Review Article

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Methylmercury neurotoxicity & antioxidant defenses

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Neurotoxicity induced by methylmercury (MeHg) increases the formation of reactive radicals and accelerates free radical reactions. This review summarizes recent findings in the MeHg- induced formation of free radicals and the role of oxidative stress in its neurotoxicity. Oxidative stress on CNS can produce damage by several interacting mechanisms, including mitochondrial damage with increase in intracellular free Ca^{2+}, activation and inhibition of enzymes, release of excitatory amino acids, metallothioneins expression, and microtubule disassembly. The nature of antioxidants is discussed and it is suggested that antioxidant enzymes and others antioxidants molecules may protect the central nervous system against neurotoxicity caused by MeHg.

Key words Anti-oxidant defenses - metallothioneins - methylmercury - microtubules network - neurotransmitters - oxidative stress

Methylmercury toxicity

Mercury is a heavy metal contaminant with potential for global mobilization following its give off from anthropogenic activities or natural processes[1]. In nature, elemental mercury (Hg^0) can be biotransformed and converted to methylmercury (MeHg), which is the most toxic form of mercury in the environment[2-4]. The studies about MeHg toxicity became ubiquitous and diversified since the outbreak of environmental catastrophes such as those in Minamata (1950s) and Niigata (1960s). In such episodes, as a consequence of MeHg exposure, the exposed individuals exhibit severe forms of neurological disease which include a collection of cognitive, sensory, and motor disturbance[5,6].

The studies on MeHg toxicity have tried to evaluate its impact on several ecosystems around the World, including places in Japan, Irak, Canada, Africa, including Brazilian Amazon, and India[2,7,8], as well as to understand its toxicological effect on biological systems. MeHg was firstly recognized as a potent neurotoxicant for the adult nervous system in studies performed on exposed workers of a chemical factory in England[9,10]. Later, its importance as a neurotoxicant for

the nervous system during development was recognized in the Minamata's outbreak[5,6]. Since then, several studies of exposed human populations as well as experiments with laboratory animals demonstrated that exposure to toxic levels of MeHg during pre- and post-natal life causes neurological abnormalities, cognitive impairment, and behavioural disturbance[11,12]. MeHg vulnerability of the developing brain reflects the ability of lipophylic MeHg to cross the placenta and to concentrate in the central nervous system (CNS) once the blood-brain barrier is not fully developed in the prenatal period[13,14].

The developing CNS is more affected by MeHg exposure and exhibits different patterns of changes than the mature CNS. In the adult brain, MeHg poisoning damages the so-called primary areas of the cerebral cortex, affecting the visual, auditory, somatic sensory, and motor cortex, as well as the hippocampus and the granule layer of the cerebellum, causing a remarkable loss of neurones in these brain regions. On the other hand, in the developing brain there is a widespread neuronal loss throughout the CNS, what has been interpreted as due to the high MeHg sensitivity of the immature CNS[11]. The neural disease due to MeHg neurotoxicity includes such symptoms as visual field constriction, somatic sensory disturbance, hearing disturbance, cerebellar ataxia, dysarthria, and mnemic deficits[15-17].

Membrane interactions and transporter-mediated MeHg toxicity

MeHg quickly diffuses across membranes without significant partitioning in lipid bilayers. Thus, it has been proposed that MeHg toxicity is mediated by MeHg membrane leakage[17]. However, it has also been suggested that the potential of MeHg to increase oxidative events leading to cell damage is controlled by MeHg binding to membrane transporters. MeHg absorption, distribution, and excretion are commonly mediated by plasma membrane protein transporters[18]. In addition, it has been possible to investigate at molecular level the mechanisms of MeHg transport through membrane transporters with broad substrate selectivity. These transporters are known as "multispecific", taking advantage of its nature to exert their toxic effects[18]. The main route for MeHg transmembrane transport seems to be the amino acid transport system L, which transports large amino acids[19].

It has been proposed that MeHg-cysteine conjugate is the pathway whereby MeHg exerts its toxicity[20]. Once the presence of such transporters is crucial for toxicity to occur at least through this mechanism, transporter inhibition is expected to be beneficial to prevent disorders caused by MeHg toxicity.

Cellular mechanism to MeHg intoxication

Calcium homeostasis: Calcium ion (Ca^{2+}) plays a critical role in CNS cell death. Ca^{2+} increase beyond physiological levels activates catabolic enzymes such as phospholipases, proteases, and endonucleases, causes mitochondrial dysfunction, and disturbes cytoskeletal organization. Several lines of evidence indicate that at low concentrations MeHg disrupts Ca^{2+} homeostasis, increasing its intracellular level in a number of experimental situations, including primary culture of cerebellar granule cells[21]. This effect has all the potential to disrupt the synaptic function and impair the neural development[22].

Ca^{2+} metabolism is altered through specific pathways which affect Ca^{2+} regulation by some organelles such as mitochondria and smooth endoplasmic reticulum (SER)[23]. Ca^{2+} channel blockers significantly delay MeHg-induced increase of Ca^{2+} levels *in vitro*[24]. In agreement with these data, blockers of voltage-dependent Ca^{2+} channels have been shown to prevent the appearance of neurological disorders in rats administered with MeHg[25]. These observations give support to the hypothesis that changes in Ca^{2+} homeostasis represent an important cellular event in the MeHg-induced CNS toxicity.

Mitochondrial damage induced by MeHg: Mitochondria are the main intracellular site for reactive oxygen production and one of the most susceptible targets for radical species to exert their actions. Importance of mitochondria for MeHg toxicity was recognized from studies performed both *in vivo* and *in vitro*. *In vivo* exposure to MeHg causes its accumulation inside mitochondria followed by a series of biochemical changes in these organelles[26]. These effects are similar to those observed in studies of mitochondria respiratory chain inhibition[27].

Under physiological conditions, the electron transport system of mitochondria consumes about 1-5 per cent of the oxygen that is converted to reactive oxygen species (ROS) such as O_2 and H_2O_2[27]. Animals exposed to MeHg display neurological symptoms after a latent period and mitochondrial function is impaired during the symptomatic period but not during the latent phase. Although MeHg concentrations are maximal during the latent period, the effects of MeHg on

mitochondria could be indirect as they are preceded by inhibition of protein synthesis[28]. Rats exposed to MeHg have reduced rates of cellular respiration and this effect is reverted by the removal of K^+, suggesting that there is an increase in K^+ permeability of the mitochondrial membrane[29]. High MeHg levels cause impairment of mitochondrial function as the organelle exhibits a membrane permeability transition state. MeHg exposure induces a decrease in the activity of enzymes of the mitochondrial energy metabolism such as cytochrome C oxidase (CCO), superoxide dismutase (SOD) and succinate dehydrogenase (SDH)[28]. This is probably due to the decrease in the respiratory rate caused by MeHg-induced inhibition of the tricarboxylic acid cycle. This is consistent with previous work showing that MeHg exposure decreases succinate dehydrogenase activity[30]. *In vitro* MeHg exposure of isolated mitochondria from rat liver inhibits electron transport and phosphorylation, increases K^+ permeability, and dissipates the mitochondrial membrane potential (MMP)[31]. Loss of MMP results in efflux of mitochondrial Ca^{+2} and inhibition of mitochondrial Ca^{+2} uptake[32]. In addition, MeHg exposure in isolated rat brain mitochondria causes ATP-dependent and -independent decrease in Ca^{+2} uptake and increase in Ca^{+2} efflux from mitochondria[33]. Although mitochondria participate in Ca^{+2} buffering at relatively elevated Ca^{+2}, the affinity of the uniport carrier for Ca^{+2} is low, and mitochondria may play only a minor role in buffering Ca^{2+} under normal conditions[32].

Increased rate of Ca^{+2} is not observed during prolonged depolarization and it seems that under normal conditions mitochondria do not contain sufficient Ca^{2+} to alter neurotransmitter release[33]. Recent evidence suggests that increased local concentration of Ca^{2+} may be required for activation of neurotransmitter release. Thus, the relatively small changes in Ca^{2+} observed upon mitochondria depolarization are likely insufficient to alter the release of neurotransmitter via normal physiological mechanisms. However, other mechanisms for MeHg toxicity could be trigger by small alterations in the internal Ca^{2+} concentrations.

Microtubules network: MeHg seems to interact with cytoplasmatic cytoskeletal components, including microtubules[34]. *In vitro* studies demonstrated that MeHg presents high affinity for tubulin sulphydryl groups (-SH), depolymerizing cerebral microtubules and directly inhibiting their assembly[34,35]. In addition, several works reported that MeHg promotes microtubule disruption in a number of cell models, including human fibroblasts[34], neuroblastoma, and glioma cells[36,37].

Neuroblastoma cells appear to be more susceptible to toxic effects of MeHg than either rat glioma or human fibroblasts[38], although the cell death mechanism produced by MeHg in these cell lines has not been well characterized[13].

The integrity of microtubule function is critical for the physiological development of the CNS including cell proliferation, migration of post-mitotic neurones to form the cortical layers of the cerebrum and cerebellum, extension and stabilization of neuritis, and axodendritic transport. Indeed, MeHg interference in microtubules is consistent with neuropathologic findings in the autoptic brains of full-term newborn human infants from the Iraqi outbreak, who were exposed *in utero* in early phases of pregnancy. These findings included brain reduction, neuronal heterotopias in the cerebral and cerebellar white matter, and abnormal patterns of neuronal arrangement and alignment in the cerebral cortex[39].

The ability of MeHg in a concentration-dependent way to inhibit neuronal migration from the external granule layer towards the internal granule layer has been demonstrated experimentally in cerebellar organotypic cultures[40]. It has been suggested that the extensive apoptotic death observed in the external granule layer following MeHg treatment is the result and not a cause of the impaired neuronal migration[40].

Interestingly, microtubule disruption provoked by MeHg does not only affect the neuronal migration and development of CNS. One of the most dangerous consequences of human exposure to relatively low levels of MeHg is DNA damage as demonstrated by detection of micronucleated cells and chromosomal aberrations[40,41]. In recent years, microtubule disruption was hypothesized as the origin of this genotoxic effect of mercury[42-44] and a recent report demonstrated that very low MeHg concentrations are able to initiate genotoxic processes in human cell lines of brain origin[45].

MeHg and neurotransmitters systems: Several metal compounds have been shown to interfere with neurotransmission. MeHg directly affects the mechanisms of neurotransmission, including release and uptake of neurotransmitters, enzymatic neurotransmitter metabolic inactivation, and post-synaptic events associated with receptor activation[46]. Some neurotoxicants indirectly interfere with neurotransmission by interacting, for example, with

energy metabolism, sodium channels, or ATPases. Furthermore, changes of any parameter of neurotransmission can be the result of neuronal death due to cytotoxic effects of the neurotoxicants[47].

The rising of extracellular glutamate levels is responsible for the constant activation of metabotropic and ionotropic glutamate receptors thus elevating Na^+ influx and Ca^{2+} release from intracellular organelles that may trigger a biochemical cascade which increases the production of ROS[47]. Oxidative stress by itself inhibits the astrocytic glutamate uptake through a direct action on the transporter proteins[48,49].

Although the toxic damage caused by MeHg might be intrinsically prevalent in neurones, many of the published evidences suggest that neuronal damage in response to MeHg most likely represents aberrant control of the extracellular milieu by the astrocytes[50]. On line with this argument it should be remarked that the neurotoxic effect of MeHg could be reverted with antagonists of N-methyl-D-aspartate (NMDA) receptor[48].

Moreover, MeHg has been described to produce increases in the spontaneous release of other neurotransmitters such as dopamine, GABA, acetylcholine, and serotonin from rat brain synaptosomes[51-53]. MeHg also inhibits astrocytic uptake of cystine and cysteine, the key precursor for glutathione biosynthesis[50].

Methylmercury and metallothioneins: Metallothioneins (MTs) constitute a family of proteins characterized by an unusual cystein abundance[54]. Under physiological conditions, MTs are unusually rich in multiple cysteine residues allowing their binding to metal centers and enabling them to serve as a heavy-metal detoxification system[55]. MTs are predominantly expressed in the central nervous system and it is important to gain new insight into how MTs are regulated in the brain in pathological injury, such as that produced by MeHg intoxication.

Some studies have reported the potential role of MTs in attenuating the cytotoxicity induced by MeHg[54,56]. Although the interaction of MTs with MeHg ions has long been established, elucidation of the binding features of MeHg-MT species has been hampered by the inherent difficulties of MeHg-thiolate chemistry, which mainly arise from the diverse co-ordination preferences of Hg (II) and the various ligation modes of the thiolate ligands[57]. Nevertheless, the analysis of MeHg binding to MTs has been intensively studied. In contrast, the chemistry of MeHg-MT complexes has attracted much less attention. Earlier reports demonstrated the inability of MT in the detoxification of MeHg and that it is unable to bind to MeHg either *in vivo* or *in vitro*[57,58]. Subsequent attempts to induce brain MT by exposure to $MeHg^+$ gave inconsistent results: MT concentrations remained unchanged in rats, whereas MT and mRNA concentrations increased in MeHg-treated rat neonatal astrocyte cultures[58]. However, there is increasing evidence that induction of MTs in astrocytes attenuates and even reverses the cytotoxicity caused by MeHg, indicating binding of MeHg by an astrocyte-specific MT isoform, MT1[59].

The MT association with heavy metals has been the subject of several studies. For example, data about geometry of mercury and metallothionein association have demonstrated that characteristics of MeHg-MT species cannot be extended to Hg-MT complexes[57]. The co-existence of digonal, trigonal-planar, and tetrahedral co-ordination geometries together with the presence of secondary mercury-sulphur interactions are common features in the chemistry of Hg(II) thiolates[57]. In contrast, MeHg shows a clear preference to form essentially linear two-coordinate Hg(II) complexes with thiolate ligands, even if, in some cases, secondary interactions at the metal center are observed[59,60]. These characteristics could reflect the differences in cellular resistances or response observed against mercury organic and inorganic toxicity[57].

Methylmercury and oxidative stress

ROS are generally very small molecules and are highly reactive due to the presence of unpaired valence shell electrons. ROS form as a natural by-product of the normal oxygen metabolism and have important roles in cell signalling. These molecules are generated continuously during oxidative metabolism and consist of inorganic molecules, such as superoxide radical anion ($O2^-$), hydrogen peroxide (H_2O_2), hydroxyl radicals (OH^-), as well as organic molecules such as alkoxyl and peroxyl radicals[61]. Some evidences suggest that the disturbance in the balance between oxidative and reductive cell processes is involved in the pathogenesis of many neurodegenerative conditions such as Alzheimer disease, amyotrophic lateral sclerosis (ALS), and Parkinson disease. Other conditions such as autoimmune and inflammatory diseases, cancer, and diabetes mellitus also seemed to be related to this Disturbance[61].

MeHg has been thought to induce ROS and generation of oxidative events leading to cell damage. Previous studies have suggested that there is a relationship between these events with dysfunction of the cellular energetic metabolism and disruption of the electron transport chain. These phenomena generate oxidative stress[62,63]. MeHg exposure increases the rate of ROS in the cerebellum (*in vivo*) and in the brain synaptosomes as well as in the cerebellum neuronal cultures, hypothalamic neuronal cell line, and mixed reaggregating cell cultures[64-66]. The formation of these species was critical to determine the damage and the cell death in distinct cell types such as astrocytes and neurones.

It seems that the intensity of MeHg exposure is a crucial factor to establish whether the neuronal death occurs by necrosis or apoptosis[67,68]. However, the mechanism of cell death induced by oxidative stress via MeHg has not been well characterized.

Methylmercury and antioxidant defenses

Many studies have already established that MeHg neurotoxicity evokes oxidative stress with formation of ROS in the CNS and that the increase of ROS induces cell damage and death in the CNS. In order to avoid the damage caused by ROS, such as DNA strand breaks, lipid peroxidation, and protein modification, mechanisms have been developed during evolution which dispose or prevent the generation of ROS[69]. However, the underlying mechanisms responsible for the protection of CNS against MeHg neurotoxicity are still poorly understood.

It is well known that cell defences against free radicals such as ROS include scavenger compounds such as glutathione, cysteine, melatonin, and enzymes with antioxidant activities as superoxide dismutase, catalase, and glutathione peroxidase[70].

It was demonstrated that MeHg induces a concentration-dependent increase in ROS formation in rat neonatal neuronal culture and astrocytes culture[48,71,72]. It was also shown that this effect can be reverted by the use of n-propyl gallate (PG), a free radical scavenger, superoxide dismutase (SOD), an antioxidant enzyme, and α-phenyl-*tert*-butyl nitrone (PBN), a lipophilic hydroxyl radical spin trapping agent[72,73].

Endogenous glutathione (GSH) is one of the most abundant and essential thiol tripeptide present in mammalian cells for scavenging reactive oxygen species[69]. The involvement of GSH in the neurotoxicity of MeHg was also evaluated, showing that the increased oxidative stress is related with the depleted intracellular GSH levels[74,75]. The excessive formation of ROS induced by MeHg exposure can be reverted under treatment with L-2-oxothiazolidine-4-carboxylic acid (OTC), which increases the amount of intracellular GSH, as well as the depletion of GSH by treatment with buthionine-L-sulphoxane (BSO) can potentiate the production of ROS induced by MeHg in rat primary cerebral astrocytes.

Recently, a human population study in the Amazon correlated the MeHg exposure with the levels of glutathione and catalase activity. Surprisingly, it was demonstrated that high blood levels of glutathione in woman exposed to high concentrations of MeHg may be explained by the increase of glutathione synthesis in response to oxidative stress or, more probably, by the inhibition of glutathione peroxidase activity[77]. In the same population the inhibition of catalase activity was also observed. These changes likely reflect adaptive responses of the Amazonian population to oxidative stress induced by MeHg.

Other studies revealed that the GSH content may vary in different regions of the CNS, demonstrating that the GSH amount is higher in cerebral cells than in cerebellar cells[78,79]. This may explain the higher susceptibility of cerebellar cells to MeHg toxicity in comparison with cerebral cells, but the reason why certain areas of CNS showed different sensitivity to MeHg toxicity, remains unclear.

In addition, MeHg poisoning can induce sympathetic ganglia toxicity and neurite outgrowth inhibition[80,81]. Compounds that possess sulphydryl (-SH) groups attenuate MeHg neurotoxicity, once at least part of MeHg effects occurs through interaction with -SH groups in cellular proteins[82]. In this context, primary neuronal cultures from avian sympathetic ganglion were used to evaluate the protective role of antioxidants agents with -SH group such as L-cysteine against MeHg toxicity. It was reported that MeHg induces massive cell death (neurite death) and that L-cysteine could fully protect (nearly 100%) the sympathetic neuron against this damage. The effect of GSH was also tested showing the same properties of cysteine[83].

The use of methionine, an antioxidant agent which does not possess -SH groups, fails to promote cell protection against MeHg intoxication, proving the relevance of -SH groups to this effect[83]. Another

antioxidant that protects the brain against oxidative stress is vitamin E, which maintains the integrity of membrane by inhibiting lipid peroxidation[84]. Recent findings reported the protective effect of the antioxidants tocopherols and tocotrienols (analogs to vitamin E) against MeHg neurotoxicity[85,86]. In cerebellar granule cells (CGC), these compounds effectively prevent cell death caused by MeHg intoxication as well as improve cell migration[87].

Evidences also suggest that the treatment with trolox (6-hydroxy-2,5,7,8- tetramethylchroman-2-carboxylic acid), other antioxidant derivate from vitamin E, might provide prevention against oxidative stress. In MeHg-treated rats, it detected many apoptotic cells in the cerebellar granule layers and the treatment with trolox clearly repressed the appearance of these apoptotic processes[76].

A number of different hypotheses have been suggested to explain the mechanism by which the antioxidants defences protect CNS against MeHg neurotoxicity, which include scavenging and removal of free radicals, reversal of glutamate uptake impairment, inhibition of cytochrome c release, and caspase activation[88].

It has been established that MeHg inhibits glutamate transport by astrocytes by an unknown mechanism which leads to the increase of ROS generation[88]. It was demonstrated that a variety of antioxidants can prevent the overproduction of ROS, in this way attenuating MeHg neurotoxicity. Some workers have focused on the effect of antioxidant agents in the impairment of EAA transport elicited by MeHg.

In primary cultures of astrocytes from cerebral cortex, MeHg treatment inhibits the net uptake of ^{3}H-D-aspartate, a glutamate analogue, that could be completely prevented by catalase activity suggesting that the amount of H_2O_2 mediates this EAA transport inhibition[89]. The mechanisms whereby H_2O_2 alters EAA transport remains elusive, although it was proposed that the excessive production of H2O2 by MeHg intoxication involves the inhibition of plasma membrane Na^+/K^+-ATPase and subsequent modification in the ratio of intracellular/extracellular Na^+ concentration necessary for EAA transport. Interestingly, others antioxidants that prevent ROS formation such as trolox and PBN did not present any effect in this transport system[89].

In previous studies it was demonstrated that intrastriatal administration of different concentrations of MeHg produces significant increase in dopamine release from rat striatum[90]. This increase is due to the interaction of this metal with dopamine transporter (DAT). Pre-treatment with both GSH and cysteine significantly decreased dopamine release induced by MeHg, with GSH being more efficient than cysteine[91].

Concluding remarks

The molecular mechanisms of MeHg damage in both adult and developing CNS is not fully understood. Early reports have described a number of possible cellular mechanisms to explain the neurotoxicity induced by MeHg. Most of these studies reported the high affinity of MeHg for thiol groups (-SH) which are present in cytoskeletal proteins, enzymes, and peptides that contain the amino acid cysteine[78].

In accordance with these results, it is well established that interactions between MeHg and -SH potentially inactivate protein functions in all cellular and subcellular compartments[42,92]. The mechanisms underlying the protective role of antioxidants molecules against CNS toxicity by MeHg will likely be related to their thiol groups (-SH) binding capacity.

Also in line with the above results, the susceptibility of neurones to MeHg intoxication has been associated to the absence or limited presence of inherent protective mechanisms such as metallothioneins, reduced glutathione, and other stress proteins[93].

This review corroborates with the major role of ROS production in mediating MeHg toxicity in the CNS (Fig.). MeHg-induced toxicity with an emphasis on the generation and role of reactive oxygen and nitrogen species is reviewed. MeHg-mediated formation of free radicals causes many modifications to DNA bases, enhanced lipid peroxidation, and altered calcium and sulphydryl homeostasis. Primary route for MeHg toxicity is depletion of glutathione and bonding to sulphydryl groups of proteins. Various studies have confirmed that metals activate signalling pathways and the cytotoxic effect of MeHg has been related to activation of mainly redoxsensitive transcription factors. Antioxidants (both enzymatic and non enzymatic) provide protection against deleterious metal-mediated free radical attacks. Vitamin E and melatonin can prevent the majority of MeHg-mediated damage both *in vitro* systems and in metal loaded animals. Toxicity produced by MeHg showed that the protective effect of vitamin E against lipid peroxidation may be associated rather with the level of non-enzymatic antioxidants than the activity of enzymatic antioxidants.

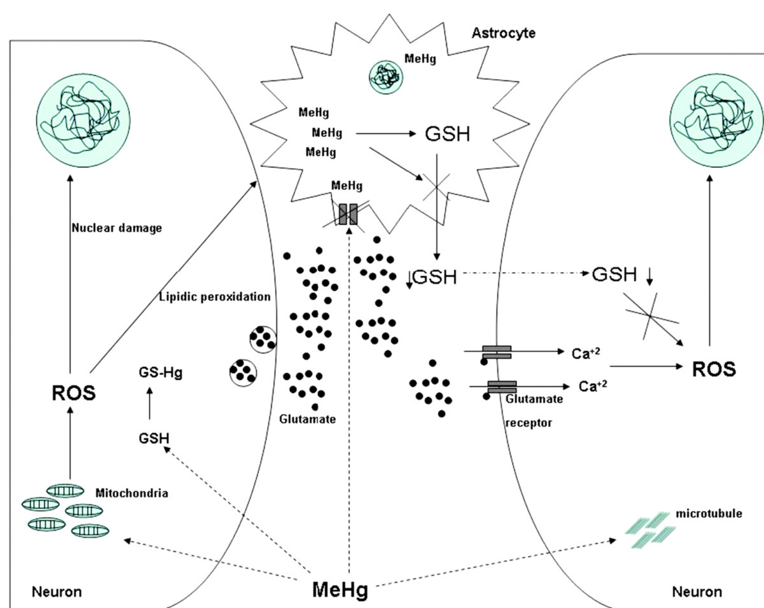

Fig. A schematic model of some of the currently proposed mechanism for cellular damage induced by MeHg in the CNS. In the extracellular environment, MeHg inhibits glutamate uptake, as well as a number of the amino acids that are associated with the synthesis of astrocytic glutathione (GSH). Accumulation of glutamate in the extracellular space and the resulting excessive activation of NMDA receptors can result in excitotoxicity, and, ultimately, cell death. Other proposed mechanisms are related with mitochondrial MeHg -associated dysfunction, including impaired cytoplasmatic Ca^{2+} homeostasis and release of ROS, metabolic inhibition that leads to impaired ATP production, lipidic peroxidation and nuclear damage. MeHg also can provoke microtubules chain disruption decreasing vesicular migration or genotoxicity.

Molecular and cellular approaches can be a strategy to critically examine the possibility of therapeutic actions such as antioxidants or chelanting agents in the treatment of neurodegeneration produced by MeHg.

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