, which also possesses biological activity. DHLA is a potent reducing agent with the capacity to reduce the oxidised forms of several important antioxidants, including vitamin C and glutathione (Jones W. et al. 2002). Although

reduced glutathione has twice the chemical reactivity in its thiol group, DHLA is superior to glutathione in regenerating vitamin C (Suh J.H. et al. 2004). ALA is a naturally occurring compound that is synthesised in small amounts by plants and animals, including humans (Smith A.R. et al. 2004). Endogenously synthesised ALA is covalently bound to specific proteins, which function as cofactors for mitochondrial dehydrogenase enzyme complexes. In addition to the physiological functions of protein-bound ALA, there is increasing scientific and medical interest in potential therapeutic uses of pharmacological doses of free ALA (Kramer K. et al. 2001). ALA exists as two enantiomers: the R-enantiomer and the S-enantiomer. Naturally occurring ALA is in the R-form, but synthetic ALA is a racemic mixture of R- and S- forms. Both forms seem to have different potencies; it was previously shown that the R-form is more potent than the S-form in its ability to stimulate glucose uptake in L6 myotubes, (Estrada D.E. et al 1996) as well as to increase insulin-stimulated glucose uptake in obese Zucker rats (Khanna S. et al. 1999). On the other hand, the S-form exerts a slightly increased affinity for glutathione reductase (Pick U. et al 1995), thus the two forms of ALA differ in the potency with which they exert various biological activities. As stated by Packer et al. (1997), an ideal therapeutic antioxidant should fulfil several criteria. ALA is unique among natural antioxidants in its ability to fulfil all of these requirements, potentially making it a highly effective therapeutic agent in a number of conditions in which oxidative damage has been implicated. The antioxidant properties of ALA comprise the following:

1. Its capacity to directly scavenge reactive oxygen species (ROS);
2. Its ability to regenerate endogenous antioxidants, such as glutathione, vitamins E and C;
3. Its metal chelating activity, resulting in reduced ROS production.

Due largely to its antioxidant properties, ALA has recently been reported to afford protection against oxidative injury in various disease processes, including

neurodegenerative disorders (Packer L. et al. 1997; Evans J.L. and Goldfine I.D. 2000). Although the ability of ALA to directly scavenge ROS appears to be responsible, at least partially, for its neuroprotective effects, it remains unknown whether the neuroprotective effects of ALA may also occur through other mechanisms, such as induction of the endogenous antioxidants and phase 2 enzymes in neuronal cells. ALA might also be able to induce endogenous antioxidants and phase 2 enzymes in neuronal cells, and the increasing endogenous defences may afford protection against oxidative/electrophilic neuronal cell injury.

ALA has neuroprotective effects in neuronal cells. One possible mechanism for the antioxidant effect of ALA is its metal chelating activity (Ou P. et al. 1995).

In a further study, Müller U. and Krieglstein J. (1995) tested whether pre-treatment with ALA can protect cultured neurons against injury caused by cyanide, glutamate, or iron ions. Neuroprotective effects were only significant when pre-treatment with ALA occurred for 24 h.

CHAPTER II

Aims

In light of what has been stated about MeHg toxicity and the lack of knowledge about the molecular mechanisms underlying its toxicity, most importantly in humans, I designed and developed the research subject of this thesis using human astrocyte cultures treated with MeHg, both at 24 h (acute treatment) and 72 h (chronic treatment), as my experimental model. The overall goal of my work was to carry out *in vitro* research using, for the first time, according to what has been reported in the literature, an experimental model that was as attributable as possible to humans, precisely human astrocytes, so as to observe the effects of MeHg on some biochemical-molecular pathways. In parallel, I used human astrocyte cell cultures, which were treated with Alpha Dihydrolipoic Acid before being subjected to the action of MeHg in order to evaluate the possible protective effect exerted by this compound with antioxidant and chelating properties on cellular metabolism subjected to stress by MeHg. Firstly, I examined the expression of some proteins capable of exhibiting possible astrocytic reactivity and the alteration of the cell cycle (GFAP, vimentin, nestin, etc) and, immediately afterwards, the protein expression of the cytoskeleton, in order to highlight a possible disintegration of the cytoskeleton (β -tubulin and actin). This research was supported by studying, in parallel, the cellular structure, which was carried out with electronic microscope analysis (SEM). The next step was to study some key markers of oxidative damage (ROS levels and GSH), and to analyse the expression of some proteins involved in the oxidative stress-induced alteration of cellular homeostasis, such as HSPs 27-60, e70KDa, iNOS, GGT1 and SOD, and the expression levels of metallothioneins. Before going on to study redox parameters in depth, I examined the expression levels of well-known markers of

apoptosis, caspase-3, AIF and Cyt C, in order to understand if programmed death was started in the cells (apoptosis), in both acute and chronic treatment. In all of the aforementioned phases, I always evaluated the effects of pre-treatment with lipoic acid by analysing the results, paying attention to possible differences between acute and chronic treatments. Therefore, encouraged and motivated by the results obtained in the first and second years, to study further the treatment-induced modulation of the cellular redox state and with the precise intention of individuating new specific targets, I went on to examine the modifications induced in both carbonyl and thiol groups, using redox proteomics. I then separated the proteins present in the cell lysate with both one-dimensional and two-dimensional electrophoresis, marking the free thiol groups with 5'-iodoacetamidofluorescein (IAF). The fluorescence images of all gels, 1D and 2D, were subsequently acquired and analysed using dedicated software.

CHAPTER III

Materials and Methods

3.1 Chemicals and Reagents

MeHg was purchased from Sigma Chemical Co. (St. Louis, MO, USA). Astrocyte Growth Supplement (AGS) was obtained from ScienCell Research Laboratories (USA). Dulbecco's Modified Eagle's Medium (DMEM), penicillin-streptomycin, trypsin-EDTA, foetal bovine serum (FBS) were obtained from GIBCO BRL (Invitrogen Corp., Carlsbad, CA, USA). ALA raceme or (R+) enantiomer were kindly provided by the School of Pharmacy, University of Camerino.

3.2 Primary Human Astrocyte Cell Line and Treatments

Primary Human Astrocytes (HA) were obtained from ScienCell. Cells were cultured and plated onto 75 cm² cell culture flasks and grown at 37 °C (humidified 5 % CO₂ and 95 % air at 1 atmosphere) in DMEM supplemented with 10 % FBS, 1 % AGS, 100 Units/ml penicillin and 100 µg/ml streptomycin. When the culture reached the sub-confluence, the cells were treated with MeHg 1.125 µM for 24 h or 72 h. This concentration was chosen based on preliminary experiments to assess toxicity (data not shown). In order to simulate acute and chronic damage conditions, two different times of exposure (24 h and 72 h) were used. The cells were plated at a constant density to obtain identical experimental conditions in the different tests and cells not exposed to MeHg were used as a reference.

Astrocyte cultures were maintained under the following experimental conditions:

- Untreated control HA non-pre-treated or pre-treated with 50 μ M ALA raceme or (R+) enantiomer for 24 h
- Chronic treatment of HA with MeHg 1.125 μ M for 24 h, non-pre-treated or pre-treated with 50 μ M ALA raceme or (R+) enantiomer for 24 h

with 0,5 mM glutamate for 24 h

- Acute treatment of HA with MeHg 1.125 μ M for 72 h, non-pre-treated or pre-treated with 50 μ M ALA raceme or (R+) enantiomer for 24 h

ALA was dissolved in 0.5 M DMSO and then diluted in the culture medium at a concentration of 1 mM. Catalase (1000 U/ml) was added and the solution was incubated at 37 °C for 30 min. This solution was diluted in the culture medium in order to obtain 50 μ M ALA final concentration and 1:10 in the culture medium in order to obtain 100 μ M ALA final concentration.

3.3 Mitochondrial functionality assessed by MTT assay

HA were treated with MeHg (1.125 μ M) for 24 h/72 h and non-pre-treated or pre-treated with 50 μ M ALA raceme or (R+) enantiomer for 24 h. The MeHg cytotoxic effect was evaluated as previously described (Malfa et al. 2010). This cell viability test is based on the conversion of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) into insoluble blue formazan crystals by mitochondrial dehydrogenases of metabolically active cells. The optical density of each well sample was measured with a microplate spectrophotometer reader (Titertek Multiskan, Flow Laboratories) at λ 550 nm. The results are reported as a percentage of cell viability with respect to untreated control cells.

3.4 Lactate dehydrogenase (LDH) release

Lactate dehydrogenase (LDH) activity, as a membrane breakdown marker, was spectrophotometrically determined, both in the culture medium and in the cell lysate, at λ 340 nm, by analysing the decrease in NADH absorbance during the pyruvate–lactate transformation as described by Murphy and coll. (Murphy T.H. And Baraban S.M. 1990). The reaction was carried out in 100 mM potassium phosphate buffer (pH 7.5); 10 mM sodium pyruvate; 1 mM NADH. Briefly, the amount of LDH released was detected for each sample in 100 μ l of supernatant medium obtained by centrifugation at 400 g for 10 min. The obtained pellet (dead cells floating in the medium) was mixed together with the corresponding cells in the dish. Cells were lysated with digitonin (2.5 mg/ml), centrifugated at 10,000 g for 15 min and endocellular LDH was measured on 30 μ l cellular lysate. Values were expressed as a percentage of LDH released in the medium with respect to total LDH, considered the sum of the enzymatic activity present in the cellular lysate (intracellular) and that in the culture medium (extracellular). The decrease in NADH absorbance (Δ min) was measured by spectrophotometer (Hitachi U-2000) at $\lambda=340$ nm.

3.5 Comet Assay

Standard Comet assay was performed utilising Trevigen (Trevigen Inc., Gaithersburg, USA) pre-treated slides. For each treatment the aliquots of HA cells were embedded in 45 μ l of low melting agarose (stabilised at 40 °C), dropped on the slides and maintained at 4 °C for 20 min. Then all the gel-supporting slides were immersed in lysis buffer (2.5 NaCl, 100 mM Na₂EDTA, 1 % Triton X100, 1 % N-laurosyl sarcosine, 10 % DMSO, pH = 10) for 1 h at 4 °C, in order to leave gel-embedded DNA in the form of nucleoids. DNA unwinding was left for 20 min in fresh electrophoresis buffer A (300 mM NaOH, 1 mM Na₂EDTA, pH = 13.1), afterwards, alkaline version of Comet assay was performed by submarine electrophoresis in the

same re-circulating pH 13.1 buffer A at 0.7 V/cm for 20 min and constant temp. (4 °C), as already reported (Renis M. et al. 2008). The samples, after the neutralisation treatment were 70 % ethanol dried and stained with SYBER GREEN (Trevigen kit) according to protocol suggestions. One hundred nucleoids per sample (50 for each of the two replicate slides) were randomly analysed using the Leica epifluorescence microscope. All the operations were kept in the dark.

The DNA damage was quantified by CASP free software utilising as values the percentage of fragmented DNA in the tail of comet (% TDNA). The data is averaged from three different experiments in which each sample was processed in duplicate.

3.6 Reactive oxygen species measurement

The assay was performed, as previously described (Renis M. et al. 2000), by using the fluorescent probe 20,70-dichlorodihydrofluorescein diacetate. 20,70-Dichlorodihydrofluorescein diacetate diffuses through the cell membrane; in the cytosol it is enzymatically hydrolysed by intracellular esterases and oxidised to the fluorescent 20,70-dichlorofluorescein in the presence of ROS.

The fluorescence (corresponding to the radical species oxidised 20,70-dichlorofluorescein) was monitored spectrofluorometrically using a Hitachi F-2000 spectrofluorimeter (Hitachi): lexcitation $\frac{1}{4}$ 488 nm, lemission $\frac{1}{4}$ 525 nm. A positive control was performed in each experiment by treating the cells with 200 mM-H₂O₂ for 20 min before adding 20,70-dichlorodihydrofluorescein diacetate to the cells. The total protein content was evaluated for each sample according to Bradford M.M. (1976). The results are reported as fluorescence intensity per mg protein and compared to the relative controls.

3.7 GSH Thiol groups determination

GSH thiol groups were measured, in 200 μ l of plasma, using a spectrophotometric assay based on the reaction of thiols with 2,2-dithio-bis-nitrobenzoic acid (DTNB) at $\lambda=412$ nm (Di Giacomo C. et al. 2003). The amount of thiol groups was calculated using an absorbance of 13,600 $\text{cm}^{-1} \text{M}^{-1}$. Results are expressed as $\mu\text{mol/ml}$ lysate.

3.8 Western Blot Analysis

HA were washed twice with ice-cold PBS, collected by lyses buffer (10 mM Tris-HCl plus 10 mM KCl, 2 mM MgCl_2 , 0.6 mM PMSF and 1 % SDS (pH 7.4), incubated for 20 min at 0 °C and then sonicated. Equal amounts of total protein (30 $\mu\text{g/lane}$), measured according to Bradford M.M. (1976), were separated by different concentrations of TGX™ precast gel electrophoresis (Bio-Rad Laboratories, Inc.) and transferred into nitrocellulose membranes (Bio-Rad Laboratories, Inc.) with a semi-dry system. After verifying the protein transfer by Ponceau S staining, the membranes were blocked with Tris buffered saline containing 0.01 % Tween 20 (TBST) and 5 % non-fat milk, washed briefly, incubated with primary antibodies at 4 °C overnight and then incubated with corresponding conjugated secondary antibodies for 2 h at room temperature. All primary monoclonal and secondary antibodies were purchased from Santa Cruz, CA, USA (NFkB: sc8008; GAPDH: sc-365062; cyclin D1: sc-718; p38 MAPK: sc-4708; nestin: sc-23927; Cyt c: sc-13561; AIF: sc-9416; caspase 3: sc-7272; GGT1: sc-20639; SOD: sc-11407; iNOS: sc-7271; HSPs 70: sc-1060; HSPs 60: sc-13966; HSPs 27: sc-13132; MT: sc-11377), except for monoclonal antibodies GFAP and vimentin (GFAP: MAB360 Clone: GA5 Chemicon society; vimentin: MAB3400 Clone: V9 Chemicon society); all the concentrations employed were set up experimentally according to the manufacturer's instructions. The complex protein-primary-secondary antibody was detected by chemiluminescence methods using the

Gel Logic 2200 Imaging System. Chemiluminescent signals were densitometrically analysed using Kodak-MI software. Values were expressed as arbitrary densitometric units (A.D.U.). The values reported have been normalised with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels.

3.9 Thiol labelling of proteins and 1D and 2D electrophoresis

Thiol groups in protein extracts of HA were labelled by adding 5'-iodoacetamide fluorescein (5'-IAF) and dimethyl sulfoxide (DMSO) to a final concentration of 800 μ M and incubating at room temperature for 2 h in the dark. Proteins were then resolved using 1DE on 12 % polyacrylamide gels (Laemmli U.K. 1970). Gels were scanned in a Typhoon9400 scanner (GE Healthcare, UK; EXmax 490–495 nm; EMmax 515–520 nm) and were subsequently stained with Coomassie G250. Equal amounts of protein were loaded in 12 wells (3 replicates for each treatment) and repeated at least 3 times.

2D-SDS PAGE analysis was performed on protein extracts of control and treated samples incubated with 5'-IAF as described above (Tedesco S. et al. 2010). Samples (180 μ g) were then precipitated with TCA/acetone and re-suspended in rehydration buffer containing 5 M urea, 2 M thiourea, 2 % CHAPS, 4 % ampholyte (Pharmalyte 3-10, Amersham-Pharmacia Biotech, Little Chalfont, Bucks., UK), 1 % Destreak reagent (Amersham-Pharmacia Biotech) and trace amounts of bromophenol blue. A final volume of 125 μ l was loaded on 7 cm IPG strips pH 3–10NL on the bench overnight. Proteins were focused on a Protean IEF Cell (Bio-rad) with linear voltage increases: 250 V for 15 min; 4000 V for 2 h; then up to 20,000 V h. After focusing, strips were equilibrated for 15 min in equilibration buffer (6 M urea, 0.375 M Tris, pH 8.8, 2 % SDS, 20 % glycerol) containing 2 % DTT and then for 15 min in equilibration buffer containing 2.5 % iodoacetamide. Equilibrated strips were electrophoresed on 12 % SDS PAGE gels at a constant voltage (150 V) at 4 °C. 2D

Gels were scanned (Typhoon9400 scanner) as described above to reveal thiol-containing proteins labelled by 5'-IAF. Gels were then visualised by silver staining (Rabilloud T. 1992) which revealed total protein. 2D-SDS PAGE gels were performed in digestive gland at least three times respectively for representative samples of control and MeHg, pre-treated or not with ALA.

For each 1DE gel, all bands detected by Typhoon9400 scanner were subsequently analysed by Quantity One image analysis software (Bio-Rad, Hercules, CA, USA) measuring the total intensity for each lane. They were quantified as arbitrary units (A.U.). All 1DE gels stained with Coomassie blue G250 were scanned in a GS-800 calibrated densitometer (Bio-Rad Laboratories) and the optical density from each lane was measured by Quantity One Image analysis software as described above. Total optical densities for each lane were normalised with those from Coomassie staining from the same gel. An average of at least three replicates from three different extracts for each treatment and tissue studied was determined.

3.10 Carbonyl labelling of proteins and 1D electrophoresis

Protein extracts (70 µg) of each sample were brought to a final volume of 100 µl and treated with 1 ml of fluorescein-5-Thiosemicarbazide (FTSC) (100 mM) and incubated for 2 h in the dark at 4 °C. The proteins were precipitated with an equal volume of cold trichloroacetic acid (TCA) to 20 % (v / v) and then centrifuged at 11,000 g for 3 minutes at 25 °C. The pellets were re-suspended and washed with ethanol/ethyl acetate (1:1) x 5 times to remove FTSC excess. The final pellet was re-suspended in rehydration buffer containing 5M Urea, Thiourea 2M, CHAPS 2 %, 4 % ampholyte (Pharmalyte 3-10, Amersham-Pharmacia Biotech, Little Chalfont, Bucks. UK) and a minimal amount of blue bromophenol (Chaudhuri A.R. et al. 2006). The proteins were separated by one-dimensional electrophoresis (1DE) on 12 % polyacrylamide gels (Laemli U.K. 1970). The fluorescent images of the gels relative to the electrophoretic run was acquired by Typhoon 9400 scanner (GE Healthcare,

UK; ExMax 490-495 nm; Emmax 515-520 nm) and normalised by referring to the pictures of the same gels stained with Coomassie G250. Each experiment was set up in triplicate and repeated 3 times. The images of all 1DE gels, acquired by Typhoon9400 scanner, were subsequently analysed using the Quantity One software (Bio-Rad, Hercules, CA, USA), to quantify the total carbonyl-marked protein content of each well, obtained from all bands in the lane and expressed as arbitrary densitometric units (A.D.U.). The same gels stained with Coomassie blue G250 1DE were analysed with a densitometer, GS-800 (Bio-Rad Laboratories) and analysed with the same Quantity One software. The A.D.U. obtained from the analysis of fluorescence were normalised with those corresponding to the same gel stained with Coomassie blue G250 and expressed as mean \pm SD.

3.11 Indirect Immunofluorescence (α -tubulin)

For the immunofluorescence staining, cells were fixed in 3.7 % paraformaldehyde in PBS for 30 min and permeabilised with 0.2 % Triton X-100 in PBS for 5 min at room temperature and blocked in 6 % BSA/PBS. After three washes in PBS, samples were incubated with 1:2000 mouse monoclonal anti α -tubulin (clone B 5-1-2; Sigma-Aldrich). After washes in 1 % BSA/PBS, samples were labelled with Alexa Fluor 594 Signal-Amplification Kit (Molecular Probes, Inc. OR, USA) for 1 h at room temperature in the dark. Finally, samples dried and mounted with mounting medium were examined with an Olympus BX50 and photographed with a Leica DC500 digital camera.

3.12 Direct Immunofluorescence (FITC- Phalloidin)

The analysis of cytoskeleton microfilaments was performed using FITC-Phalloidin (Sigma-Aldrich, Inc., St. Louis, MO, USA). Fluorescein-labelled phalloidin was used as described by Dejana E. et al. (1988). Samples dried and mounted with mounting medium (Calbiochem) were visualised with an Olympus BX50 and

photographed with a Leica DC500 digital camera.

3.13 Scanning Electron Microscopy (SEM)

The morphological analysis was carried out on cell samples allowed to adhere to circular glass coverslips, 13 mm \varnothing (EMS: Electron Microscopy Sciences, Fort Washington, PA, USA). HA were fixed in 2 % glutaraldehyde in 0.1 M sodium-cacodylate (EMS) buffer, pH 7.2, for 1 h at 4 °C and then postfixed in 1 % osmium tetroxide (EMS) for 1 h at 4 °C. After dehydration in graded ethanol and critical point drying using CO₂ (Emscope-CPD 750), the coverslips were coated with vacuum evaporated gold (Emscope-SM 300) and observed using a Hitachi S4000 Field Emission Scanning Electron Microscope.

3.14 Statistical analysis

Each experiment was repeated three times in triplicate and the means and standard deviations for each value were calculated. Statistical analysis of results was performed using paired Student's t-test with the statistical software OriginPro 8.5. Differences were considered significant at $p \leq 0.05$.

CHAPTER IV

Results

4.1 MeHg Cytotoxicity and Genotoxicity in Human Astrocytes pre-treated with ALA

We preliminarily analysed whether MeHg treatment and ALA pre-treatment have an impact on HA survival, evaluating the cytotoxic and protective effects by MTT.

Figure 12 reports the results of cell viability showing that 24 h and 72 h after MeHg treatment cell survival was significantly reduced by approximately 40 % ($p < 0.05$ vs control). Conversely, HA pre-treated with 50 μ M ALA raceme or (R+) enantiomer for 24 h show significantly increased cell survival with respect to the non-pre-treated cells. It is clear that ALA, in particular or (R+) enantiomer, exerted a protective effect on HA treated with MeHg for 24 h and 72 h. Figure 13 shows the data of the release of lactate dehydrogenase in HA treated with MeHg compared with the untreated control and with astrocytes pre-treated with 50 μ M ALA raceme or (R+) enantiomer. The results clearly indicate that methylmercury, at the concentration used, did not induce cytolysis in human astrocytes. The values, in fact, are completely superimposable in respect to the untreated control cells or those pre-treated with 50 μ M ALA raceme or (R+) enantiomer.

DNA damage, assessed by alkaline comet assay and shown in Table 1, is expressed as % TDNA, or rather the percentage of DNA in the tail.

The results show that MeHg at 1.125 μ M does not exhibit any genotoxic activity for 24 h and 72h, and also in the samples pre-treated with 50 μ M ALA raceme or (R+) enantiomer.

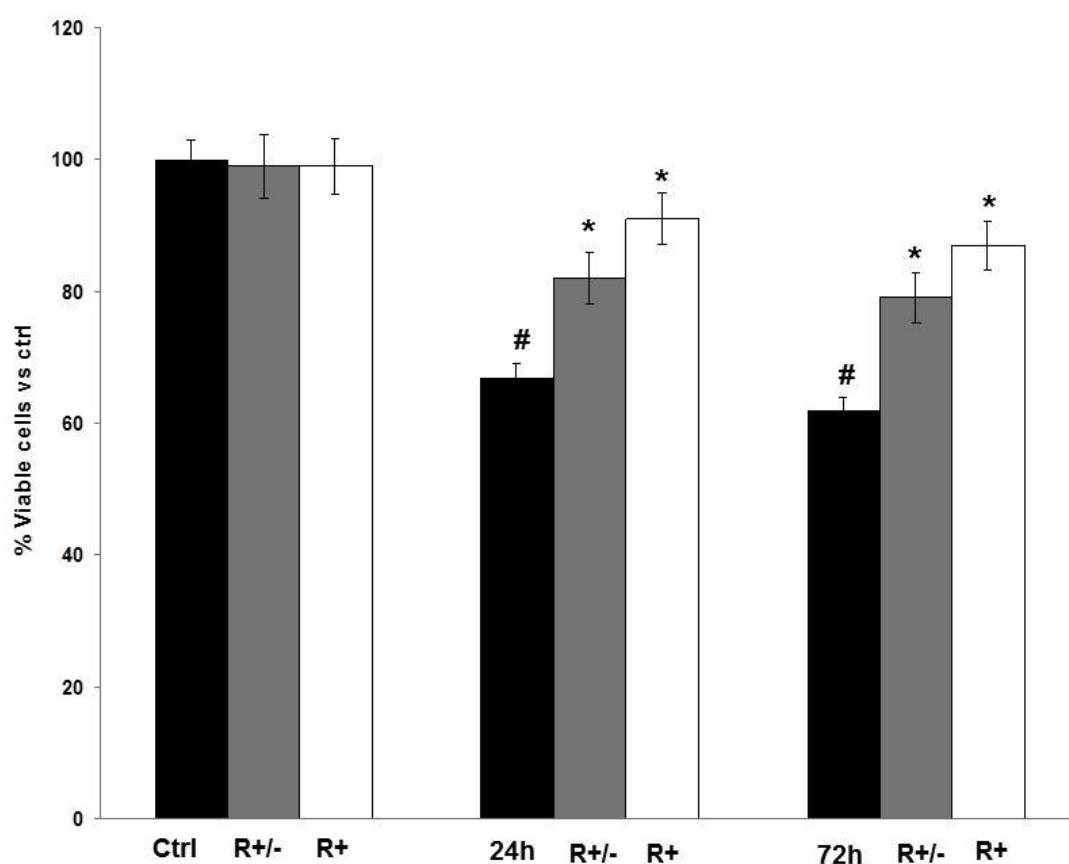


Figure. 12. Cell viability analysis (MTT assay) performed on primary human astrocytes (HA) seeded in 96-well plates and treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h. The results are expressed as a percentage of the control value. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 h MeHg treatment (P<0.05) respectively.

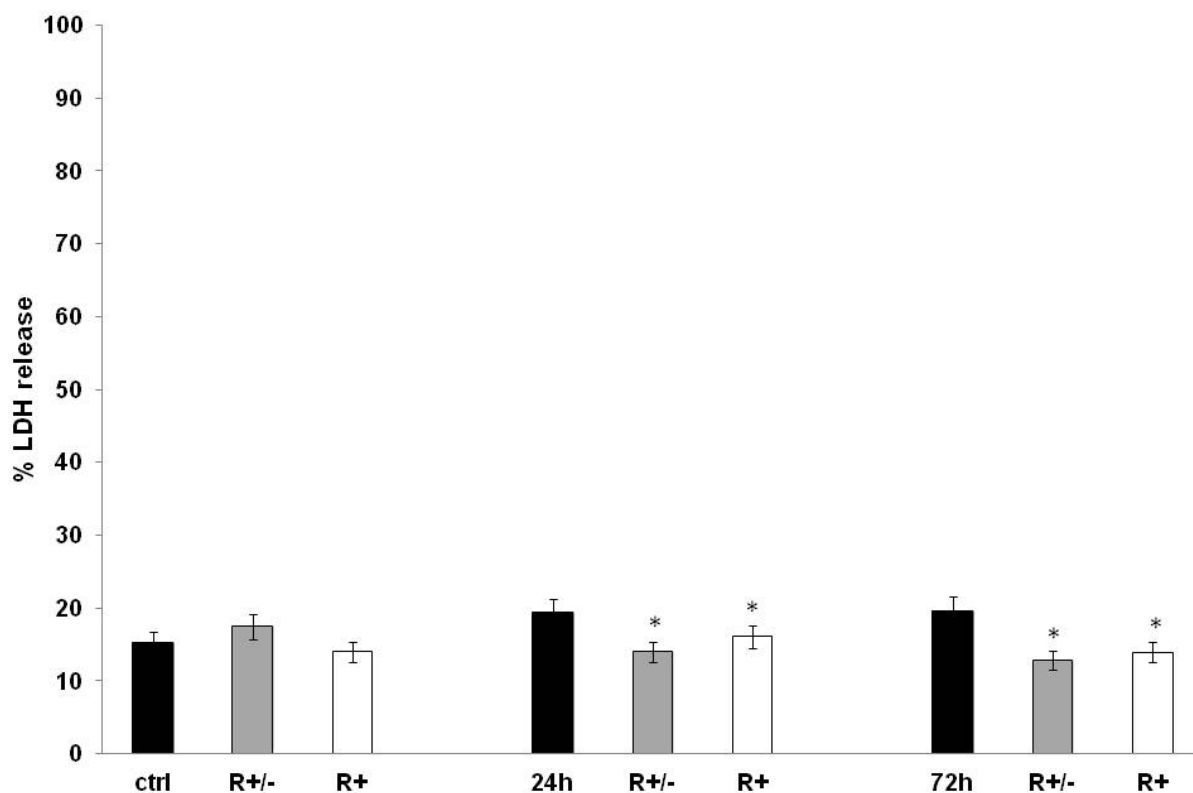


Figure 13. LDH levels release in cultured primary human astrocytes (HA) treated with MeHg 1.125 μ M for 24 h or for 72 h in combination with a pre-treatment with Lipoic Acid (R \pm or R+). The results are expressed as % release as Mean \pm SD of three independent experiments each performed in duplicate. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment (P<0.05) respectively.

Ctrl	R+/-	R+		24h	R+/-	R+		72h	R+/-	R+
11,63±6,1	4,85±2,15	3,69±1,9		8,25±4,6	9,40±4,2	3,33±2,02		8,94±3,9	9,12±4,8	10,15±4,4

Table 1. The data shown the results of the Comet assay at pH > 13 performed on primary human astrocytes (HA) treated with MeHg 1.125 μ M for 24 h or for 72 in combination with a pre-treatment with Lipoic Acid (R \pm or R+). The results are expressed as % TDNA and represent the mean \pm SD of three experiments performed in duplicate. Any significant differences are observed

4.2 Apoptosis Assessment after MeHg exposure of Human Astrocytes pre-treated with ALA

The histograms related to the expression levels of the following proteins: Caspase 3, AIF and Cytochrome c are represented in figures 14, 15, 16 respectively. The results clearly indicate that methylmercury, at the concentration used, did not induce caspase 3 expression. In fact, there are no significant differences in expression levels in HA treated with MeHg for 24 h and 72 h in respect to the untreated control cells or those pre-treated with 50 μ M ALA raceme or (R+) enantiomer (Fig. 14).

In figure 15, AIF expression is significantly down-regulated only at 72 h in respect to the untreated control cells (Fig 5, $p < 0.05$ vs control). Moreover, pre-treatment with ALA raceme or (R+) enantiomer did not change protein levels in respect to the untreated control cells or those treated with MeHg for 24 h and 72 h.

Figure 16 demonstrates that cytochrome c protein showed a peculiar expression trend: it reached the highest levels 24 h after MeHg treatment, but does not persist until 72 h when cytochrome c levels were slightly significant in respect to the control values ($p < 0.05$). ALA raceme or (R+) enantiomer pre-treatment attenuates the up-regulation of cytochrome c at 24 h of MeHg treatment and markedly decreases levels of the protein at 72 h of MeHg treatment to below the control levels.

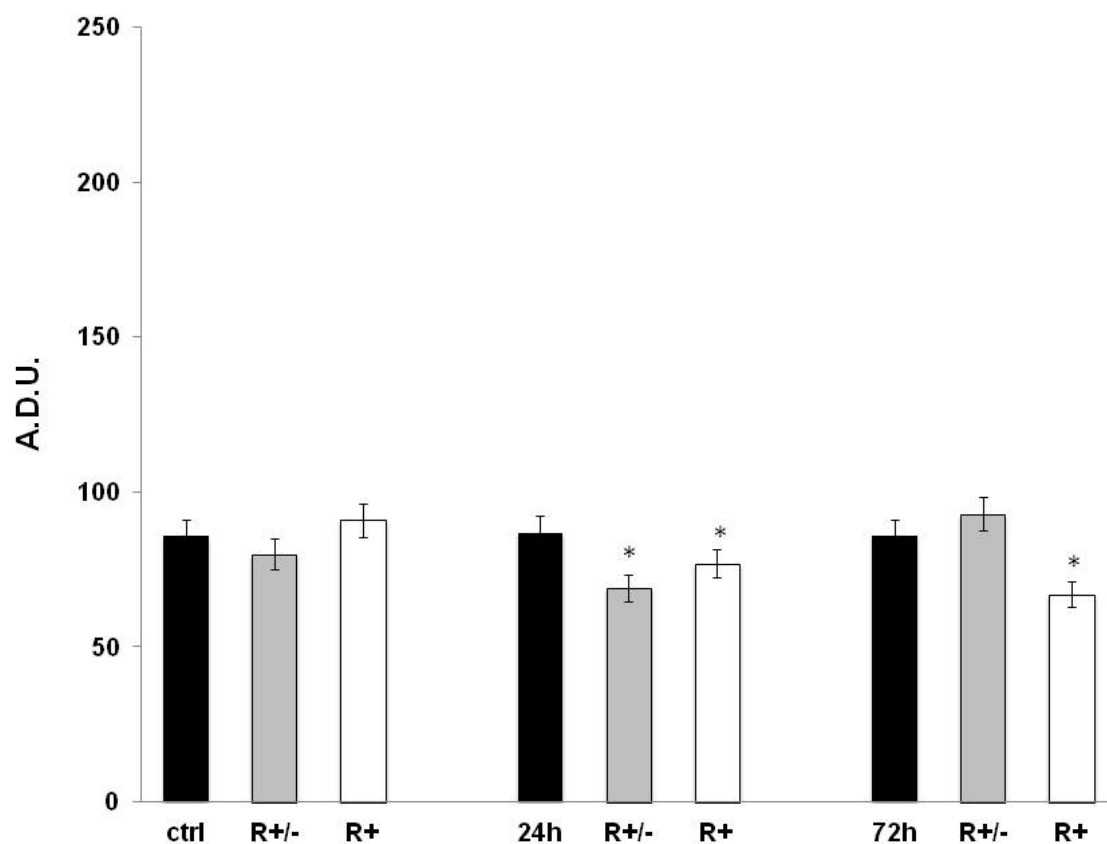


Figure 14. Immunoblotting analysis for Caspase 3, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment ($P < 0.05$) respectively.

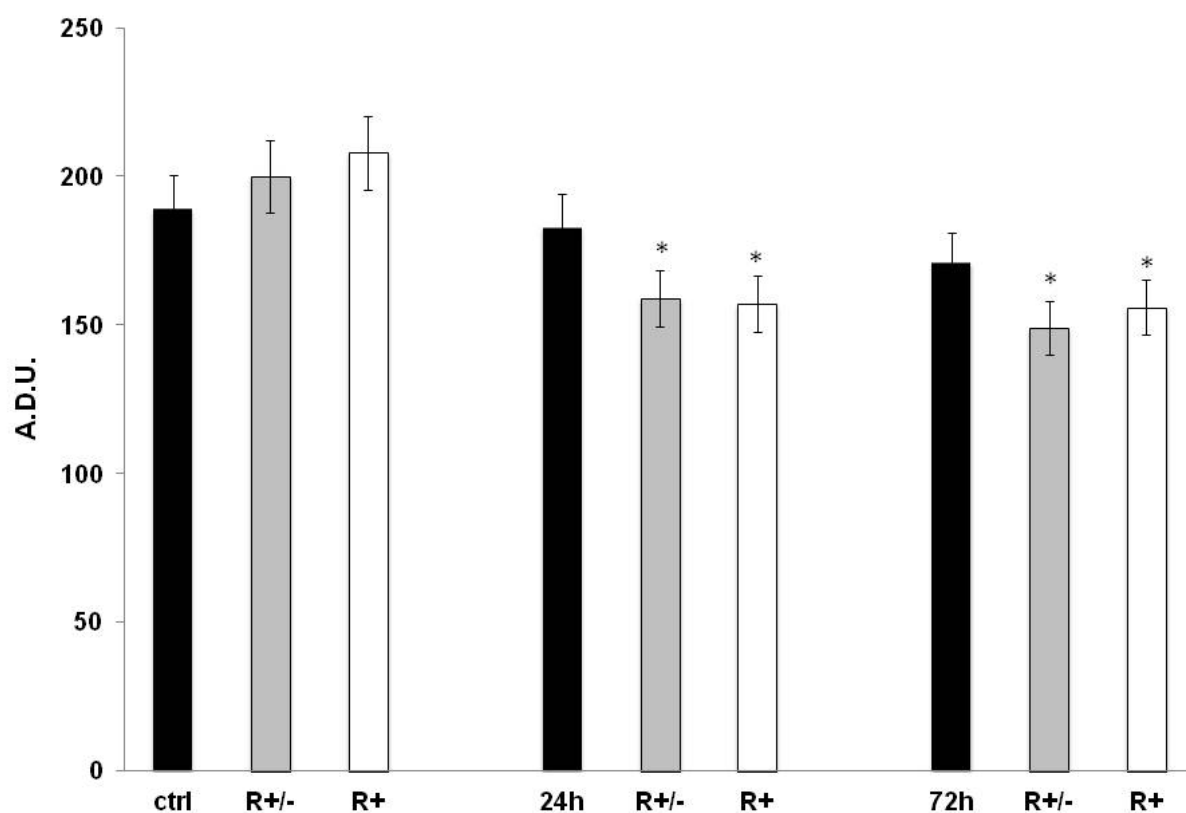


Figure 15. Immunoblotting analysis for AIF, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R $+$) either for 24 hr or for 72 hr. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment (P<0.05) respectively.

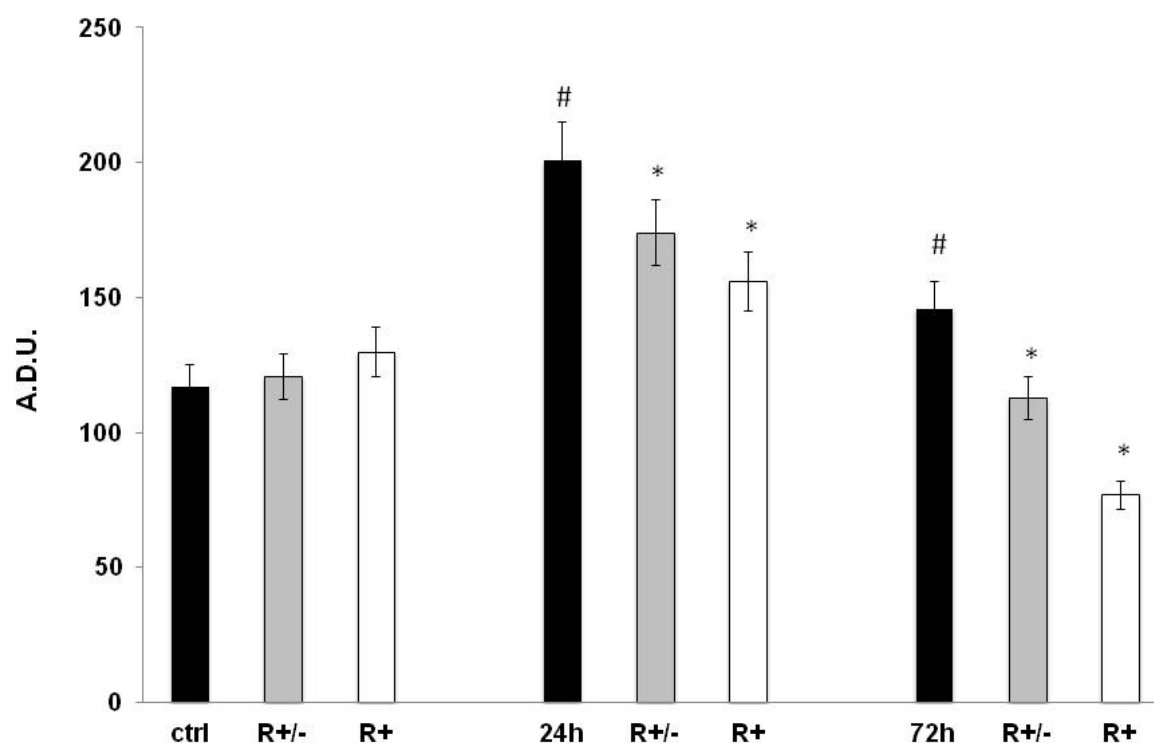


Figure 16. Immunoblotting analysis for Cyt C, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 hr or for 72 hr. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment (P<0.05) respectively.

4.3 Intermediate Filaments (IF) expression increased with pre-treatment with ALA R+

One of the main events of astrocyte reactivity is hypertrophy of astrocytic processes sustained by a filamentous network, composed of microfilaments (MF), microtubules (MT) and intermediate filaments (IF), mostly consisting of vimentin and GFAP (Bramanti et al. 2010a). In our experimental model, GFAP protein expression was markedly decreased after 24 h (p<0.05 vs control), whereas a recovery of its

expression levels 72 h after metal exposure was observed (Fig. 17, $p < 0.05$ vs 24 h; $p = 0.09$ vs control). Conversely, GFAP expression was significantly increased after pre-treatment (24 h) with R(+)-ALA and, in particular, after treatment with MeHg at 72 h. The pre-treatment with (R+) enantiomer showed higher GFAP levels in respect to the control cells (Fig. 17). Differently, vimentin expression is down-regulated both at 24 h and 72 h but pre-treatment with (R+) enantiomer significantly increased protein levels (Fig 18, $p < 0.05$ vs control). Moreover, MeHg treatment resulted in down-regulation of nestin, another IF protein generally re-expressed in reactive astrocytes (Lin et al., 1995); only after 72 h, and for both treatments with MeHg, the pre-treatment with (R+) enantiomer significantly increased nestin levels (Fig. 19, $p < 0.05$ vs control and 24 h).

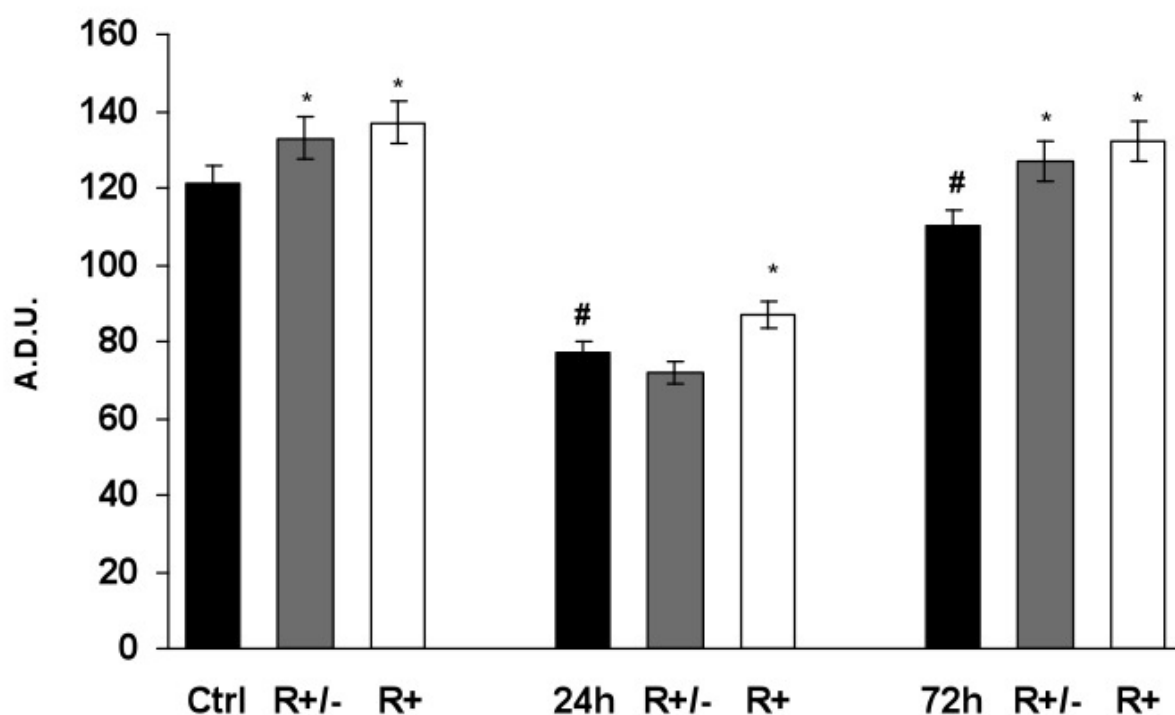


Figure 17. Immunoblotting analysis for GFAP, performed on untreated HA and HA treated with MeHg 1.125 μM in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD.

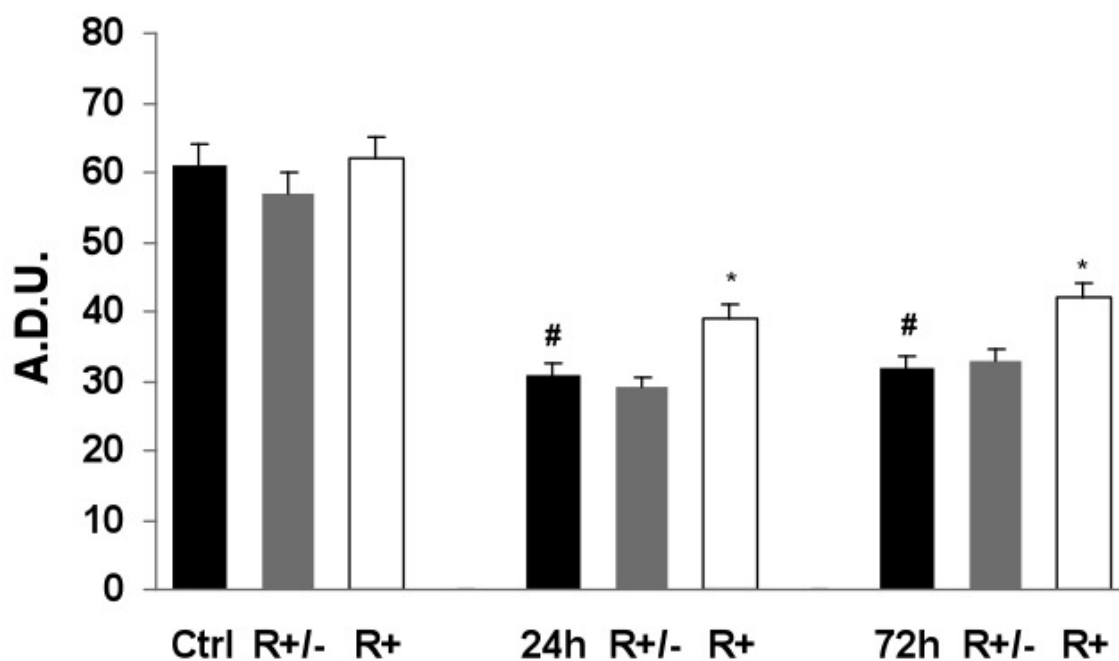


Figure 18. Immunoblotting analysis for vimentin, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R $+$) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment (P<0.05) respectively.

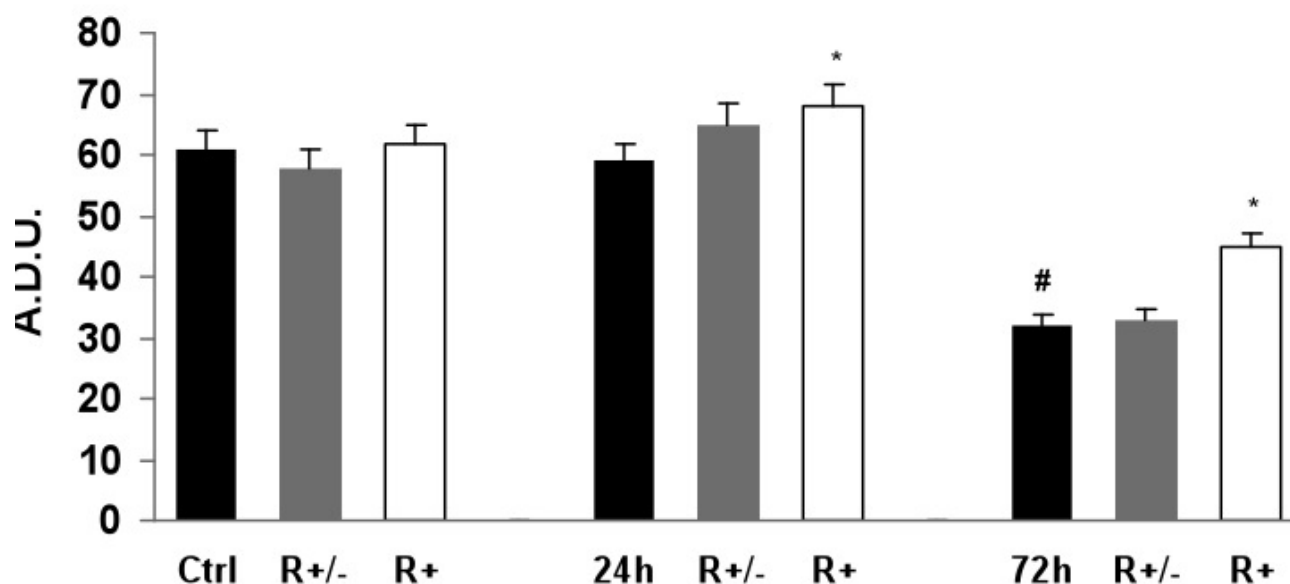


Figure 19. Immunoblotting analysis for nestin, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R $+$) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment ($P < 0.05$) respectively.

4.4 Destabilisation of Human Astrocyte Cytoarchitecture as target of MeHg neurotoxicity

Similar to the modulation of IF proteins, we observed a time-dependent down-regulation of β -tubulin, the main constituent of the MT network (Fig. 20A, $p < 0.05$ vs control and 24 h). Pre-treatment with ALA raceme or (R $+$) enantiomer does not change protein levels respect to the control cells untreated or treated with MeHg for 24 h and 72 h. Moreover, to better understand the extent of the damage to the MT

network, Indirect Immunofluorescence analysis for α -tubulin was performed. Figure 20B demonstrates that untreated astrocytes predominantly showed a typical *in vitro* organisation, with a thin and well-ordered MT network pervading the whole cytoplasm and spreading out to cellular processes. Figures 20C and D respectively show that MeHg treatment for 24 h and 72 h resulted in disruption of the representative cytoarchitecture; in fact, two areas could be detected: a central and more compact cell area and another consisting of a thicker microtubules network in the periphery of astrocytic cell bodies. Microtubules, especially, were no longer organised in a network but often aggregated to form a felt-like structure in the perinuclear area. Moreover, pre-treatment with ALA raceme or (R+) enantiomer presents no changes from the results mentioned above in respect to the untreated control cells or those treated with MeHg for 24 h and 72 h.

Next, to further demonstrate cytoarchitecture destabilisation, we analysed the MF system focusing on actin protein. As shown in figure 20E, untreated astrocytes were characterised by very thin stress fibres (arrow) with numerous evident fluorescent spots at the end (arrow heads) indicating adhesion areas on the substrate. After incubation for 24 h with MeHg (Fig. 20F), the HA MF network was composed of very *thick bundles of microfilaments*, which were often fragmented (Fig 20F white arrows), and very fluorescent clusters in the perinuclear area were frequently present (Fig 20F yellow circles). Analysis at 72 h revealed that astrocyte microfilaments, united to form bundles or thick stress fibres, are disorganised and irregular (Fig. 20G, yellow arrows). We believe that MeHg may exert its neurotoxic action on the cytoskeleton, inducing time-dependent changes in the MF system (Hunter et al., 2000): first, actin is accumulated around the nucleus (100X photo not shown) and afterwards is clustered in large microfilament bundles (Fig 20C and D). Pre-treatment with ALA raceme or (R+) enantiomer presents no changes from the results mentioned above in respect to the untreated control cells or those treated with MeHg for 24 h and 72 h (figures not shown).

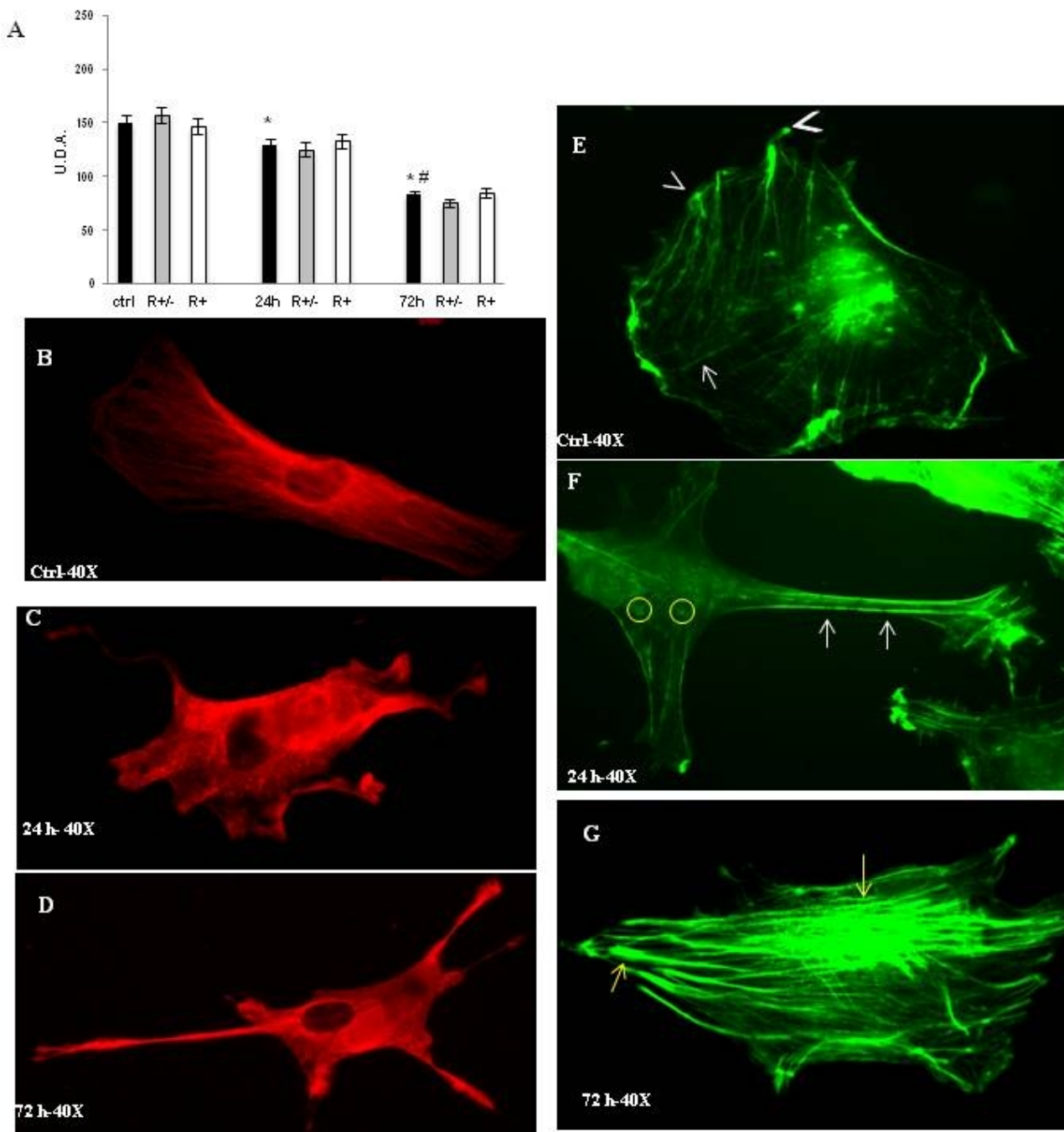


Figure 20. MeHg toxicity resulted in modification of the representative HA cytoarchitecture. Immunoblotting analysis for β -tubulin (A) performed in HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h, shows a time-dependent down-regulation of the main constituent of the MT network. Values were expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm S.D. Representative western blots from three independent experiments are shown. Indirect Immunofluorescence analysis for α -tubulin was performed on untreated HA. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment ($P < 0.05$) respectively. (B), treated with MeHg 1.125 μ M for 24 h (C), treated with MeHg 1.125 μ M for 72 h (D). MeHg treatments resulted in destabilisation of the representative cytoarchitecture. The analysis of

cytoskeleton microfilaments (MF) with FITC-Phalloidin performed in untreated HA (E, arrow: thin stress fibres; arrow heads: adhesion areas on the substrate) and treated with MeHg 1.125 μ M either for 24 h (F, white arrows: *thick bundles* of fragmented *microfilaments*; yellow circles: fluorescent clusters) or 72 h (G, yellow arrows: bundles or thick stress fibres of disorganised microfilaments). MeHg exerts its neurotoxic action inducing time-dependent changes in the MF system.

Since MeHg mainly affects cytoskeleton organisation, we addressed whether this alteration results in morphological changes to the astrocytes by performing SEM analysis. Figure 21A demonstrates that untreated HA show uniform features: cells appear very flattened and arranged in a single layer, with a heterogeneous shape rich in microvilli (head arrows) and numerous specialised cell surfaces (white arrows), typical of glia cells. Twenty four hours after treatment, cells maintain a more or less flattened shape but have some cellular areas in which the number of microvilli are apparently reduced compared to untreated cells (Fig. 21B). HA treated for 72 h still have a regular shape even if their surfaces are decidedly compromised by treatment. Interestingly, the microvilli and other plasma membrane specialisations also become smaller in number and in length (Fig. 21C). Together, these results indicate that MeHg promotes disruption of cytoskeleton organisation that, in turn, compromises the normal development of all cellular events related to its correct assemblage.

Pre-treatment with ALA raceme or (R⁺) enantiomer presents no changes from the results mentioned above in respect to the untreated control cells and those treated with MeHg for 24 h and 72 h (figures not shown).

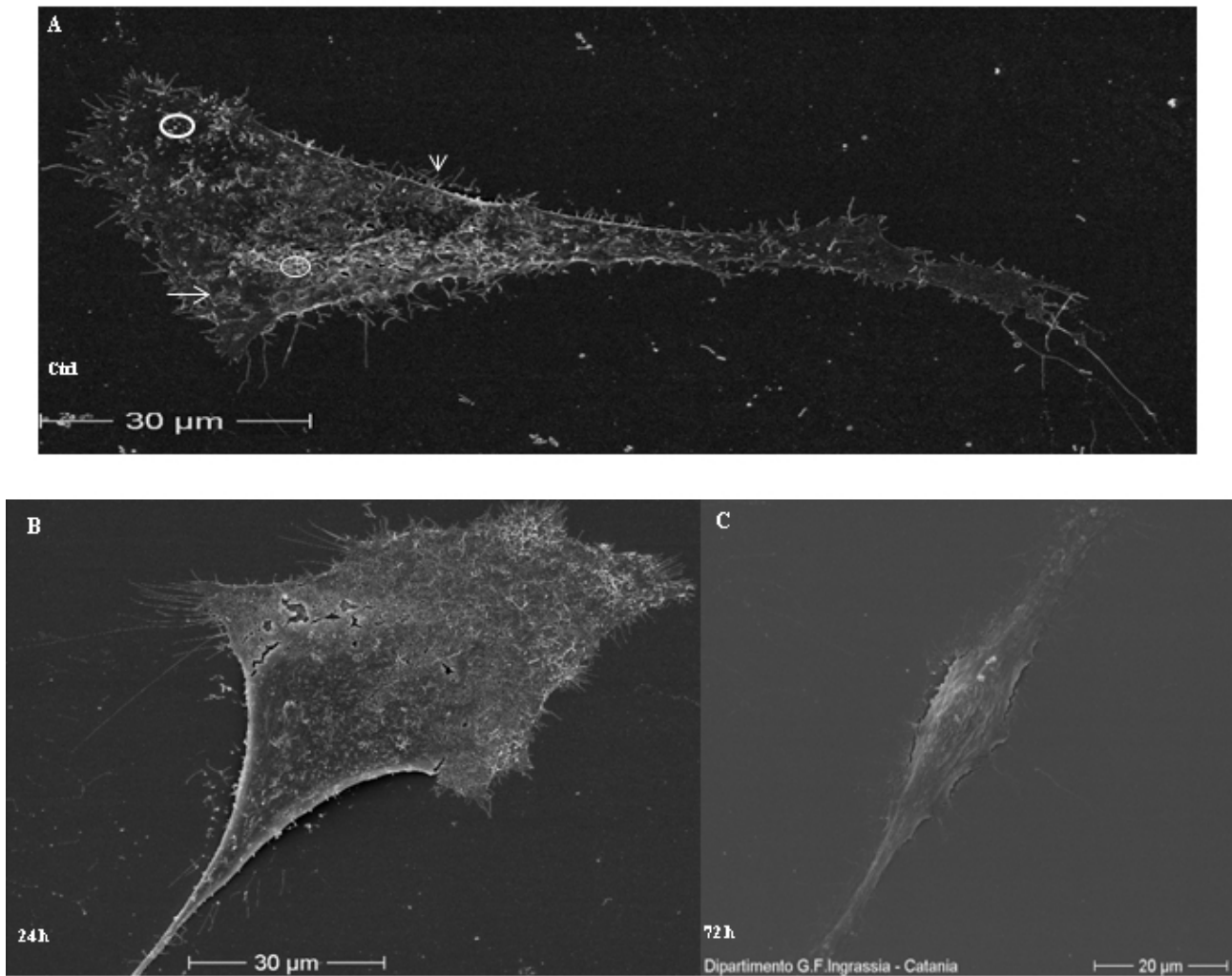


Figure 21. SEM images representative of morphological features of untreated HA (A) head arrows: microvilli; white arrows: specialised cell surfaces and treated with MeHg 1.125 μM for 24 h (B) or treated with MeHg 1.125 μM for 72 h (C), show that HA treated with MeHg still have a regular shape even if their surface is compromised by treatment.

4.5 ALA enhances Cyclin D1 and MAPK expression and down-regulates NFkB signalling pathways in MeHg induced-cytotoxicity

It is known that reactive astrocytes proliferate, depending on the nature, severity of the injury and the time of exposure to the toxicant, thus producing proinflammatory cytokine by activation of MAPK and NFkB signalling pathways (Wu et al., 2012; Block et al., 2007).

Analysis of cyclin D1 protein expression revealed that MeHg significantly increased its levels after 24 h ($p < 0.05$ vs control) until reaching a maximum (approximately 2.5 fold of control levels) after 72 h ($p < 0.05$ vs control and 24 h) (Fig. 22). ALA raceme or (R+) enantiomer pre-treatment follows the same expression trend. Figure 23 demonstrates that MAPK protein showed a peculiar expression trend: it reached the lowest levels 24 h after MeHg treatment, then started to increase until 72 h with a level lower than the untreated control ($p < 0.05$). ALA raceme or (R+) enantiomer pre-treatment attenuates the down-regulation of MAPK at 24 h of MeHg treatment and markedly increases levels of the protein higher than the untreated control cells.

The inflammatory process activation was evaluated by NFkB expression (Fig. 24). Both acute and chronic treatments significantly up-regulate its expression compared to the untreated control cells ($p < 0.05$), confirming the inflammatory status induced by MeHg. ALA (R+) enantiomer pre-treatment significantly down-regulates NFkB expression at both 24 h and 72 h of MeHg exposure while no marked differences for ALA raceme pre-treatment were highlighted.

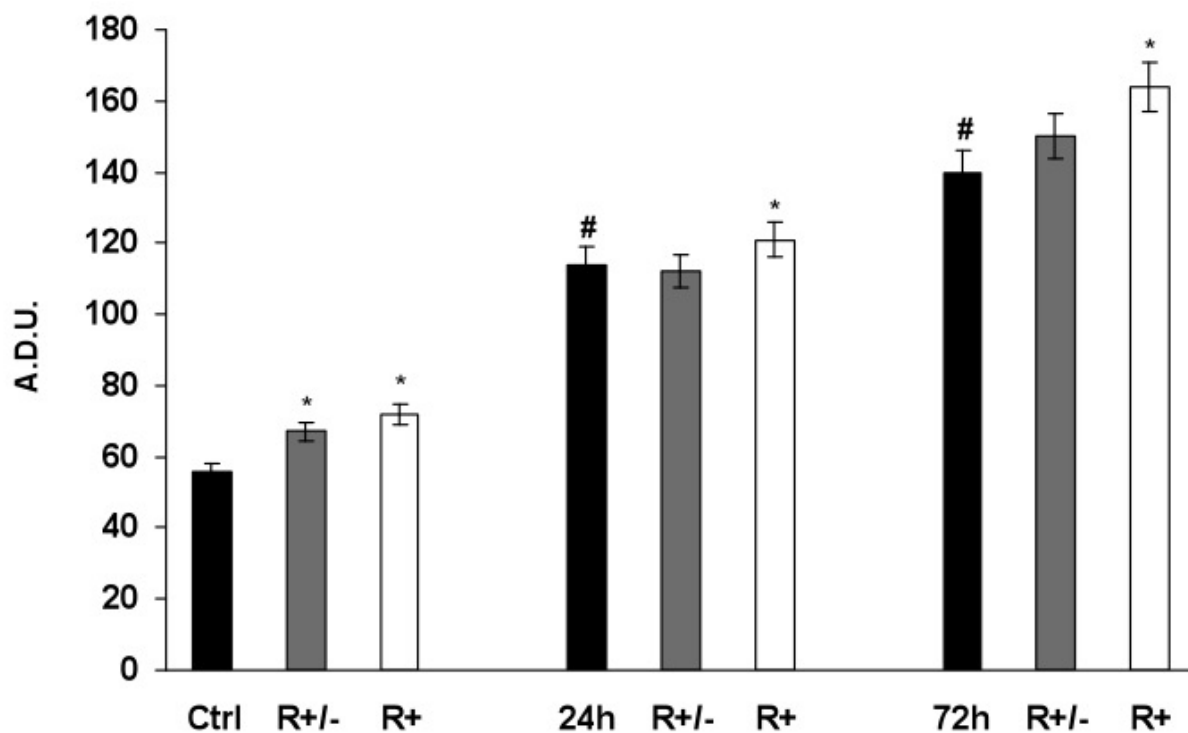


Figure 22. Immunoblotting analysis for cyclin D1, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment ($P < 0.05$) respectively.

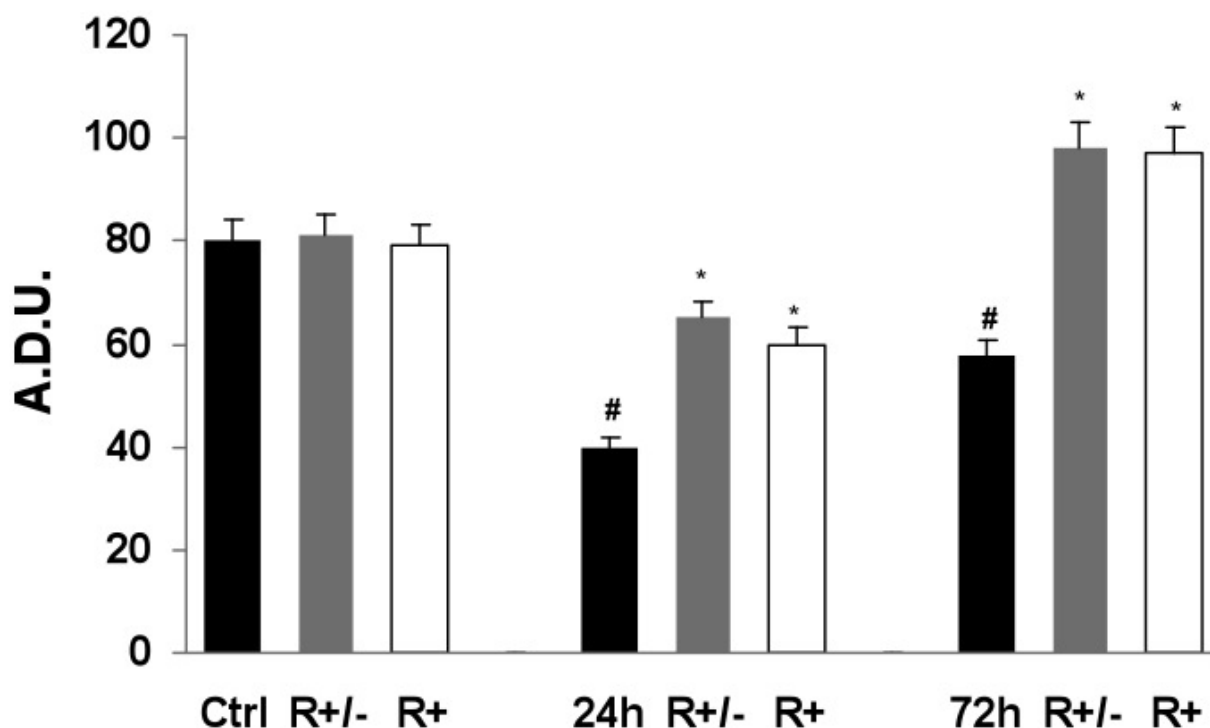


Figure 23. Immunoblotting analysis for MAPK, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R $+$) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment (P<0.05) respectively.

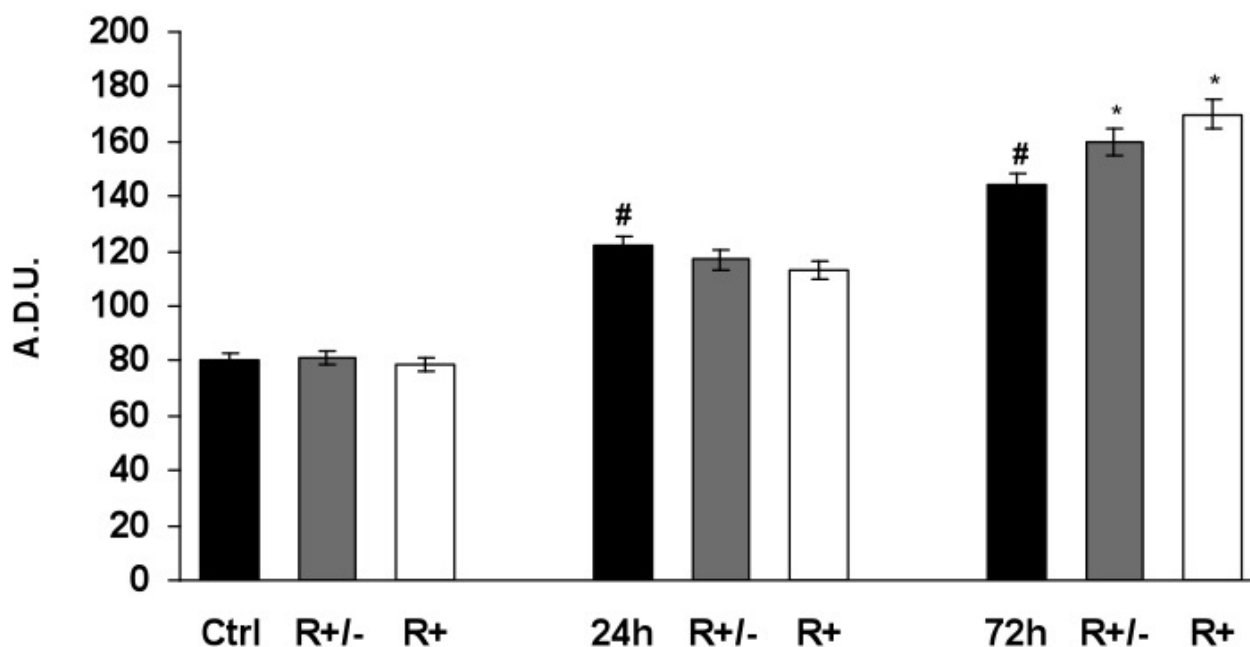


Figure 24. Immunoblotting analysis for NFkB, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R $+$) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment (P<0.05) respectively.

4.6 ALA exerts a protective effect on some Redox parameters in HA exposed to MeHg

ROS have been shown to mediate MeHg-induced neurotoxicity in multiple experimental models. The redox state analysis showed a sustained increase in radical oxygen species (ROS) for both treatments with MeHg (Fig. 25). The pre-treatment with 50 μ M ALA raceme or (R $+$) enantiomer at 24 h showed a significant decrease in

ROS levels in respect to cells treated only with MeHg at both 24 h and 72 h.

GSH levels are increased only after 24 h of treatment, in response to an oxidative insult; conversely, we found a depletion of glutathione content after 72 h of MeHg exposure, with values below the control (Fig. 26). ALA pre-treatments with raceme or (R+) enantiomer for 24 h increased GSH levels in respect to the control and MeHg-treated cells. (R+) enantiomer particularly exerts a stronger effect on GSH amount.

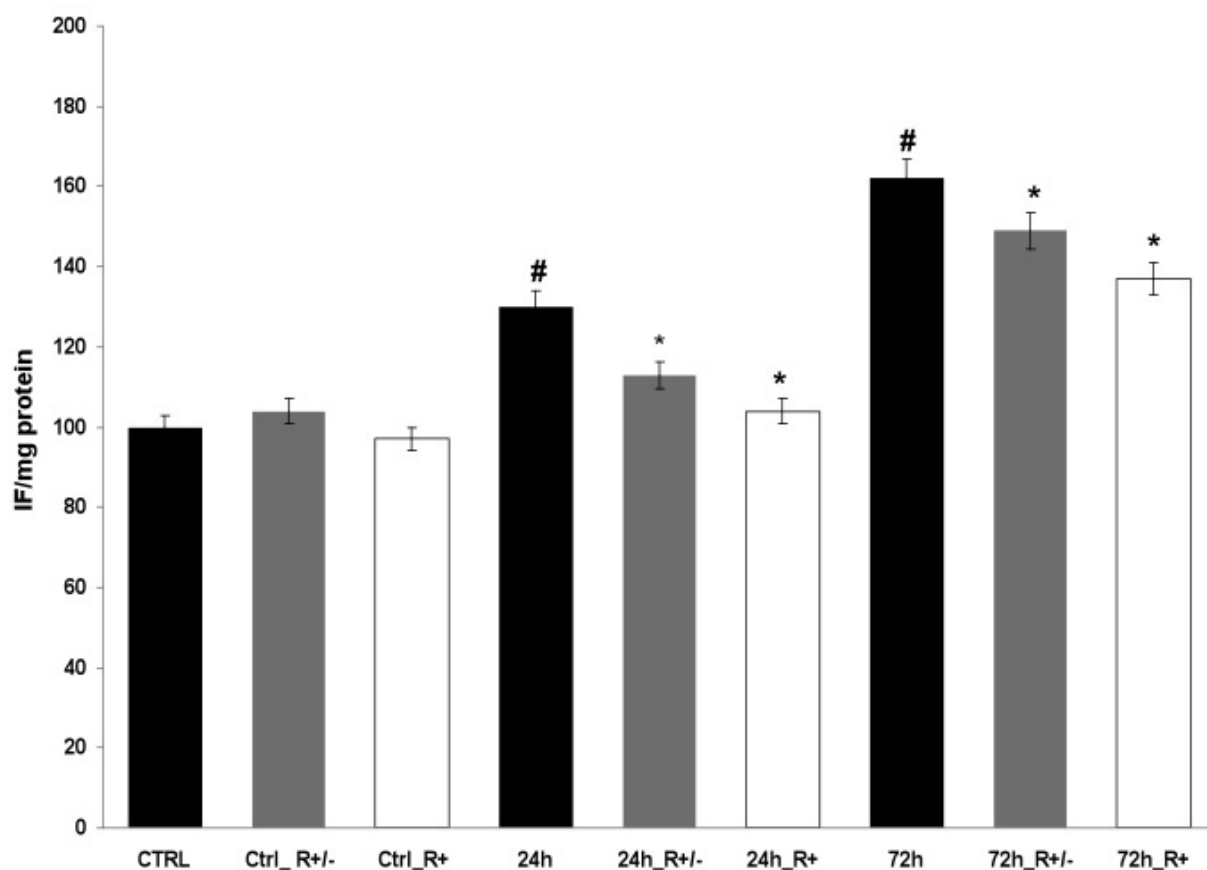


Figure 25. Reactive oxygen species analysis of differently treated primary human astrocytes (HA) treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h. Results are presented as fluorescence intensity normalised for mg protein with each value representing the mean of at least three experiments, each one performed in triplicate (three independent measures). #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 h MeHg treatment (P<0.05) respectively.

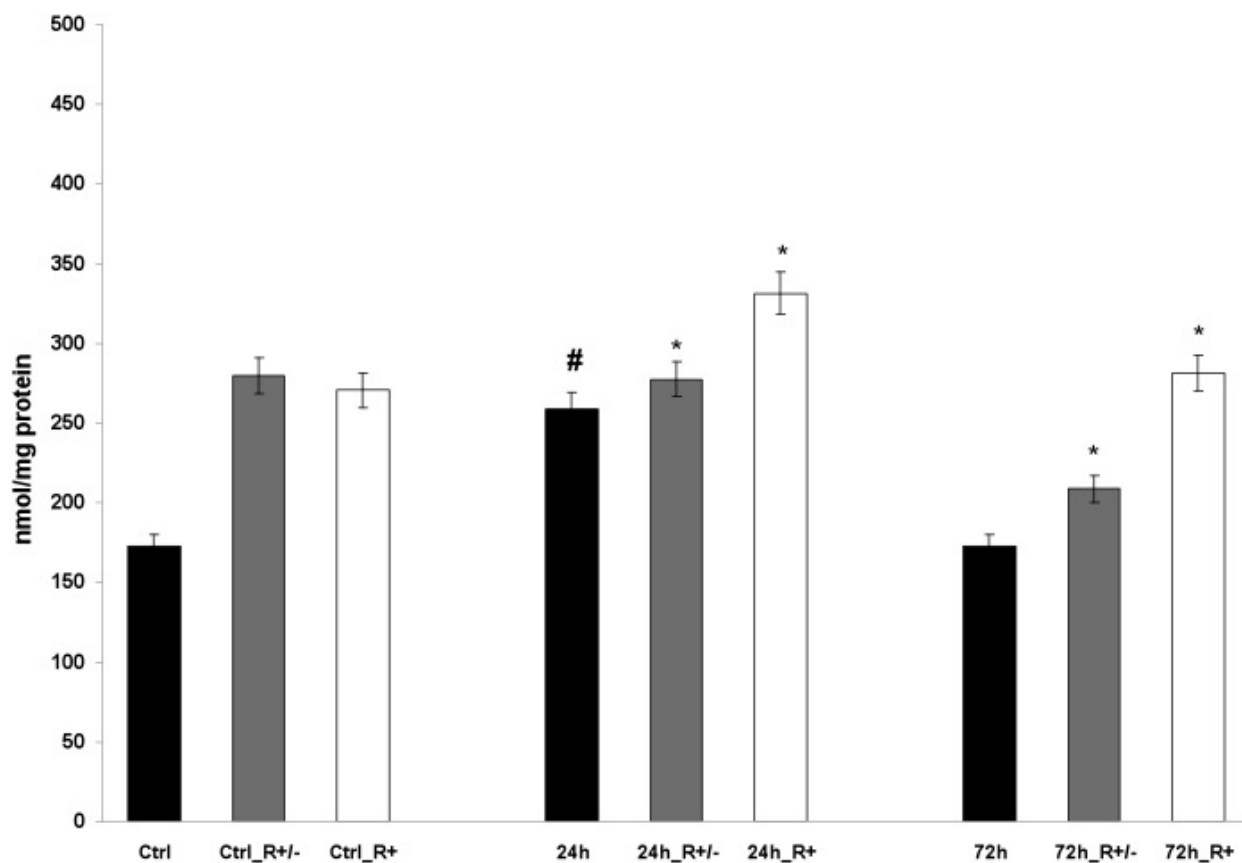


Figure 26. Determination of total intracellular GSH levels in primary human astrocytes (HA) treated with MeHg 1.125 IM in combination with Lipoic Acid (R \pm or R $+$) either for 24 h or for 72 h. The values, reported as nmol of GSH normalised for mg of total protein, are the mean \pm SEM of two experiments performed in triplicate. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 h MeHg treatment (P<0.05) respectively.

Treatment with MeHg already determines an increase in SOD expression after 24 h compared to untreated cells, while levels of the proteins are reduced in respect to control values after 72 h of treatment (Fig. 27). ALA pre-treatments with raceme or (R $+$) enantiomer at 24 h increased SOD levels in respect to the control and MeHg-treated cells.

Treatments with MeHg, both acute and chronic, significantly up-regulate GGT1

expression compared to the untreated control cells ($p < 0.05$), immediately after 24 h (Fig. 28). Pre-treatment with ALA raceme or (R+) enantiomer does not affect GGT1 levels in the untreated control cells, as in MeHg-treated cells at 72 h. Only pre-treatment with (R+) enantiomer significantly increases GGT1 expression in MeHg treated cells at 24 h.

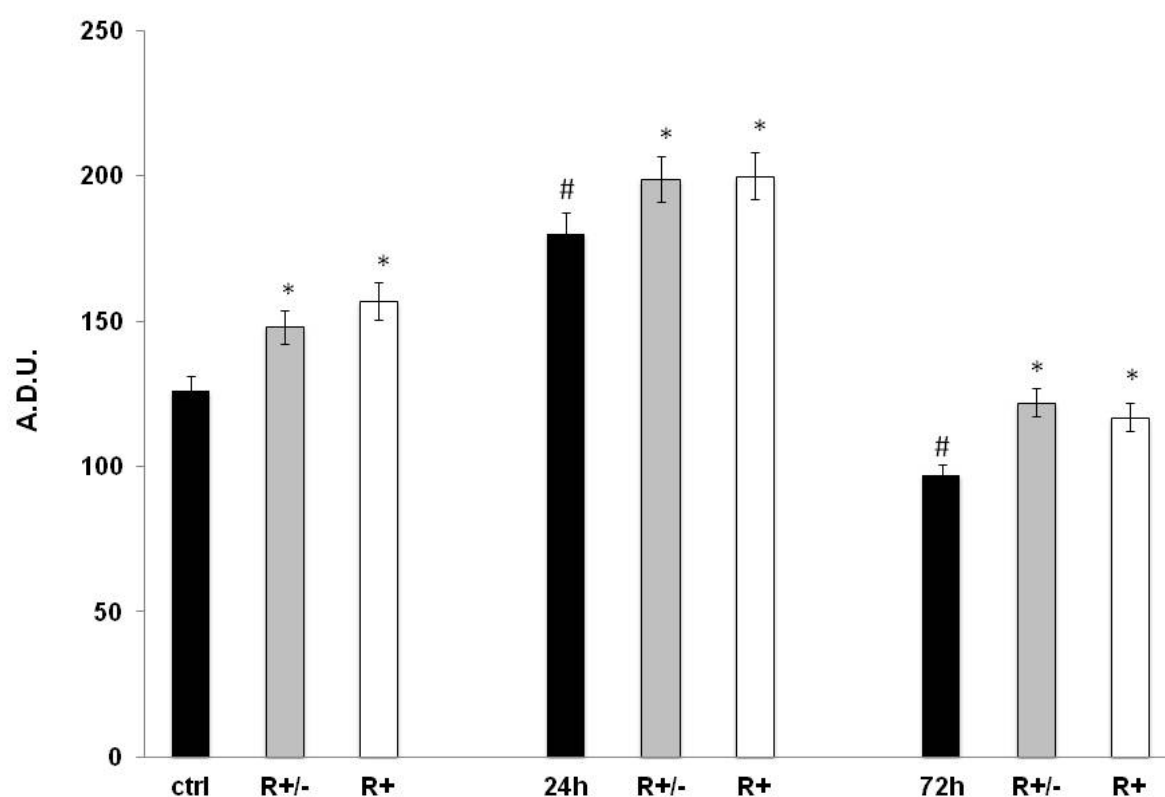


Figure 27. Immunoblotting analysis for SOD, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment ($P < 0.05$) respectively.

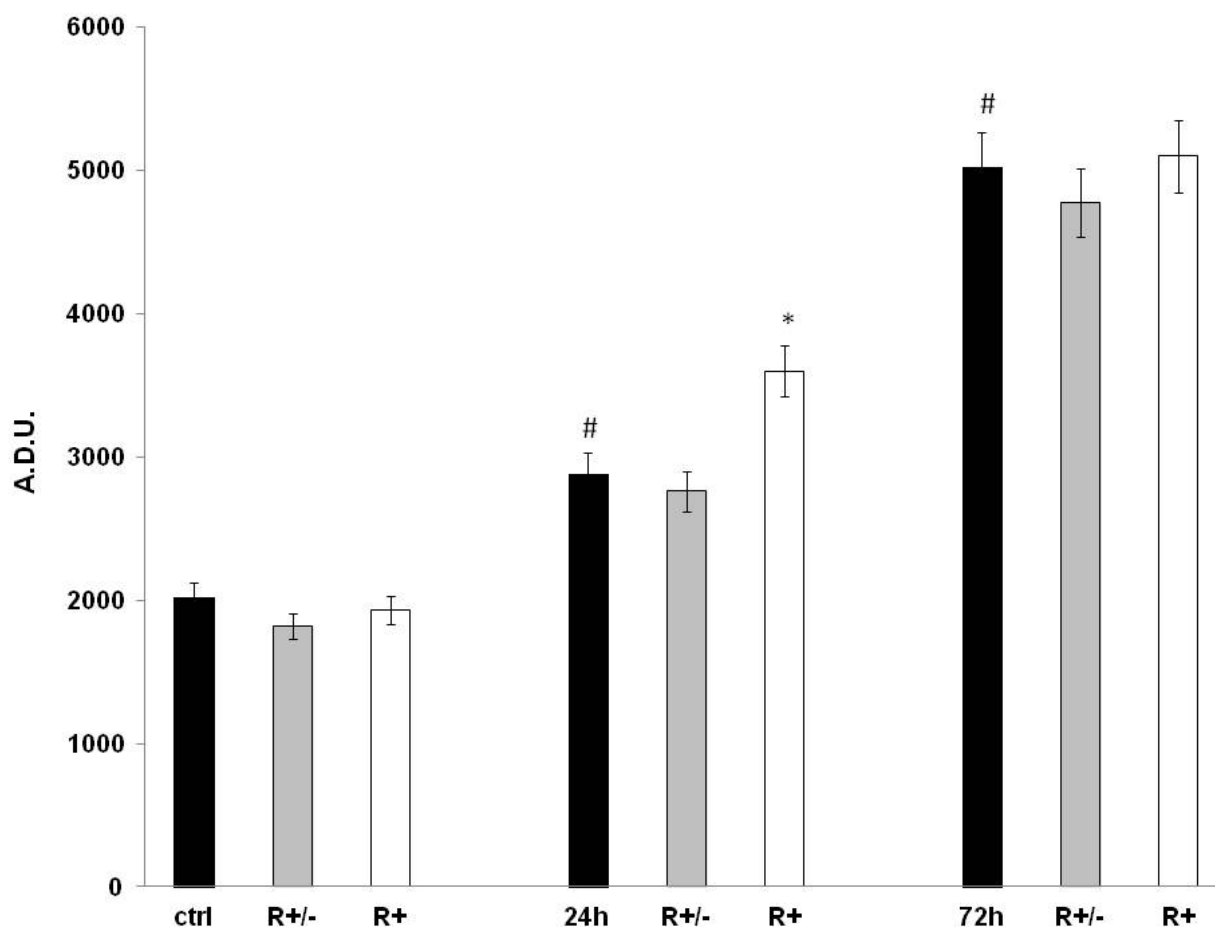


Figure 28. Immunoblotting analysis for GGT1, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment ($P < 0.05$) respectively.

4.7 Human Astrocyte stress response after MeHg treatments

The histograms of expression levels for the following proteins: HSPs70, HSPs60, HSPs27, metallothioneins and iNOS are shown in figures 29, 30, 31, 32 and 33. Values are expressed as arbitrary densitometric units and have been normalised with respect to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels. From figure

29, we observe that members of the heat shock proteins family respond differently to treatment with MeHg. In particular, levels of HSPs70 are significantly down-regulated in a time-dependent treatment.

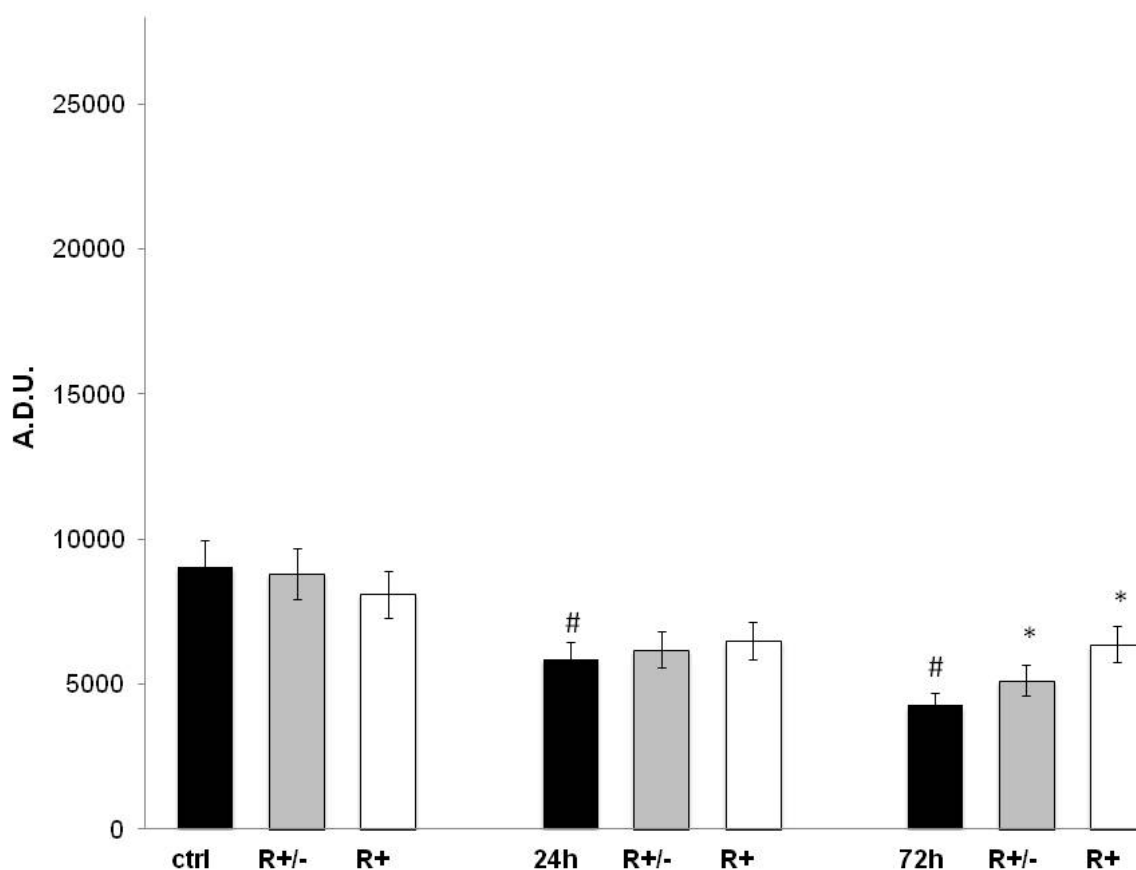


Figure 29. Immunoblotting analysis for HSPs 70, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment (P<0.05) respectively.

On the other hand, the other two proteins analysed, HSPs60 (Fig. 30) and HSPs27 (Fig. 31), are modulated differently. These are, in fact, up-regulated after 24 h of

treatment, while a significant decrease in expression levels is observed at 72 h with values that fall below those measured in untreated control cells. ALA pre-treatments with raceme and, in particular, (R+) enantiomer at 24 h and 72 h of MeHg treatment, decreased HSPs60 and HSPs27 levels in respect to the control and MeHg-treated cells.

The HSPs27 have the highest expression levels in astrocytes when compared with the other two members of the HSPs family.

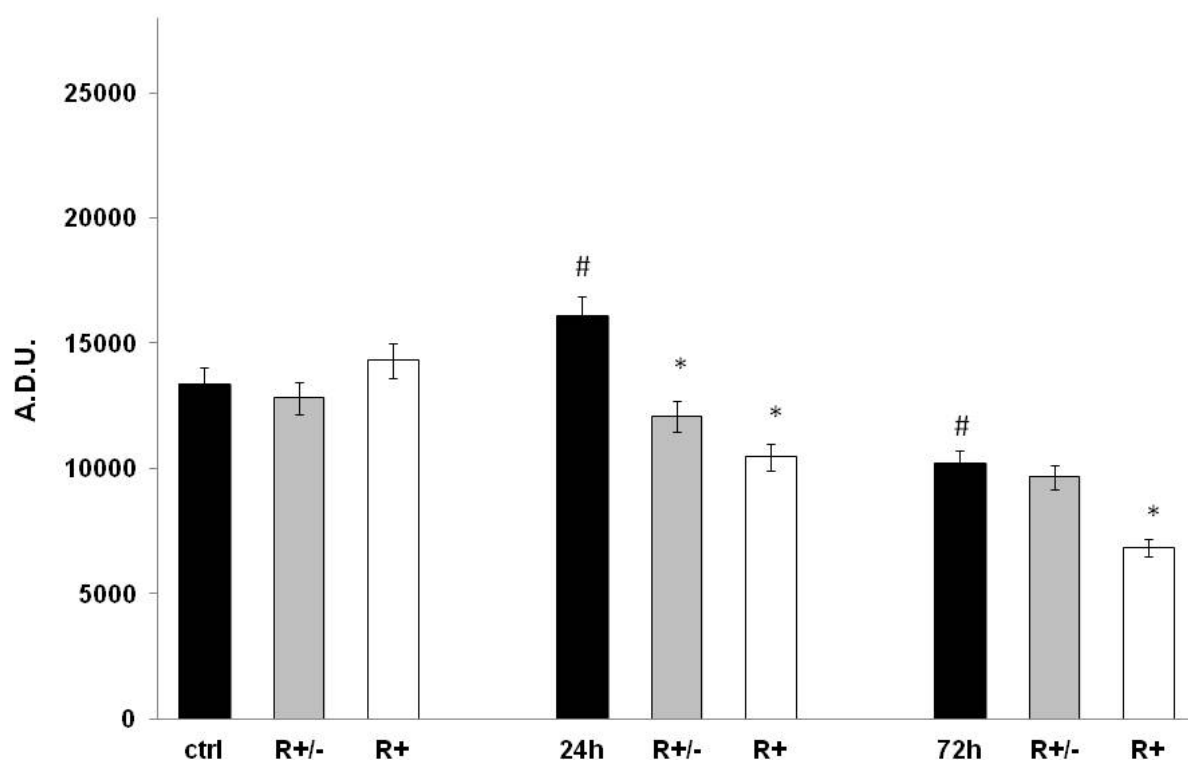


Figure 30. Immunoblotting analysis for HSPs 60, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment (P<0.05) respectively.

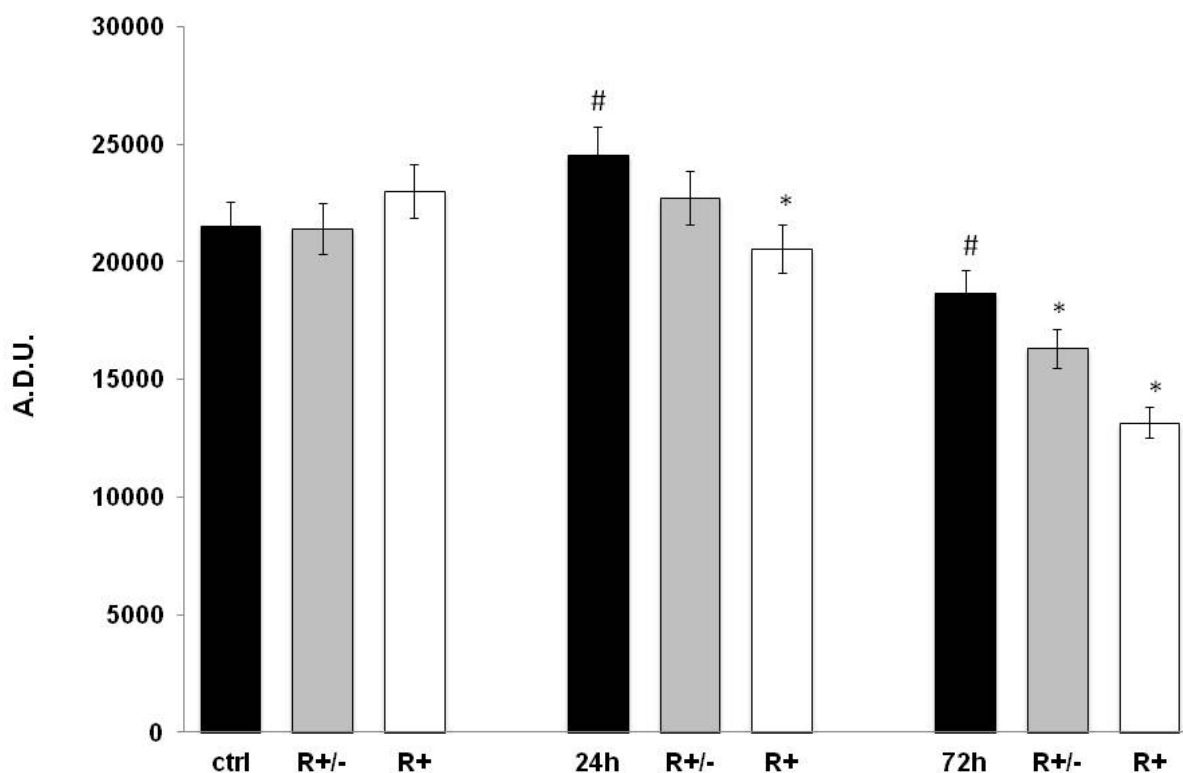


Figure 31. Immunoblotting analysis for HSPs 27, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R $+$) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment (P<0.05) respectively.

Treatment with MeHg determines an increase in the expression of metallothioneins immediately after 24 h and levels remain high and unchanged even after 72 h (Fig 32). ALA pre-treatments with raceme and (R $+$) enantiomer at 24 h increased metallothionein levels in respect to the control and MeHg-treated cells. In particular (R $+$) enantiomer significantly increases protein levels at both 24 h and 72 h.

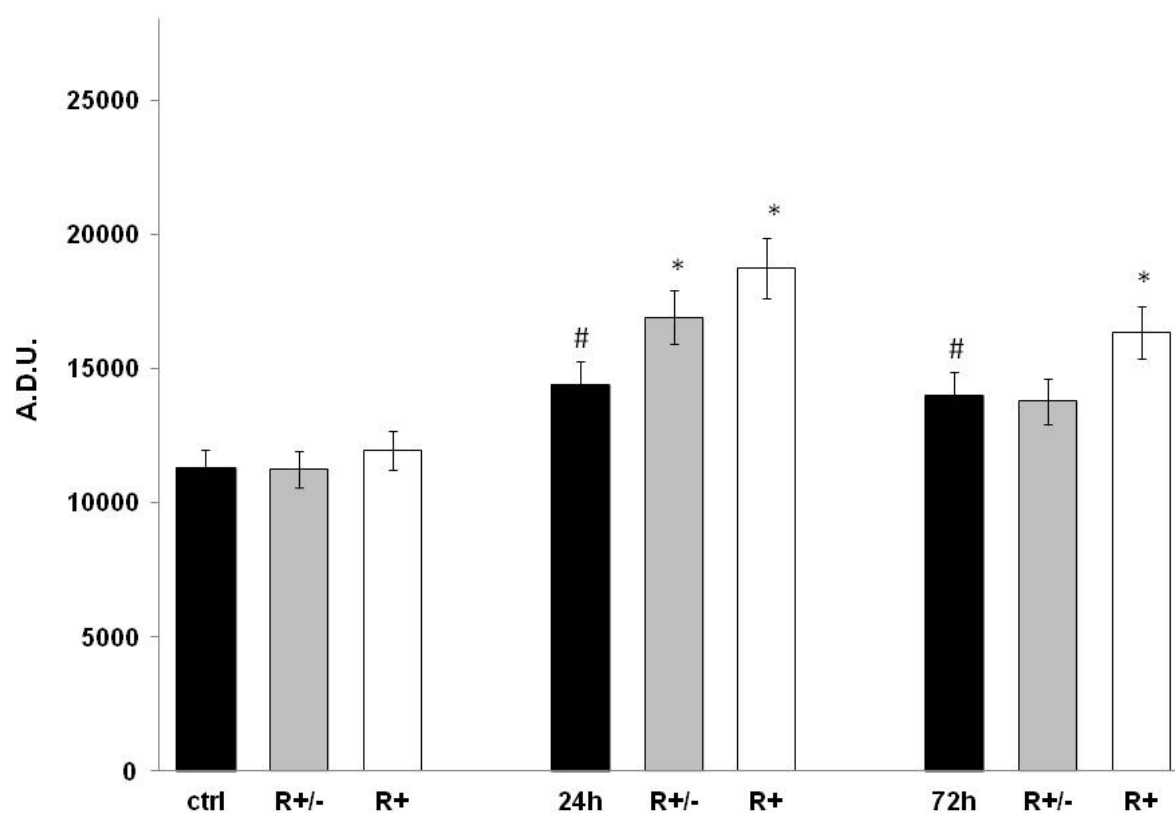


Figure 32. Immunoblotting analysis for metallothioneins, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment ($P < 0.05$) respectively.

Moreover, treatment with MeHg determines a decrease in the expression of iNOS to below control values after 24 h. The decrease in expression remains unchanged, compared to the control, even in cells undergoing treatment at 72 h (Fig. 33). ALA pre-treatments with raceme and (R+) enantiomer significantly increased iNOS levels in respect to the control and MeHg-treated cells only at 24 h.

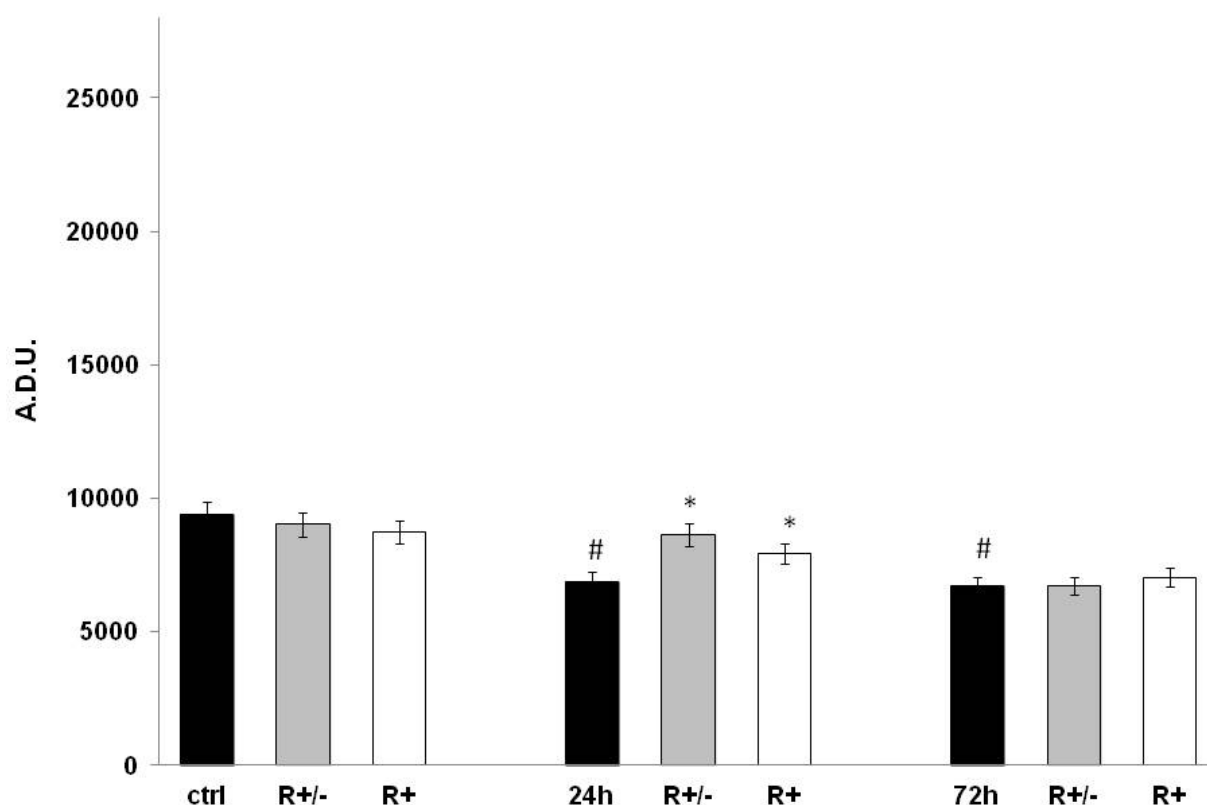


Figure 33. Immunoblotting analysis for iNOS, performed on untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h. Values are expressed as arbitrary densitometric units (A.D.U.) corresponding to signal intensity and represent the mean of three independent experiments \pm SD. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment ($P < 0.05$) respectively.

4.8 Measurement of Carbonyl and Thiol proteins in HA treated with MeHg

The data in fig. 34 shows how the amount of carbonyl residues, resulting from the oxidation of side chain groups present in some amino acids, detected via the covalent bond with FTSC, do not undergo significant changes in response to treatment with

methylmercury at 24 h and 72 h. ALA pre-treatments with raceme and (R+) enantiomer do not change carbonyl levels in respect to the control and MeHg-treated cells.

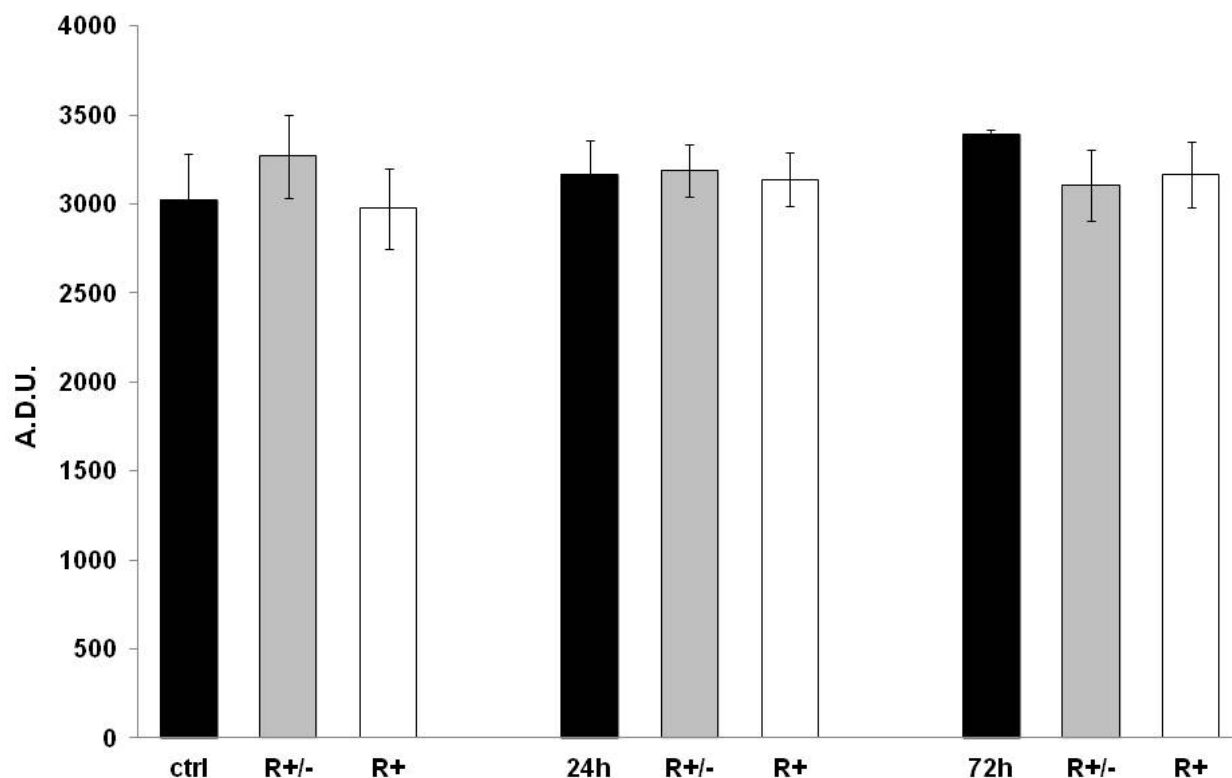


Figure 34. Levels of carbonyl groups present in proteins in primary cultures of untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or for 72 h. The values shown are mean \pm SD and are expressed as total UDA obtained from all bands present in a lane. The analyses were performed in triplicate.

The one-dimensional electrophoresis revealed that the amount of free thiol groups present in cellular protein extracts from astrocytes treated with methylmercury for 24 h and/or 72 h, is significantly decreased in a time-dependent treatment (Fig 35). ALA pre-treatments with raceme and (R+) enantiomer for 72 h increased thiol groups levels in respect to the control and MeHg-treated cells.

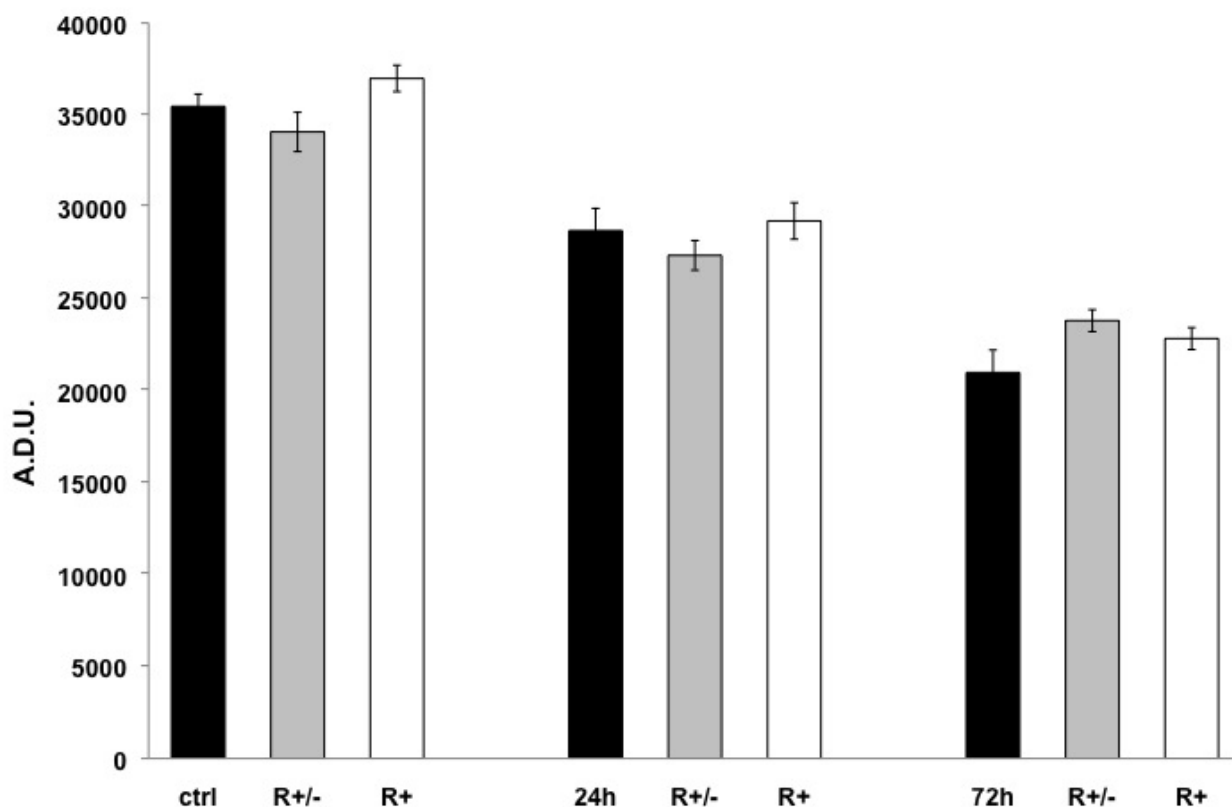


Figure 35. Levels of free thiol groups present in proteins in primary cultures of untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R+) either for 24 h or 72 h. The values shown are mean \pm SD and are expressed as total UDA obtained from all bands present in a lane. The analyses were performed in triplicate. #, * Mean values were significantly different from those of the control group or the corresponding 24 /72 hr MeHg treatment (P<0.05) respectively

This decrease in free -SH groups induced by MeHg and observed in the whole proteome, was further investigated by 2D electrophoresis SDS-PAGE. The analysis of the spots corresponding to the fluorescent proteins, which have free thiol residues 5'-IAF labelled, revealed that several spots disappeared or decreased following treatment with MeHg (Fig. 36).

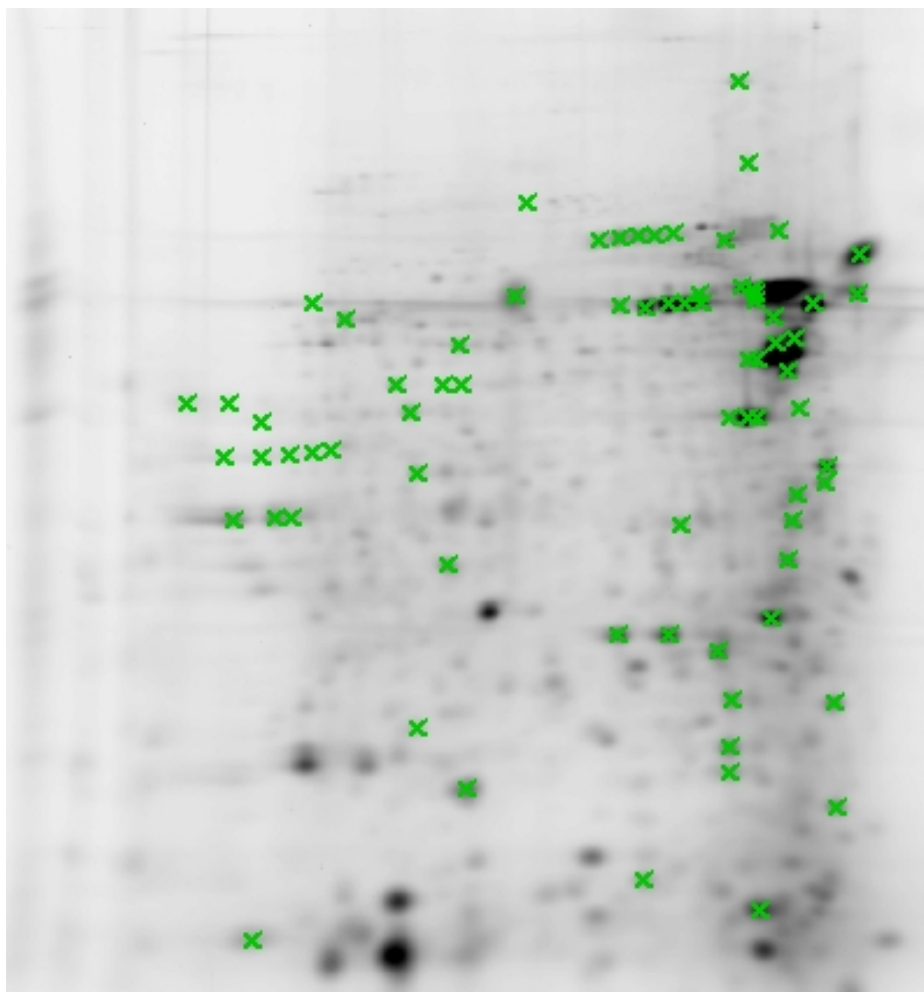
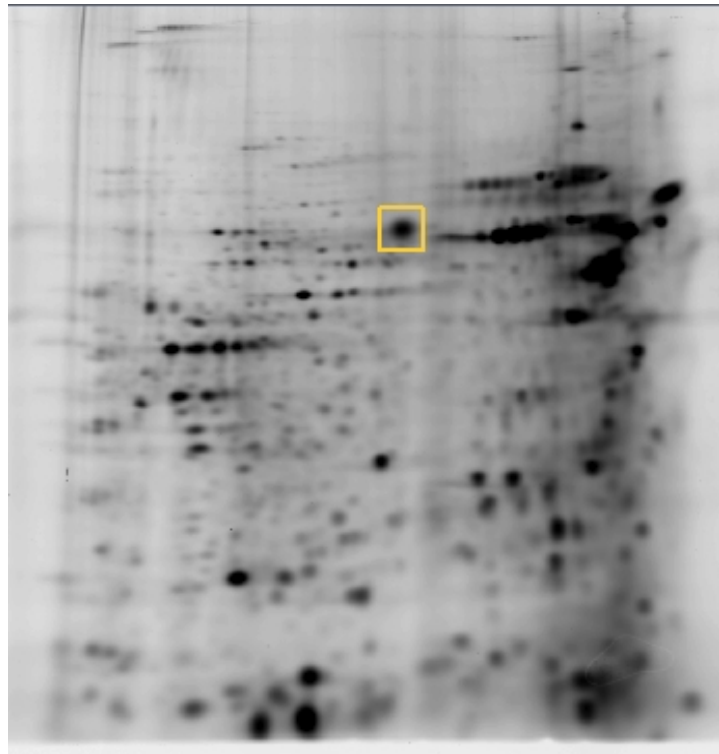
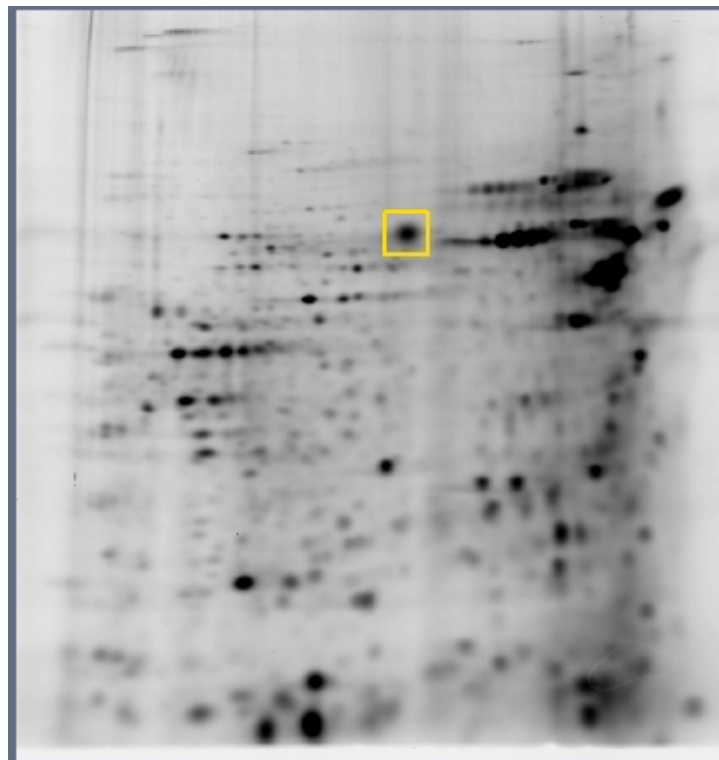


Figure 36. Two-dimensional gel representative of the free -SH groups labelled with 5'-IAF protein present in the extract of untreated HA and HA treated with MeHg 1.125 μ M in combination with Lipoic Acid (R \pm or R $+$) either for 24 h or for 72 h. The crosses in green indicate the spots present in control and that have changed following treatment with MeHg. For each sample the experiment was repeated 3 times.

The effect is particularly evident at 72 h, suggesting that the oxidising or chelating action of MeHg on -SH groups increases in a time-dependent manner (Fig. 37). Pre-treatment with ALA raceme or (R $+$) enantiomer does not noticeably change protein levels in respect to the control cells untreated or treated with MeHg for 24 h and 72 h.



A



B

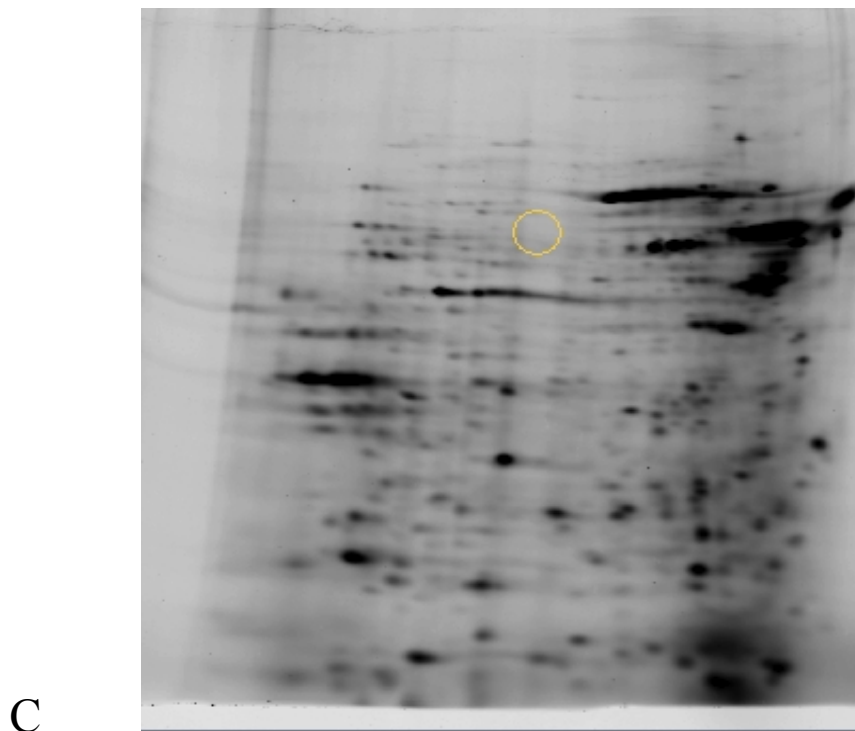


Figure 37. Two-dimensional gel representative of the free SH groups labelled with 5'-IAF protein present in the extract of astrocytes: A) control, B) treated with MeHg for 24 h and c) treated with MeHg for 72 h. The yellow square indicates a spot present in the untreated control (A)and in HA treated with MeHg for 24 h, which disappears completely after 72 h of treatment (yellow circle C). For each sample the experiment was repeated 3 times.

CHAPTER V

Discussion

It is well-known that astrocytes play a crucial role in CNS homeostasis and in the response induced by several toxic substances or by brain injuries through astrocyte activation and, consequently, the development of a reactive gliosis state (O'Callaghan, 1993; Norton et al., 1992). In this context, the use of MeHg-treated primary cultures of HA revealed that this neurotoxin, while reducing mitochondrial functionality without cytolysis, as the data of LDH release shows (Fig. 13), impaired astrocytic reactivity primarily in one of its three main aspects, hypertrophy, without suppressing inflammatory and proliferative signals (Malfa et al. 2014). These effects are largely impeded by pre-treatment with lipoic acid, an endogenous, antioxidant and chelating molecule. In fact, both the racemic mixture and (R+) enantiomer exert a clear protective effect on mitochondrial activity, as observed in the values of MTT (Fig.12). The analysis of DNA status, carried out with Comet Assay, demonstrated that MeHg, at the chosen concentrations and times of treatment, does not explain a genotoxic action as with pre-treatment with ALA raceme and (R+) enantiomer (Table. 1). According to some authors, signalling cascades leading to MeHg-induced cell death involve the release of mitochondrial factors, such as cytochrome c and AIF with subsequent caspase-dependent or -independent apoptosis (Ceccatelli S et al. 2010). In our experimental model we did not observe apoptotic involvement in relation to the pathway activated by Cyt c release. Following an injury to the CNS, the hallmark of reactive gliosis is the characteristic hypertrophy of astrocytic cellular processes, which is expressed by the up-regulation of proteins of intermediate filaments (IF): GFAP, vimentin and nestin (Pekny and Nilsson, 2005). These biomarkers were all negatively modulated in our experimental model (Malfa et al.

2014). But we observed that lipoic acid is able to prevent the fall in expression levels of the mentioned proteins, especially at 72 h of MeHg treatment. In particular, the increase in GFAP after 72 h of MeHg treatment appears to be more pronounced in the presence of lipoic acid, which may be associated with activation of the reactive response. In fact, fibrillary acidic protein (GFAP) represents the best marker of a reactive state (Pekny and Nilsson 2005), as well as astrocyte differentiation in normal cellular functioning and also responses to any CNS injuries (Bramanti et al., 2010a). Usually, a reactive state in astrocytes is associated with a proliferative index sustained by the high expression of the cell cycle proteins family, such as CDKs (Wu et al., 2012). Therefore the increased expression of cyclin D1 observed after 72 h MeHg treatment (Fig. 23) may, in our opinion, be associated with the onset of a proliferative state. In fact, it was demonstrated that cyclin D1 levels in actively cycling cells also increase during G2 phase, are maintained during mitosis and G1 phase, and decline when DNA synthesis begins (Yang et al., 2006). However, even if this proliferative signalling should stimulate cell proliferation, the cell cycle progression may be impeded through antimitotic effects (Fig 1) exerted by MeHg-induced cytoskeletal instability (Fig. 18, 19 and Fig 21A-G) (Curle et al., 1987; Hunter et al., 2000; Crespo-López et al., 2009; Choi et al., 1980). In line with what has been said above, we observed that lipoic acid exerts a protective effect on the expression of intermediate filaments, significantly increasing cyclin D1 expression at 72 h, promoting cell proliferation. The closest relative of CDKs is the MAPKs family (Bramanti et al., 2010b). These are serine/threonine protein kinases mainly involved in cellular responses to different stimuli, such as mitogen, osmotic stress, heat shock and proinflammatory cytokines (Kaminska et al., 2009). In our experimental model, a decrease of p38 MAPK expression was observed after 24 h and 72 h, allowing us to hypothesise about the absence of a crosstalk between p38 MAPK and cyclin D1. This statement is contradicted by the data obtained in pre-treatment with lipoic acid, which demonstrate a marked increase, with performance similar to 72 h in the

expression levels of cyclin D1 and MAPK, even exceeding the levels of the control. We therefore hypothesise that there may be, at least in this case, the establishment of a crosstalk between the two proteins. Furthermore, astrocytic reactivity is associated with increased levels of proinflammatory cytokines and NFκB expression, an ubiquitous transcription factor involved in the immune response (Brambilla et al., 2009; 2005). We observed a time-dependent increase in NFκB, which may be indicative of the initiation and maintenance of an astrocytic inflammatory state. This is significantly reduced in cells pre-treated with (R+) lipoic acid, giving this molecule a definite anti-inflammatory action.

Furthermore, evaluating the level of radical species and glutathione content we observed that lipoic acid, in both its forms (racemic and enantiomer), when added to cell cultures before MeHg treatment, significantly reduces the amount of radical species and preserves GSH reserves inside the cell, in agreement with data present in the literature (Packer et al. 1997, Evans J.L. Goldfine I.D. 2000). Moreover, treatment with ALA increases the expression levels of SOD, clearly exerting a protective action on cellular redox state.

We believe that this is, in part, the expression of the antioxidant action exerted by the two -SH residues present in the molecule and, in part, the chelating action of lipoic acid against the heavy metal, sparing glutathione an attack by MeHg (Ou P. et al. 1995). The analysis of GGT1 expression shows, in our experimental conditions, that MeHg stimulates the expression of this enzyme in a time-dependent manner, an effect which we relate to the fall in GSH levels. Pre-treatment with ALA does not seem to exert any effect, apart from expression levels of R(+) at 24 h of MeHg treatment, which are particularly significant.

Numerous proteins are involved in the response to stressors such as MeHg, amongst which there are the thermic shock proteins (HSPs) and metallothioneins (MT) families. The MTs are small proteins, rich in cysteine residues capable of binding

metals and, therefore, are involved in metal detoxification, homeostasis and protection from oxidative stress induced by the metal (Maret W. 2008). Because of their high cysteine content, MTs have a strong affinity for MeHg. It has also been demonstrated that MeHg induces the expression of MTs (Tsui M.T. and Wang W.X. 2005).

The preliminary results I obtained indicate, for treatment with MeHg, a down-regulation of HSPs 70 in a time-dependent manner, unlike what has been reported by other authors who have demonstrated an up-regulation of these proteins following Hg exposure. In particular, Han et al. demonstrate, in bronchial epithelium cultures treated with Hg at concentrations of 0-50 micromolars, an up-regulation of HSPs 70, an effect that was observed, however, at 50 micromolars (Han S.G. et al. 2007).

Differently, other authors, while studying the toxic effect of various heavy metals, including Hg, on chicken embryos, observed that cellular levels of these proteins, at different exposure times, increase initially (2-4 h), and then return to levels of values observed in the control at 24 h (Papaconstantinou A.D. et al. 2003). However, it should be noted that these results were obtained using much lower Hg concentrations than those used by us, and are probably not sufficiently useful for observing an effect on HSPs 70.

Similarly, it can be considered that the treatment times we selected do not allow us to highlight an increase in these proteins since they are probably involved in an immediate response to stress, and at 24 h are not capable of showing the expected response. Furthermore, we also think that a hormetic response or cellular adaptation to stress is triggered and so, after a rapid initial response that can occur within the first few hours after treatment, they are down-regulated to give way to other proteins which are involved in successive phases of the response to stress. In particular, hormesis refers to a process in which exposition to a sub lethal stress factor makes an organism resistant to successive stresses.

This effect has been demonstrated in many models, from *in vitro* cell cultures to *in*

vivo human models under stress conditions, such as dietary restrictions, physical exercise, radiation and exposure to chemical substances, including metals, and heat (Cypser J.R. et al. 2006; Le Bourg E. 2009). Even though the mechanisms of hormesis are unknown, various studies have clarified that HSPs, and, in particular, the HSPs 70 family and MTs can be involved. In fact, both can be up-regulated following exposition to heavy metals (Damelin L.H. et al. 2000). Furthermore, hormesis has also been implicated in explaining the time delay, which is observed between MeHg exposition and the manifestation of symptoms of toxicity.

The other HSPs proteins we studied show, conversely, a trend, in response to treatment, similar to what has already been reported in the literature. In fact, HSPs 60 and 27 show an increase at 24 h, and then return to control values at 72 h. Metallothioneins, instead, undergo an increase at 24 h in response to treatment. All of this suggests that these three proteins are involved in a second phase of stress response, which attempts to protect the cell from the toxic action of the metal either directly, as with the MTs, which bind the metal by sequestering it from the cellular environment, or, in the case of HSPs 60 and 27, by triggering other cellular responses to counteract the action of the metal.

Pre-treatment with ALA increases expression levels of HSPs 70 and MTs, while it negatively modulates HSPs 60 and 27, suggesting ALA exerts its role particularly on the proteins mainly involved in an early stress response. The proteomics study indicated to us that there do not seem to be significant effects on the protein carbonyl groups, while for the reduced thiol residues we have demonstrated the presence of a negative modulation induced by MeHg in a time-dependent manner. This decrease was partially counteracted by both ALA treatments, and was particularly significant after 72 h of treatment with MeHg. This data was obtained with a method for labelling of thiols with IAF, which results specific only for free thiols and not for oxidised variants such as the sulfenic groups, sulfinic acids, disulfides or nitrosothiols (Baty J.W. Et al. 2002).

The one-dimensional protein analysis of total free -SH was later followed by the two-dimensional analysis which, resolving the whole protein pool, confirms that MeHg (1.125 μ M) at 24 h and 72 h significantly alters the total free thiol residue content of the proteome in a time-dependent manner. The phenomenon may have been exerted by MeHg both directly, by binding with the free -SH groups, and, indirectly, through oxidation linked to the increase in ROS. To further clarify the action mechanism, I went on to study the proteome state by means of 2D-SDS PAGE. This analysis allowed me to individuate specific effects exerted by the heavy metal on single proteins for which the fluorescence signals were reduced considerably, or, as in some cases, disappeared completely (data reported in Fig. 36, 37). This was highlighted, in particular, for a protein with a molecular weight of between 70 and 90kDa, as shown in Fig. 37. In conclusion, our data evidences that ALA exerts a distinct protective power towards human astrocyte cells treated with MeHg, evidencing clear indications of the use of this molecule in the field of prevention and prophylaxis against oxidative and neurodegenerative damage in the CNS. (R+) enantiomer, in particular, is more active in producing the effects, both antioxidant and protective, and it is this molecule that should be used *in vivo*.

CHAPTER VI

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Chapter VI:References

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Appendix A: Publications

GRASSO S., BRAMANTI V., TOMASSONI D., BRONZI D., **MALFA G.**, TRAINI E., NAPOLI M., RENIS M., AMENTA F., AVOLA R. *Effect of lipoic acid and α -glyceryl-phosphoryl-choline on astroglial cell proliferation and differentiation in primary culture.* J Neurosci Res. 2014 Jan;92(1):86-94. Doi: 10.1002/jnr.23289. Epub 2013 Oct 26.

MALFA GA., TOMASELLO B., SINATRA F., VILLAGGIO G., AMENTA F., AVOLA R., RENIS M. *"Reactive" response evaluation of primary human astrocytes after methylmercury exposure.* J Neurosci Res. 2014 Jan;92(1):95-103. Doi: 10.1002/jnr.23290. Epub 2013 Oct 3.

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TOMASELLO B., **MALFA G.**, GALVANO F. AND RENIS M. *Dna damage in Normal-weight obese syndrome measured by Comet assay.* Mediterr J Nutr Metab. DOI 10.1007/s12349-010-0035-6 2010.

Appendix A: Publications

MALFA G., TOMASELLO B., SPATAFORA C., CARDILEV., SCIFO C., TRINGALI C., RENIS M. *Chemotherapeutic effects of resveratrol and its analogue 3,5,4'-trans-trimethoxystilbene on DU145.* Trends in Cancer Research, vol 6 pp 45-54, 2010.