FUNCTIONAL NEUROTOXICITY OF ARSENIC AND MANGANESE AS ENVIRONMENTAL AGENTS AND THE POSSIBLE PROTECTIVE ROLE OF NATURAL ANTIOXIDANTS

Summary of PhD Thesis

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INTRODUCTION and AIMS

Oxidative stress apparently plays a major role in the effect of several toxicants, including heavy metals, of environmental or occupational origin. It means inequilibrium between metabolic reactions producing reactive oxygen and nitrogen species (ROS and RNS), and the capacity of enzymatic and non-enzymatic reactions to neutralize these. In itself, oxidative stress is a side effect of oxidative energy production, as some electrons "leak" from the transport chain in the mitochondria too early and react directly with the O_2 molecule. The immediate product is the superoxide anion ($O_2^{\bullet -}$), leading to generation of hydroxyl ($^{\bullet}OH$) and hydroperoxyl ($HOO^{\bullet}$) radicals. Reactive nitrogen species (RNS) are also known, the most prominent being nitrogen monoxide ($NO^{\bullet}$) an important signal molecule. Abnormally increased oxidative stress can be caused by exposure to a number of environmental xenobiotics, metals and others. Non-neutralized ROS and other radicals damage the nervous system, liver, kidneys etc. Free radicals are more and more held responsible for various chronic non-communicable diseases, and for aging. The central and peripheral nervous system is prone to oxidative damage, due to highly active mitochondrial energy production, to abundance of (unsaturated) structural lipids, and to low antioxidant defence capacity in the brain; and ROS may constitute the final common pathway for several neurotoxicants because oxidative damage to membrane lipids may lead to alterations of the membrane-bound functions crucial to the functioning of neurons.

The list of environmental toxicants causing oxidative stress includes a number of heavy metals. Continuous exposure to metals may create a "silent pandemic" in modern societies, being responsible for decrease in IQ, increased risk of antisocial behavior, neurodevelopmental disorders and brain dysfunctions. Heavy metals are newly defined on the basis of their place in the periodic table because this determines their chemical properties. The biologically most relevant two properties are the existence of more than one oxidation states and the tendency for making coordinate bonds. The two heavy metals included in this thesis are arsenic (As) and manganese (Mn).

Arsenic is abundant in the Earth's crust and ubiquitous in trace amounts. Its increased presence in certain rocks may lead to human exposure from, e.g., mining and smelting non-ferrous metal ores. Arsenic used to have numerous practical applications (pigments, wood preservatives, pesticides, even medicines) mostly discontinued by now because of the health concerns. Its modern applications, e.g. in semiconductor manufacturing, are apparently safe.

The most important source of human As exposure is drinking water. The presence of As in subsurface waters is due to geochemical factors. The problem of As in drinking water has been quite serious in regions of Asia and South America. In South-East Hungary and the adjacent Romanian and Serbian regions the problem has become less severe by now but municipal drinking waters with over 10 μg/L As still exist. Arsenic may be a micronutrient but in any higher amount it is poisonous, and is among the ten most important chemicals of major public health concern (WHO). As occurs in four oxidation states and in inorganic or organic forms. Inorganic As^{III} is more toxic than As^{V} while organic arsenicals are practically nontoxic. Arsenite strongly binds to the –SH group of proteins, inactivating various enzymes and promoting generation of ROS. It also increases oxidative stress by depleting reduced glutathione and thioredoxin and generating H_2O_2. The best known consequences of chronic exposure to inorganic arsenic include cardiovascular, hepatic and renal diseases, but nervous system damage is also known. High-As water of a private well caused encephalopathy, ending with coma, in a family. In schoolchildren exposed to As, problems of cognitive development were significantly associated with elevated urinary As. Electrophysiological data on As neurotoxicity are scarce, but in As-exposed workers of a copper smelter, altered EEG, visual evoked potentials, and peripheral nerve activity were detected. In the same subjects, signs of oxidative stress were also found and both that and the extent of the electrophysiological alterations were correlated to the internal As burden.

Manganese (Mn) can have oxidation states from -3 to 7+, indicating its propensity to redox reactions. This, and its capacity to form complexes, has biological and toxicological relevance. Manganese and its compounds have had numerous technical applications. Mn is an essential trace element for plants, animals and humans. It is a cofactor for several enzymes such as Mn-dependent glutamine synthetase (in astrocytes, acting in the turnover of glutamate) superoxide dismutase (protecting mitochondria against oxidative stress) etc. Despite its essentiality, Mn in high amounts is toxic, among others, neurotoxic. The population can be exposed to Mn when there is an elevated level of Mn in food or drinking water, but typical neurotoxic manifestations are rare. Occupational Mn exposure is primarily inhalational, mostly in the metal industry from welding etc. and often leads to neurological problems. Mn can disturb neurotransmission (glutamatergic, cholinergic, dopaminergic, GABAergic), can cause mitochondrial dysfunction and cellular energy shortage, and can inhibit voltage-gated Ca-channels in neurons. Oxidative stress is involved in the toxicity, including neurotoxicity, of Mn. Mn induces oxidative stress via the oxidation of dopamine and other catecholamines and by mitochondrial damage; and the activity of Mn-SOD and

glutathion peroxidase is decreased by local overdose of Mn. The typical human neurological disorder caused by Mn, manganism, is a Parkinson-like syndrome. Disorders with electrophysiological signs after Mn exposure include myoclonus and epileptic activity. EEG and visual evoked potential alterations were also observed in heavily exposed workers. Mn inhalation in workplace settings is due to presence of aerosol particles containing Mn, including so-called nanoparticles (NPs, with at least one dimension smaller than 100 nm) which are of especial concern in terms of health hazard. NPs are supposed to be involved in various chronic non-communicable diseases of humans. Inhaled NPs are either deposited in the nasopharynx or get down to the alveoli. Their entrance into and mobility within the organism is facilitated by their small size, enabling them to easily penetrate physiological barriers. Their huge and reactive surface induces generation of ROS. The relationship between Mn exposure and neurologic-neuropsychiatric dysfunctions has been established both in massive workplace exposures, and in chronic low-level exposure among workers and in the general public. Motor and olfactory dysfunction, as well as abnormal behavior plus IQ drop, was observed among exposed schoolchildren.

Living organisms have several biochemical mechanisms to protect themselves against endogenous or xenobiotic-induced oxidative stress. The small biomolecules involved are collective called antioxidants (in the strict sense, only exogenous, foodborne factors like vitamin E, vitamin C for primates, flavonoids etc. are called antioxidants). Depending on the actual redox conditions, the same compound can act as a reducing (antioxidant) or an oxidizing (pro-oxidant) agent. The two most important endogenous small antioxidant molecules are glutathione and uric acid, further molecules with antioxidant activity include lipoic acid and melatonin. Exogenous, foodborne antioxidants are micronutrients, required to be present in food in sufficient amounts. Some are considered essential, with known recommended daily intake and known health consequences of insufficient or absent supply. The antioxidants used in the present work belong to this category. Vitamin C or ascorbic acid is an essential factor only for humans, other primates, and a few more mammalians while all other species synthesize it. Ascorbic acid is able to scavenge ROS, improving this way mitochondrial function and protecting membrane lipids. It also recycles oxidized glutathione. Intestinal uptake is limited and apparently depends on the actual need; uptake to the brain is also an active process. Depending on the immediate chemical environment, ascorbate can also have pro-oxidant effect. Vitamin C is a well-known and well-described natural substance, present in various foods and drinks, and recognized as a leading natural nutrient and antioxidant. Rutin (quercetin-3-rutinoside) is a flavonoid present in citrus fruits, rhubarb etc.

It acts both as free radical scavenger and as chelator for metals prone to act as pro-oxidants. Health benefits and pharmacological properties ascribed to rutin include antioxidative, anti-inflammatory, antiallergic, antiviral, anticarcinogenic and anti-hypertensive effects. Bioavailability of rutin is limited due to poor intestinal absorption and degradation by the gut microflora. Neuroprotective effect of rutin has been documented in animal models of brain ischemia and Alzheimer's disease. Green tea flavonoids, first of all catechins, constitute 35–40% weight of fresh leaves of the tea shrub. Green tea is prepared by brewing the harvested but not fermented leaves. Tea is a popular drink worldwide and is a major source of total flavonoid intake in many populations. The neuroprotective effect of catechins is based on antioxidant and metal chelating activity; but they also activate transcription factors and antioxidant enzymes. Regarding the prevalence of chronic degenerative central nervous diseases and the popularity of tea, this protective effect has been extensively studied. Curcumin is a yellow substance was isolated from the rhizomes of turmeric, with multiple pharmacological properties (anti-inflammatory, anti-carcinogenic, anti-mutagenic, anti-ischemic, hypotensive and antioxidant effects). It was found effective in animal models of Alzheimer's dementia and chemically induced neurotoxicity. Curcumin is a powerful ROS scavenger and metal chelator. Being lipophylic, curcumin easily crosses the blood brain barrier. In the last decade, interest in the potential health benefits of exogenous, foorborne, antioxidants has been increasing. Epidemiological studies suggested that long term consumption of diets rich in plant polyphenols can protect against chronic non-communicable diseases including the neurodegenerative ones. Ideas have been published to create “functional drinks” rich in antioxidants. However, the beneficial effects observed in cell cultures and experimental animals have been verified in humans, by now, only to a rather limited extent.

Exposure to metals and metalloids remains a source of health damage for the population. Oxidative stress plays a significant role in the mechanism of metal-induced damages, especially in the nervous system which has limited capacity to counteract an oxidative attack. By supporting antioxidant defence via supplying the exposed organism with exogenous antioxidants, metal-induced nervous system damages could possibly be diminished. The general aim of this thesis was to observe, in experiments done in subacutely treated rats, the neuro-functional damage caused by exposure to arsenic and manganese and the possible protective effect of certain antioxidants of natural origin.

The particular questions to be answered on the basis of the expected results were as follows:

- Can the neurotoxic, and other toxic, effects of the used physicochemical form of As and Mn be investigated in identically built-up experiments or together?
- Have the antioxidants included in the experiments any effect on the alterations induced by the two metals?
- What differences, qualitative or quantitative, can be seen between the effects of the antioxidants?
- Which of the antioxidants might be an optimal choice, considering also protection of human health?

MATERIALS and METHODS

These aims have been realized in three experiments, performed on young adult male Wistar rats. They were treated with Mn and As, and the natural antioxidants ascorbic acid (vitamin C), rutin, curcumin, and green tea brew as given in the table below. With physicochemical form and way of application, imitation of real life human exposure was attempted.

	Experiment 1	**Experiment 2**	**Experiment 3**
Duration, agents, application	4 weeks of MnO_2 NPs, 4 mg/kg b.w. intratracheal then 1 week of antioxidants (ascorbic acid, curcumin, rutin or their vehicles), 100 mg/kg b.w. by gavage	6 weeks As 5 and 10 mg/kg b.w. ($NaAsO_2$,) by gavage; and ascorbic acid, 1 g/L, green tea brew, 2.5 g/500 mL, via drinking fluid	6 weeks As 10 mg/kg b.w. ($NaAsO_2$,) by gavage; or MnO_2 NPs, 4 mg/kg b.w. intratracheal and ascorbic acid, 2 g/L, rutin, 1 g/L, green tea brew, 2.5 g/500 mL, via drinking fluid
Investigations	• Body weight • Organ weight • Open field test • Electrophysiology	• Body weight • Food and water consumption • Organ weight • Open field test • Electrophysiology • Biochemical measurements • Tissue metal level	• Body weight • Food and water consumption • Organ weight • Open field test • Electrophysiology • Biochemical measurements • Tissue metal level

The animals were housed with three or four rats in one cage under GLP equivalent conditions (22±1ºC, 40-60% relative humidity, 12-h light/dark cycle with light on at 06:00), and had free access to standard rodent pellet and drinking fluid (plain tapwater or a treatment solution).

For Experiment 1, MnO_2 NPs were produced at the Department of Physical Chemistry and Materials Science, University of Szeged Faculty of Science and Informatics. Synthesis was done in wet reaction in aqueous alkaline medium containing polyacrylic acid and ethanol (as reducing agent). $KMnO_4$ solution was dripped in, and a sol containing MnO_2 NPs of 25-30 nm diameter was generated. For Experiment 3, the MnO_2 NPs were synthesized at the Department of Applied Chemistry, University of Szeged Faculty of Science and Informatics. Aqueous $KMnO_4$ solution was mixed with ethylene glycol and sonicated with an ultrasound device. The resulting suspension was heated at 200ºC for 16 hour then allowed to cool to room temperature naturally. The precipitate was filtered, washed, and dried at 100 ºC for 1 hour. Chemical purity and particle size (25-30 nm) were checked by electron microscopy and X-ray diffraction. For administration, the MnO_2 NPs were suspended in 1% hydroxyethyl cellulose dissolved in phosphate buffered saline (PBS; pH=7.4). The MnO_2 NPs were instilled intratracheally (it.).

For treatment of the rats with arsenic, crystalline $NaAsO_2$ was dissolved in distilled water and was administered by gavage.

The antioxidants were formulated and applied differently. In Experiment 1, the antioxidants were given by gavage once a day for 1 week; ascorbic acid was dissolved in distilled water while rutin and curcumin were dissolved in sunflower oil. In Experiments 2 and 3, antioxidants were administered via drinking water for 6 weeks. For vitamin C tapwater was boiled and cooled to room temperature to eliminate dissolved chlorine and oxygen. The pH was set to 7.5 by adding $NaHCO_3$ to diminish breakdown of ascorbic acid. In Experiment 3, rutin powder was added to the prepared tapwater (1 g/L) under moderate stirring, then $NaHCO_3$ was added until pH≈9.8 where rutin completely dissolved, finally 20% acetic acid was added to bring back pH to 7.8. In Experiment 2 and 3, green tea infusion was prepared by brewing 2.5 g tea leaves in 500 ml boiled tapwater.

General toxicology and chemical-biochemical measurements: The rats' body weight was registered, as well as their general health state observed, daily in all experiments. Food and water consumption were also measured in Experiment 2 and 3. Drinking fluid consumption was measured to calculate antioxidant intake. After all behavioral and electrophysiological recordings (see below) had been done, the rats were sacrificed by an overdose of urethane, and were dissected. The animals were transcardially perfused with 500 ml PBS to remove

blood from the organs. Organs were removed and weighed, and the relative organ weights were calculated. For metal level determination and biochemical measurements, tissue samples were taken from 3 randomly chosen rats per group. Brain, liver, kidneys, and 2-3 ml of red blood cells were frozen in liquid nitrogen, and stored at -20°C. Level of As and Mn was determined by inductively coupled plasma mass spectrometry after acid digestion of the tissue samples. Indicators of oxidative stress (H_2O_2, $ONOO^-$, TBARS) were determined from cerebral cortex and RBCs, from the same samples as metal level were, in Experiment 2 and 3.

Behavioral investigation: The rats' spontaneous motility was tested in an open field (OF) apparatus (Conducta 1.3). The OF test was usually done on the day following the last treatment (in Experiment 1, also one week before the end of treatment period).

The OF box was equipped with infrared beam gates at floor level and at 12 cm height. From the beam interruptions, event counts and summed time of the basic activity forms (ambulation, local activity, rearing, immobility), as well as run length of ambulation, were computed.

Electrophysiological investigation: On the day following the last OF test, the rats were anesthetized with urethane (1000 mg/kg b.w. ip.), and the left hemisphere was exposed by removing the soft tissues and temporal bone along the inner circumference. After at least 30 min recovery, the rat was placed into the stereotaxic frame of the electrophysiological apparatus. Ball-tipped silver recording electrodes were positioned on the dura over the primary somatosensory (SS) projection area of the whisker pad (barrel field), and over the primary visual (VIS) and auditory (AUD) area. SS stimulation was done by delivering square electric pulses into the contralateral whisker pad. VIS stimuli were flashes to the contralateral eye of the rat, and for acoustic stimulation, clicks were applied into the ear of the animal. Compound action potentials (CAP) of the tail nerve were also recorded. The recording sequence started with six minutes recording of spontaneous activity (electrocorticogram, ECoG) from the three sensory cortical areas. From that, the relative spectral power by frequency bands was determined. Evoked potentials (EPs) were recorded from the same cortical areas via the same surface electrodes. EPs and CAPs were averaged automatically, and latency and duration of the responses was measured manually. The complete recording and evaluation was done by the software NEUROSYS 1.11.

Statistical evaluation: The distribution of data was checked for normality by means of the Kolmogorov-Smirnov test. Analysis was done by parametric one-way ANOVA using SPSS 17.0. Post hoc analysis of group differences was performed by Scheffe's test, with probability

level at $p<0.05$. Linear correlations between tissue metal levels and neurotoxicological parameters were checked by the "linear fit" function of MS Excel.

The procedures used in the experiments were approved by the Ethical Committee for the Protection of Animals in Research of the University (licenses No. XXI./02039/001/2006 and XXI./151/2013)

RESULTS and CONCLUSION

Both As and nano-Mn caused reduced body weight gain. This was paralleled partly by reduced food intake in As treatment (Experiment 2 and 3) but almost fully in Mn treatment (Experiment 3). Protective effect of the used antioxidants against the metals' action of body weight was weak or absent, with rutin showing the relatively clearest effect. In rats instilled with MnO_2 NPs (Experiment 1 and 3) relative weight of the lungs increased massively and this was not influenced by the antioxidants. Else, only the weight of the adrenals reacted on As and Mn exposure, with an inconsistent increase which was weakly influenced by the antioxidants. Elevated As level was measured in the treated rats' red blood cell, cortex, liver and kidney samples (strongest in the latter organ). Antioxidants reduced As deposition but only moderately. In Experiment 3, also Mn was deposited in the tissues but the antioxidants had no effect on that.

In the OF test, the antioxidants alone had no effect on parameters of motility. As reduced motility slightly in Experiment 2 but more significantly in Experiment 3, with increased local activity and immobility but decreased rearing. The OF effect of Mn was similar (Experiment 1 and 3). Green tea had minimal influence on the OF effect of either As or Mn. In Experiment 3, rutin counteracted the effect of As more clearly than that of Mn while ascorbic acid influenced only the OF effect of Mn. Rutin also reversed the Mn-dependent increased preference of the rats to the corner zones of the OF box in Experiment 1.

Electrophysiological effects: The band spectrum of the electrocorticogram was influenced only by nano-Mn exposure, not by As. The shift to higher frequencies was moderate in Experiment 1 but more pronounced in Experiment 3. The decrease of low-frequency activity was abolished more strongly by green tea than by rutin but not at all by ascorbic acid. There were more marked changes in the parameters of evoked activity.

Latency of the cortical evoked potentials was negligibly influenced by the antioxidants alone. Administration of Mn NPs caused significant increase of the latency in each modality, which effect was counteracted by rutin and green tea, less strongly by ascorbic acid, but not at all by curcumin. The effect of As, and the counter effects of the antioxidants on that, was similar. Slowing of the conduction velocity in the tail nerve, seen on treatment with both As and Mn, was counteracted most strongly by rutin, less by green tea and only weakly by ascorbic acid. In Experiment 1, curcumin also had a weak protective effect against decrease of conduction velocity by Mn.

Biochemical effects: The intensity of TBARS reaction and level of $ONOO^-$ were altered by exposure to As and Mn, and by antioxidant application, in a way which showed effect and counter effect. The effect of As was most clearly reversed by rutin, and that of Mn, by ascorbic acid. The causal relationship between inner metal doses (tissue As and Mn levels) and the observed neuro-functional and biochemical changes was tested searching for correlations. In Experiment 2 cortical and peripheral electrophysiological changes, and cortical TBARS level, was significantly correlated to cortical As concentration. In Experiment 3, body weight gain was correlated to liver level of As or Mn, while certain electrophysiological and behavioural parameters, and TBARS, to cortical metal levels but also to each other. The effects of As, nano-Mn, and the antioxidants are summarized in the table in the next page (Legend: Single arrow, increase/decrease; double arrow, significant increase/decrease; ⊕, ∅, effect or counter effect present/absent. Rut, rutin; VitC, ascorbic acid; Kur, curcumin; Tea, green tea brew.)

In the investigated functional alterations of the treated rats' nervous system, there were some general trends, such as decreased OF motility or slowed electrophysiological responses, indicating possible common mechanisms in the background. Oxidative stress may be one such mechanism, as both As and Mn, as inorganic chemical agents, are know to induce the generation of reactive oxygen species in living tissue, and such effect has been described also in exposed humans. The negative effect of oxidative stress on CNS functionality has also been reported for both metals.

		As	Counter effects of the antioxidants	nano-Mn	Counter effects of the antioxidants
Exp. 1	Body weight gain			⇓	⊕ weak: Rut>VitC ∅ Kur
	Organ weights			Lungs ⇑ Adrenals↑	∅
	OF motility			Local act.↑ Immobility ⇑ Rearing ⇓	⊕ Rut ∅ VitC, Kur
	ECoG			Frequency shift to higher ↑	∅
	EPs			Latency ⇑	⊕ Rut>VitC ∅ Kur
	CAP tail nerve			Cond. vel. ⇓ Rel. refr. ⇑	⊕ Rut>Kur ∅ VitC
	Correlations			SS EP lat., OF rearing time to Body weight gain	
Exp. 2	Body weight gain	⇓	∅		
	Organ weights	∅			
	OF motility	Local, immob. ↑	⊕ partial: VitC, Tea		
	ECoG	∅	---		
	EPs	Latency ⇑	⊕ Tea>VitC		
	CAP tail nerve	Cond. vel. ⇓	⊕ Tea>VitC		
	Metal level	Cortex ⇑ Liver ↑ Kidney ⇑	∅		
	Oxidative stress: TBARS	RBCs ⇑ Cortex ⇑	⊕ Tea ∅ VitC		
	Correlations	SS EP lat, Cond. vel., Cortex TBARS to Cortex As level			
Exp. 3	Body weight gain	⇓	⊕ Rut>Tea>VitC	⇓	∅
	Organ weights	Adrenals ⇑	⊕ Rut	Adrenals ⇑ Liver ⇓	⊕ Rut weak effect
	OF motility	Local, immob. ⇑ Rearing ⇓	⊕ Rut ∅ Tea, VitC	Local, immob. ⇑ Amb. ⇓	⊕ VitC>Rut
	ECoG	∅	---	Freq. shift to higher ⇑	⊕ Tea>Rut ∅ VitC
	EPs	Latency ⇑	⊕ Rut~Tea>VitC	Latency ⇑	⊕ Rut~Tea>VitC
	CAP tail nerve	Cond. vel. ⇓	⊕ Rut>Tea>VitC	Cond. vel. ⇓	⊕ Rut~Tea>VitC
	Metal level	Cortex ⇑ Liver ⇑ Kidney ⇑	⊕ Rut>Tea~VitC ⊕ Rut>Tea>VitC ∅	Cortex ⇑ Liver ⇑	∅
	Oxidative stress: TBARS	RBCs ↑ Cortex ↑	⊕ Rut, Tea ⊕ Rut, Tea	RBCs ⇑ Cortex ↑	∅ ⊕ Tea, VitC
	Correlations	Body weight gain to Liver As; OF local act., SS EP lat., Cortex TBARS to Cortex As; OF local act. to Cortex TBARS		Body weight to Liver Mn; OF amb., SS EP lat., Cortex TBARS to Cortex Mn; OF amb. to Cortex TBARS	

The antioxidants tested in this study had some counter effect on the electrophysiological and/or behavioral alterations induced by As and nano-Mn, but to a dissimilar extent. The effect of ascorbic acid was in most cases less than that of rutin or green tea brew, although the measured antioxidant capacity of ascorbic acid solution was higher. Protective actions, independent of direct reduction of oxidized biomolecules, of rutin and green tea flavonoids include first of all chelation of metal ions.

All antioxidants tested are natural, foodborne compounds potentially available for general use in populations exposed to As or Mn, but also to other environmental toxicants, but the real life efficacy of this kind of preventive measures has not yet been verified, so the ideas like "functional drinks in neurodegenerative diseases" still await implementation.

The particular questions of the study can be answered as follows:

- In Experiment 3, the used physicochemical form of As and Mn were investigated in terms of neurotoxicity and general toxicity together in the same experiment. Identical but separate experiments with the two metals were not made, but the answer to the first question is essentially positive.
- The antioxidants included in the experiments had some clearly detectable effects on the alterations induced by the two metals, regarding both nervous system effects (electrophysiological and behavioral) and general toxicity, but:
- There were marked differences between the effects of the antioxidants: 1/ Curcumin had practically no effect. 2/ The protective effect of vitamin C was weaker than that of the applied flavonoids – rutin and green tea constituents. The cause of the difference was apparently not the antioxidant capacity but probably the metal chelating ability of the flavonoids and/or their better local availability in the CNS.
- The examined antioxidants are all easily available natural compounds. As a chemically defined compound, rutin showed better effect than ascorbic acid. Green tea infusion (or an extract or concentrate) is chemically complex but the main constituents have been chemically identified. An "optimal" choice of antioxidant has to consider, beyond verified in vivo protective effect, also technical (sources, processing, formulation) and social (acceptance in the population to be protected) aspects, and requires further studies both within and outside the scope of environmental neurotoxicology.

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