EFFECTS OF THREE NATURAL ANTIOXIDANTS ON THE GENERAL AND NERVOUS SYSTEM TOXICITY OF MANGANESE NANOPARTICLES IN RATS

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ABSTRACT: Nanoparticles are frequently present in the occupational and residential environment, and affect health by several mechanisms including induction of oxidative stress and toxicity of the particles' constituents. Manganese content of the studied nanoparticles is likely to increase oxidative stress which is especially harmful to neurons due to their high energy demand and to the sensitivity of membrane lipids. Rats were treated for 4 weeks with intratracheally applied MnO_2 nanoparticles, and for further 1 week with the natural antioxidants ascorbic acid, curcumin and rutin. In the nanoparticle-treated rats, body weigh gain was retarded, motility in the open field apparatus was reduced, cortical evoked potential was delayed and nerve conduction velocity slowed. Rutin counteracted all these effects, while ascorbic acid was effective only on the cortical evoked potential, and curcumin showed no corrective action on the effects of MnO_2 nanoparticles. Antioxidants might have a role in the prevention or treatment of nervous system damage caused by Mn but the choice of substance and dose needs further experimental work.

KEY WORDS: antioxidants, manganese, neurotoxicity, rat

INTRODUCTION

The presence of nanoparticles (NPs) in the occupational and residential environment and their negative health effects have been more and more recognized in the last two decades (Oberdörster et al., 2005). In high-temperature industrial procedures involving metals, such as casting or welding, metal-containing NPs are typically produced. In welding fumes, the nanoparticulate form of manganese (Mn) and its oxides or other compounds are typically present (Antonini, 2003) and their etio-

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Abbreviations:
NPs = nanoparticles
Mn = manganese
ROS = reactive oxygen species
OF = open field
SS = somatosensory
EPs = evoked potentials

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logical role in workplace-acquired neurological diseases, including a Parkinson-like syndrome, has been described (Bowler et al., 2006).

Oxidative stress is apparently one of the main features of the interaction between NPs and living cells. The (disproportionately large) surface of the NPs with a wide spectrum of chemical components is generating reactive oxygen species (ROS) in the extra- or juxtacellular space; while after being internalized the NPs tend to be accumulated in mitochondria and disturb their function. The chemical components of the NPs can have their own oxidative stress generating capacity like in the case of transition metals including Mn (Oberdörster et al., 2005; Flora et al., 2008). The toxic mechanisms of Mn involve ROS generation because of its capacity to redox cycling and Fenton reaction (Jomova and Valko, 2011) and because of mitochondrial damage, more exactly inhibition of complex II (Malecki, 2001) and complex III (Zhang et al., 2003).

Oxidative stress is especially deleterious to nerve cells because of their high rate of oxidative energy production, and because of the numerous functions bound to the neuronal membrane and hence to the integrity of oxidation sensitive membrane lipids. This, and the oxidative stress caused by NPs as described above, suggested that the nervous system effects in rats treated with various NPs, seen in previous experiments (Sárközi et al., 2008; Oszlánczi et al., 2010; Horváth et al., 2012) might be partly due to excessive oxidation. In the present work it was tested whether certain compounds of herbal origin, known to have antioxidant properties, can reduce or abolish the neuro-functional damages induced by application of Mn-containing NPs to rats.

MATERIALS AND METHODS

Animals and treatment

Young adult male Wistar rats (7 weeks old, weighing 220 ± 15 g) were obtained from the breeding centre of the university and were housed in a GLP-rated animal house (22 ± 1°C, 30–60% relative humidity, 12-h light/dark cycle with light on at 06:00), with free access to tap water and standard pellet. There were 8 rats in each treatment group (see *Table 1*) except the untreated control (6 rats).

To observe the general and neurological effects of Mn NP exposure, the rats in groups *Mn*, *Mn+VitC*, *Mn+Cur* and *Mn+Rut* were treated by intratracheal instillation of the NP suspension for 4 weeks as described in *Table 1*. The effects themselves, and that they are reliably induced during this exposure period, were known from previous research (Oszlánczi et al., 2010; Horváth et al., 2012). Then three antioxidants, vitamin C (ascorbic acid), curcumin, and rutin, were administered orally by gavage for another week to see if they can influence, or counteract, the effects of Mn NP exposure.

Vitamin C is a major natural antioxidant and cofactor in various enzymatic reactions, acting as a one-electron donor. In the brain, these functions of ascorbic acid, together with its role in catecholamine synthesis and anti-excitotoxic effect, are of especial importance (Harrison and May, 2009).

TABLE 1. Treatment groups and doses

Group	*Code*	*Dose and application*	*n*
Untreated control	*Con*	None	6
Vehicle control 1 (viscous medium, intratracheal application)	*VCon*	Viscous suspension medium: 1.7% polyacrylic acid (MW= 5000) in saline, pH=7.5, applied once daily by intratracheal instillation, 5 days a week for 5 weeks	8
Vehicle control 2 (oil, oral application)	*OCon*	Sunflower oil (1 ml/kg b.w.), once daily by gavage, 5 days a week for 5 weeks	8
Mn nanoparticles	*Mn*	MnO_2 nanoparticles, 4 mg/kg b.w., suspended in the viscous medium, applied once daily by intratracheal instillation, 5 days a week for 5 weeks	8
Vitamin C	*VitC*	Ascorbic acid, 100 mg/kg b.w., dissolved in distilled water, applied once daily by gavage, 5 days a week for 5 weeks	8
Curcumin	*Cur*	Curcumin, 100 mg/kg b.w., dissolved in sunflower oil (1 ml/kg b.w.), applied once daily by gavage, 5 days a week for 5 weeks	8
Rutin	*Rut*	Rutin, 100 mg/kg b.w., dissolved in sunflower oil, applied once daily by gavage, 5 days a week for 5 weeks	8
Mn nanoparticles + vitamin C	*Mn+VitC*	Mn nanoparticles for 4 weeks, then ascorbic acid for 1 week, doses and application as above	8
Mn nanoparticles + curcumin	*Mn+Cur*	Mn nanoparticles for 4 weeks, then curcumin for 1 week, doses and application as above	8
Mn nanoparticles + rutin	*Mn+Rut*	Mn nanoparticles for 4 weeks, then rutin for 1 week, doses and application as above	8

Curcumin is the main active ingredient of turmeric, and rutin is found e.g. in citrus fruits, apples, rhubarb and cranberries. Both belong to the flavonoid group of phytochemicals, and are known to enter redox reactions within the animal or human organism with the potential of reducing oxidative stress. Curcumin was found to scavenge hydroxyl and superoxide radicals (Kunchandy and Rao, 1990). With quercetin, the aglycone of rutin, oxidative damage and cognitive impairment in aluminium-treated rats could be reduced (Sharma et al., 2013).

MnO_2 NPs for intratracheal administration were synthesized (at the Department of Physical Chemistry and Materials Science) by wet reaction in aqueous alkaline medium containing polyacrylic acid (PAA; MW 5000) and ethanol (as reducing agent). Stoichiometric amount of $KMnO_4$ solution was dripped into this medium under stirring, and a sol containing MnO_2 NPs of 10-20 nm diameter was generated. It was administered to rats after adjustment of concentration and pH. For vehicle control, the starting medium was completed with KOH and NaOH, and pH was set to 7.5. The chemical purity of the nanoparticles was checked by X-ray diffraction, and their particle size, by X-ray diffraction and transmission electron microscopy.

General toxicology

The rats' body weight was measured every workday, and weekly weight gains (presented in *Fig. 1*) were calculated Monday to Monday. After the electrophysiological recording (described below) the rats were sacrificed with an overdose (2 g/kg b.w.) of urethane and were dissected. The weight of the brain, heart, lungs, liver, kidney, adrenals and thymus was measured and relative organ weights, related to brain weight, were calculated (brain weight being a more stable calculation basis than body weight, as shown by our data and stated in Schärer, 1977).

Behavioural investigation

The rats' spontaneous motility was tested in an open field (OF) apparatus (Conducta 1.3 System; Experimetria, Budapest) after the 4^{th} and the 5^{th} (final) week of treatment. The OF box used was of 48×48×40 cm size and was equipped with two arrays of infrared beam gates at floor level and at 12 cm height. The animals were put into the centre of the OF box one by one for a 10 min session. From the beam interruptions caused by the rat moving around, event counts and summed time of the basic activity forms (ambulation, local activity, rearing, immobility), as well as run length of ambulation, were computed as follows: more than 40 mm shift in the location of interrupted beams at the floor level during a time unit of 1 s was interpreted as horizontal activity; less shift, as local activity; and no shift at all, as immobility. Rearing was recorded if beams at both floor level and higher level were interrupted simultaneously. In earlier works (Vezér et al., 2007) this method was found sensitive to metal-induced changes of motor activity.

Electrophysiological investigation

On the day following the 5th week OF session, the rats were prepared for electrophysiological recording in urethane (1000 mg/kg b.w. ip.; Maggi and Meli, 1985) anaesthesia. The left hemisphere was surgically exposed (lidocaine spray was applied on the wounds) and spontaneous electrical activity (electrocorticogram, ECoG) was recorded from the primary somatosensory (SS) areas for 6 minutes using a ball-tipped silver wire surface electrode. From this, band spectrum according to the standard human EEG bands (delta to gamma; Kandel and Schwartz, 1985) was calculated. Then, evoked potentials (EPs) were recorded from the same site by applying electric square pulses (3-4 V; 0.05 ms) to the contralateral whisker pad, through a pair of needles. One train of 50 stimuli each was applied with 1, 2 and 10 Hz frequency. Onset latency of the EPs was measured after averaging the 50 individual records. From the tail nerve, compound action potential was recorded by inserting a pair of needle electrodes at the base of the tail for stimulation, and another pair 50 mm distally for recording. Conduction velocity was calculated from this distance and the onset latency of the action potential. Relative refractory period was measured by double stimuli with 1–10 ms inter-stimulus interval, from the extra delay of the second potential. The complete electrophysiological work was performed by means of the software Neurosys 1.11 (Experimetria Ltd., Budapest, Hungary).

During the whole study, the principles of the Ethical Committee for the Protection of Animals in Research of the University were strictly followed. The methods used in the experiments were licensed by the authority competent in animal welfare issues under No. XXI./02039/001/2006.

Statistical analysis

Group differences were analyzed by one-way ANOVA and post hoc Scheffe's test, with $p<0.05$ as limit of significance. The possible linear correlation between data sets was tested by the "linear fit" function of MS Excel.

RESULTS

General toxicity

Body weight gain was practically identical in the untreated and treated controls and was not significantly influenced by the antioxidants given alone (*Fig. 1*). In the Mn NP treated rats (group *Mn*), however, there was a significant reduction of body weight gain already after one week of treatment. This effect of Mn, a sign of general toxicity, was not influenced by vitamin C and curcumin (*Mn+VitC*, *Mn+Cur*), but in the group *Mn+Rut* body weight gain was nearly normal.

The relative organ weights also showed that the effect of Mn NP exposure could be reduced by the antioxidants studied but the changes, except for relative weight of the lungs, were not significant.

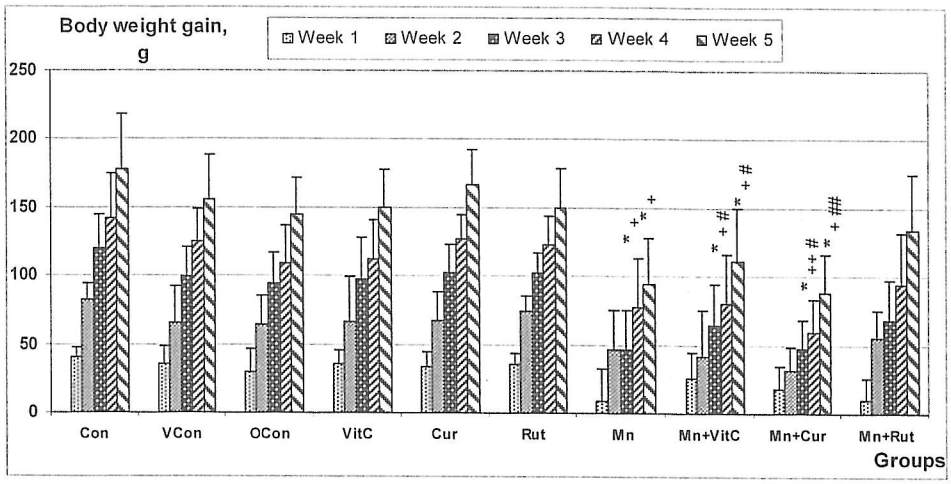

Fig. 1. Body weight gain of the control and treated rats during the five weeks of the experiment. The values are group means (+SD) of the individual weight gains, starting from the day preceding the first administration
**: $p<0.05$ vs. VCon; $^{+}$, $^{++}$: $p<0.05$, 0.01 vs. OCon; $^{\#}$, $^{\#\#}$: $p<0.05$, 0.01 vs. value of the corresponding antioxidant given alone (significance for the first 3 weeks is omitted in order not to make the graph too crowded*

Open field behaviour

The distribution of the summed time spent with ambulation (locomotion), local activity (grooming etc.), immobility and rearing was, at the end of the 4th week, nearly identical in the controls and in the groups receiving the antioxidants alone (*Fig. 2*). In the Mn-treated groups, however (*Mn*, *Mn+VitC*, *Mn+Cur*, *Mn+Rut*; all these received only Mn NP treatment in the first 4 weeks) decreased rearing was already observed. After the 5th week, the effect of Mn was more pronounced (decreased ambulation was also seen), and the effects of Mn were largely reversed by the applied dose of rutin, but not by that of vitamin C or curcumin.

Electrophysiology

The antioxidants alone had no effect on the latency of SS EPs (*Fig. 3*). Treatment with Mn NPs for five weeks (group *Mn*) caused a significant latency increase and made also the frequency dependence of latency more intense. Curcumin had no influence on the effect of Mn but rutin and vitamin C brought the latency values back nearly to control level.

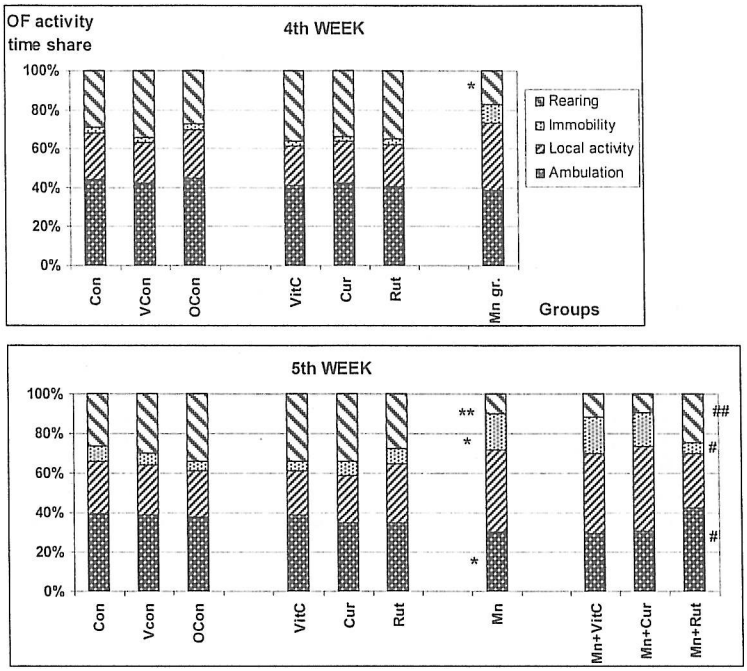

Fig. 2. Time spent by the rats in the activity forms indicated after the 4th (top) and 5th (bottom) week. Group mean values, n=8 (6 for Con). Mn gr. is the grand total of all groups receiving Mn NP treatment in the first 4 weeks
*, **: $p<0.05$, 0.01 vs. VCon; #, ##: $p<0.05$, 0.01 vs. Mn

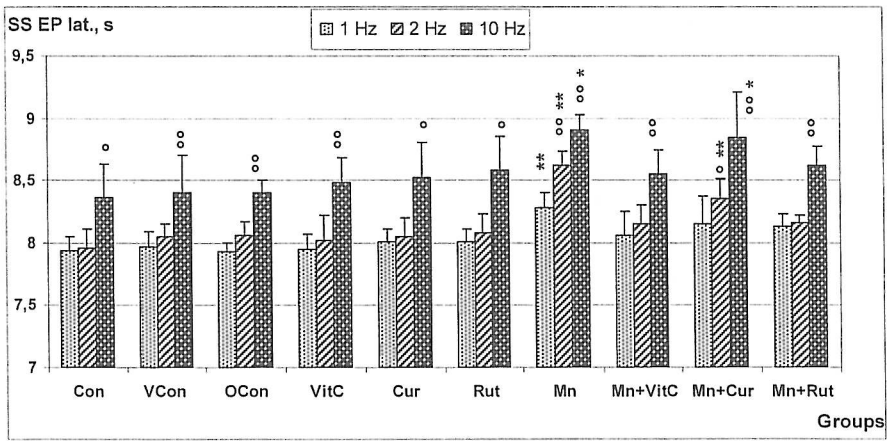

Fig. 3. Latency of the somatosensory evoked potential. Group mean + SD, n=8 (6 for Con). Insert: frequency of stimulation
*, **: $p<0.05$, 0.01 vs. VCon; °, °°: $p<0.05$, 0.01 vs. 1 Hz stimulation in the same group

The changes of the tail nerve parameters, conduction velocity and relative refractory period, were highly similar to those of the SS EP (*Fig. 4*).

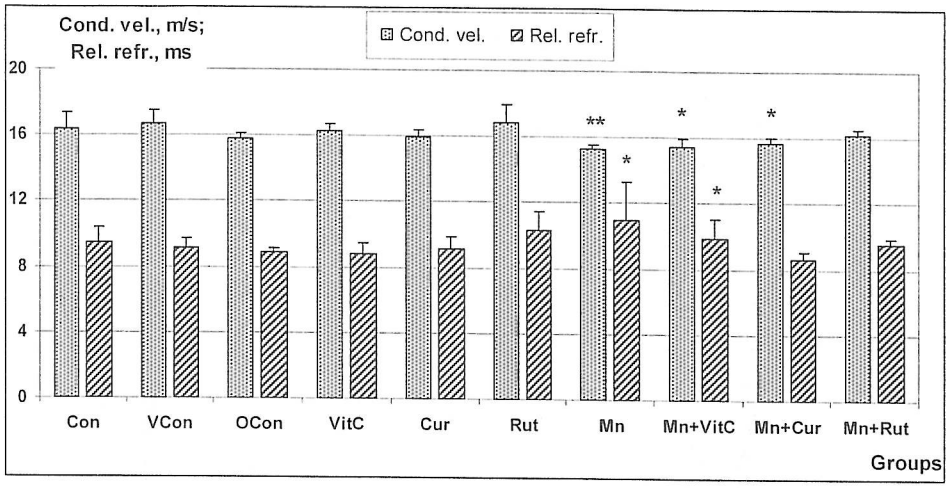

Fig. 4. Conduction velocity and relative refractory period (see insert) of the tail nerve Group mean + SD, n=8 (6 for Con)
**, **: p<0.05, 0.01 vs. VCon*

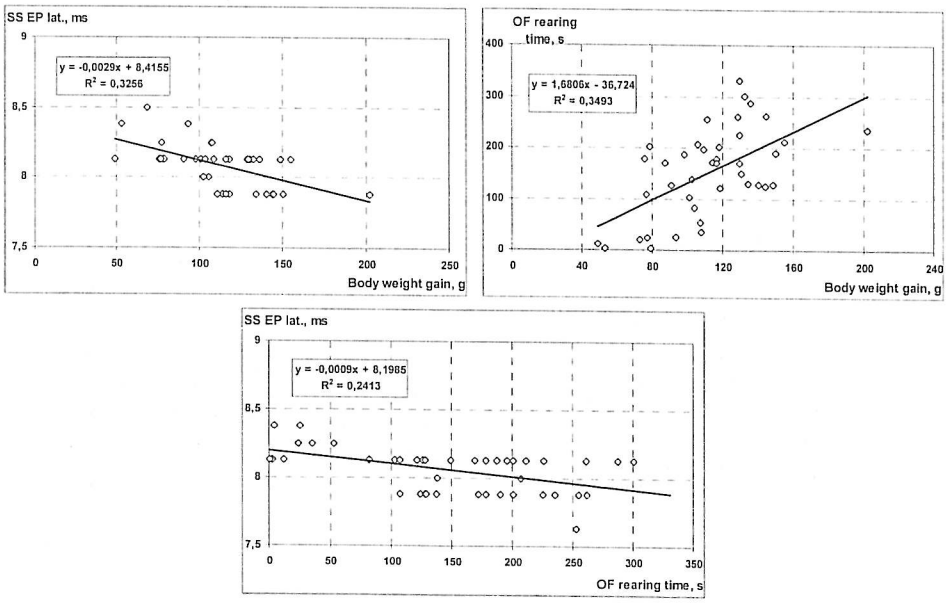

Fig. 5. Correlation between body weight gain and SS EP latency (top left) and OF rearing time (top right), and between the two neuro-functional parameters (bottom)

Correlations

The effects of the various treatments on body weight, OF motility and EP latency had apparently the similar trend, suggesting common mechanisms in the background. As a further proof of this, the correlation of these parameters was tested as described in Methods. As seen in *Fig. 5 (on previous page)*, the correlation between body weight gain in the last 4 weeks (including 3 weeks Mn NP exposure and 1 week of antioxidant treatment) with the neuro-functional parameters OF rearing time and SS EP latency was fair, which argued for all three being caused by the treatments applied.

DISCUSSION

The significant alterations in the parameters of OF motility and SS EP latency, and also in body weight gain, in the Mn NP-treated rats indicated that the toxic effects of the NPs in fact took place. The present experiment did not include metal level determination in tissue samples, but in previous comparable experiments (Oszlánczi et al., 2010) significantly elevated Mn levels (represented by the NPs and/or by dissolved Mn^{2+}) in brain and other tissue samples were measured.

In the literature, several mechanisms have been suggested for the general and organ-specific toxicity of Mn, one being the induction of oxidative stress via mitochondrial inhibition and redox cycling (Erikson et al., 2004). The ability of Mn-containing welding fumes to induce oxidative stress was proven by McNeilly et al. (2004). There is also increasing body of evidence on the role of oxidative damage in the pathomechanism of chronic CNS diseases – among them Parkinson's disease, to which manganism, the human syndrome caused by chronic Mn exposure, bears high resemblance (Aschner et al., 2009). In both, the proper function of dopaminergic motor control is being lost. Dopaminergic neurons are especially vulnerable to oxidative stress due to the auto-oxidizing tendency of dopamine and to the presence of monoamine oxidase producing hydrogen peroxide (Alexi et al., 2000). Decreased motility of the Mn NP-treated rats in the present experiment, first of all decreased vertical activity (rearing) indicated dopaminergic damage (Sedelis et al., 2001).

The effects which may be responsible for the observed electrophysiological alterations, and are related to oxidative stress, include first of all membrane damage. ROS generation and membrane lipid peroxidation was seen in rats after oral Mn exposure (Avila et al., 2008). Damage to these important functional units of the cell membrane results, in turn, in changes of fluidity and probably in altered membrane-bound functions including action potential propagation and synaptic transmission. Impaired spike propagation was reflected in the slowed conduction velocity in the treated rats' tail nerve and in the increased cortical evoked potential latency. The latter was also probably influenced by the damage done by ROS to the astrocytic glutamate transporter (Erikson et al., 2004). Generally, it is more and more accepted that oxidative stress plays a role in the pathomechanism of various CNS

disorders and that antioxidants may have a role in their prevention and therapy (Pandya et al., 2013).

Ascorbic acid (Vitamin C, an essential nutrient for humans), is actively accumulated in brain tissue to a level well-regulated against decreased or increased external supply, and acts there both as antioxidant and as cofactor in the synthesis of, e.g., catecholamines (Harrison and May, 2009). It has been supposed that insufficient cellular antioxidant defence, among others by vitamin C, promotes the neurotoxic effect of Mn (Desole et al., 1995), and the lipid protecting effect of vitamin C *in vivo* has been described (Chen et al., 2000). One could expect that external ascorbic acid increases the supply for the transporter and helps replenish the amount consumed in the brain for neutralizing the oxidative effects of Mn, and may so counteract Mn-induced functional alterations. However, such an effect was seen – with the dose applied in the present work – only on the SS EP latency (and slightly on the body weight gain, not directly related to nervous system effects).

Flavonoids are exogenous for animals and humans but their antioxidant effect can be considerable compared to ascorbic acid (Yen et al., 2002). They are among the major groups of phytochemicals which are known or supposed to protect against oxidative stress caused by xenobiotics, and their potential in prevention and therapy is intensively investigated. Quercetin (the aglycone of rutin) was found to be neuroprotective in PCB-treated rats (Bavithra et al., 2011). With curcumin, Agarwal et al. (2010) could diminish the level of Hg and oxidative stress indicators in acutely Hg-treated rats. Chtourou et al. (2010) used silymarin to reduce the oxidation-related biochemical changes in the brain of rats receiving Mn orally. In case of oxidative stress caused by transition metals (Mn is one of them) the metal chelating ability of flavonoids (curcumin: Daniel et al., 2004; rutin: Valko et al., 2006; general review: Flora et al., 2008) can add to the true antioxidant reactivity in counteracting metal-induced oxidative stress.

Why then curcumin had finally no effect in our work remains an open question, but it might be related to lack of water solubility and low bioavailability (Fan et al., 2013). Rutin, in contrast, has also water solubility which might have facilitated its absorption, as stated in Andlauer et al. (2001).

The results presented above indicate that antioxidants might have a role in the prevention or treatment of nervous system damage caused by Mn but the choice of substance and dose needs further experimental work.

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