Biometals (2010) 23:985–996
DOI 10.1007/s10534-010-9345-x

Silymarin, a natural antioxidant, protects cerebral cortex against manganese-induced neurotoxicity in adult rats

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Received: 21 January 2010 / Accepted: 12 May 2010 / Published online: 26 May 2010

Abstract Manganese (Mn) is an essential element for biological systems, nevertheless occupational exposure to high levels of Mn can lead to neurodegenerative disorders, characterized by serious oxidative and neurotoxic effects with similarities to Parkinson's disease. The aim of this study was to investigate the potential effects of silymarin (SIL), an antioxidant flavonoid, against manganese chloride induced neurotoxicity both in vivo (cerebral cortex of rats) and in vitro (Neuro2a cells). Twenty-eight male Wistar rats were randomly divided into four groups: the first group (C) received vehicle solution (i.p.) served as controls. The second group (Mn) received orally manganese chloride (20 mg/ml). The third group (Mn + SIL) received both Mn and SIL. The fourth group (SIL) received only SIL (100 mg/kg/day, i.p.). Animals exposed to Manganese chloride showed a significant increase in TBARS, NO, AOPP and PCO levels in cerebral cortex. These changes were accompanied by a decrease of enzymatic (SOD, CAT, GPx) and non-enzymatic (GSH, NpSH, Vit C) antioxidants. Co-administration of silymarin to Mn-treated rats significantly improved antioxidant enzyme activities and attenuated oxidative damages observed in brain tissue. The potential effect of SIL to prevent Mn induced neurotoxicity was also reflected by the microscopic study, indicative of its neuroprotective effects. We concluded that silymarin possesses neuroprotective potential, thus validating its use in alleviating manganese-induced neurodegenerative effects.

Keywords Manganese toxicity · Cerebral cortex · Oxidative stress · Silymarin

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Introduction

Mn is an essential trace element required for normal growth, development, cellular homeostasis and many ubiquitous enzymatic reactions involved in the neurotransmitter synthesis and metabolism (Aschner and Aschner 2005; Pine et al. 2005; Golub et al. 2005; Dobson et al. 2004). Although small amounts of Mn are a nutritional necessity for normal brain functioning, it has been considered as an environmental neurotoxic at high doses (HaMai and Bondy 2004; Mergler et al. 1999; Pamphlett et al. 2001). This

metal is widely used in pesticide formulations (Belpoggi et al. 2002), in water purification as bactericide and fungicide agents and as an antiknock agent in gasoline (Zayed et al. 1999). According to previous findings, excess exposure to high levels of Mn in occupational or environmental settings and therapeutic or disease conditions (Krieger et al. 1995; Spahr et al. 1996; Layrargues et al. 1998) leads to excessive Mn accumulation in the nervous system (Erikson et al. 2007). Thus induces a decrease in dopamine (DA) levels and cell death, syndrome commonly referred to manganism (Barbeau 1984; Sloot et al. 1994).

The cellular and molecular mechanisms of Mn neurotoxicity are not well understood. Generally, Mn is alleged to exert cellular toxicity via a number of mechanisms, including a direct or an indirect formation of reactive oxygen species (ROS) (Ali et al. 1995; Brouillet et al. 1993; Milatovic et al. 2007), oxidation of biological molecules (Archibald and Tyree 1987), and the disruption of Ca^{2+} and iron homeostasis (Gavin et al. 1990; Kwik-Uribe et al. 2000; Zheng and Zhao 2001). An imbalance between ROS generation and the antioxidant defense mechanisms with subsequent oxidative stress (Betteridge 2000) can initiate apoptosis and/or necrosis in several tissues (Orrenius et al. 2007). Oxidative stress is in fact one of the putative mechanisms by which Mn induces neuronal damages (Dukhande et al. 2006). Efforts have been made to minimize the severity of manganese toxicity via its enhanced sequestration and elimination using different agents. Considering the relationship between manganese exposure and oxidative stress, it is reasonable that administration of some antioxidant and natural biomolecules should be an important therapeutic approach in manganese intoxication. To our knowledge, the effect of silymarin against Mn-toxicity constitutes nowadays the first study.

Silymarin (SIL), is one of the most frequently studied bioflavonoid in the class of flavonols. It is a polyphenolic flavonoid antioxidant isolated from the fruits and seeds of milk thistle *Silybum marianum* (L.) containing a mixture of several flavonolignans such as silibinin, isosilibinin, silidianin and silichristin (Valenzuela and Garrido 1994). Silymarin has been used clinically in alcoholic disease (Saller et al. 2001). It is also used against various hepatotoxicants including carbon tetrachloride, concanavalin A (Schümann et al. 2003) and aflatoxin B1 (Rastogi et al. 2000). Various studies also indicate that silymarin exhibits cancer preventive effects (Zi et al. 1998; Bhatia et al. 1999). Moreover, silymarin possess number of additional biological effects, such as an antioxidative activity (Valenzuela et al. 1986), anti-inflammatory effects (Manna et al. 1999) and an inhibitory action on tumor necrosis factor-α (TNFα) expression (Zi et al. 1997). Although, a number of compounds, such as trolox and *N*-acetylcysteine have been used to prevent manganese-induced toxicity in vitro and in vivo (Marreilha dos Santos et al. 2008; Hazell et al. 2006), no studies yet were interested to evaluate the protective effects of silymarin antioxidant flavonoid against Mn-induced neurotoxicity and brain damages. Thus, the aim of this study was to determine first whether changes in the activities of antioxidant enzymes occurred in the cerebral cortex of rats exposed to manganese and second to determine if such changes were associated with oxidative stress by means of lipid peroxidation, protein oxidation and peroxide hydrogen analysis. Furthermore, we have also evaluated whether co-administration of silymarin could modulate these parameters, in vivo and in vitro, and thereby rendered protection to the brain during manganese exposure.

Materials and methods

Chemicals and reagents

Silymarin and all other chemicals, required for all biochemical assays, were obtained from Sigma Chemicals Co. (St. Louis, France).

Animals

Twenty-eight male rats of Wistar strain weighing 190 ± 23 g were obtained from Central Pharmacy (SIPHAT, Tunisia). They were fed pellet diet, purchased from the Industrial Society of rodents diet (SICO, Sfax, Tunisia). Diet composition was detailed by Fetoui et al. (2006, 2007). All animal procedures were conducted in strict conformation with the local Institute Ethical Committee Guidelines for the Care and Use of laboratory animals of our Institution.

Experimental design

After 1 week of acclimatization in a room with controlled temperature (22 ± 3°C) and lighting (12-h light/dark cycle), rats were randomly divided into four groups of seven animals each: The first group of rats served as the control, received ad libitum distilled water and 0.5 ml vehicle solution of silymarin given daily by intraperitoneal (i.p.) injection. The second group (Mn) received, through drinking water, a solution of manganese chloride (20 mg/ml corresponding to 100 mM of Mn^{2+} (Calabresi et al. 2001; Morello et al. 2007). Animals in the third group (Mn + SIL) were given a single i.p. injection of silymarin (100 mg/kg bw per day), 24 h after the administration of manganese chloride solution. The dose of silymarin used in our study and others (Mansour et al. 2006) gave good protection against the toxicity. The fourth group (SIL) was given daily a single dose of silymarin (100 mg/kg bw). At the end of the experimental period (30 days), the animals in different groups were sacrificed by cervical decapitation to avoid stress conditions. The brain tissue was immediately removed and dissected over ice-cold glass slides and cerebral cortex region was collected and homogenized (10%, w/v) in appropriate phosphate buffer saline (0.1 M, pH 7.4) and centrifuged at 10,000×*g* for 15 min at 4°C. The resulting supernatants were used for immediate lipid peroxidation and protein oxidation assays. Homogenate aliquots were stored at −80°C for further biochemical assays.

Cell culture and peroxide hydrogen production

Cell culture

Neuro-2a mouse neuroblastoma cell line, obtained from the ATCC (CCL-131), was routinely grown in 75 cm^2 flasks (Nunc, Denmark) and maintained in minimum essential medium (MEM) (Invitrogen, Glasgow, UK) supplemented with 10% foetal bovine serum (FBS, Hyclone, Logan, UT), 100 units/ml penicillin and 100 μg/ml streptomycin. Cultures were maintained in a humidified atmosphere with 5% CO_2 at 37°C. Cell dissociation was achieved with 0.05% trypsin-0.02% EDTA. Briefly, cells were seeded on 24-well culture plates in medium at an approximate density of 10^5 cells/cm^2 and, after 24 h stabilization, neuronal cells were co-cultured with medium containing various concentrations of $MnCl_2$ (200 and 800 μM) and silymarin (10, 50 and 100 μM) for 24 h. The concentration of $MnCl_2$ was selected based on previously reported cytotoxic levels in cultured cells (dos Santos et al. 2010). For stock solution, $MnCl_2$ was dissolved in MilliQ Plus sterilized water at the concentration of 800 mM and silymarin was dissolved in dimethylsulfoxide (DMSO) to obtain a 100 mM. The experimental concentrations were freshly prepared in the basal medium with a final DMSO concentration of 0.1%.

Measurement of H_2O_2

Measurement of H_2O_2 was carried out by the ferrous ion oxidation xylenol orange (FOX1) method (Ou and Wolff 1996). The FOX1 reagent consisted of 25 mM sulphuric acid, 250 μM ferrous ammonium sulfate, 100 μM xylenol orange and 0.1 M sorbitol. Briefly, 100 μl of culture medium were added to 900 μl of FOX1 reagent vortexed and incubated during 30 min at room temperature. Solutions were then centrifuged at 12000×*g* for 10 min, the amount of H_2O_2 in the supernatants was determined using spectrophotometer at 560 nm.

Biochemical assays

Enzymatic antioxidants

Catalase (CAT) activity was assayed by the decomposition of hydrogen peroxide according to the method of Aebi (1984). A decrease in absorbance due to H_2O_2 degradation was monitored at 240 nm for 1 min and the enzyme activity was expressed as μmol H_2O_2 consumed/min/mg protein.

Total superoxide dismutase activity (SOD) was evaluated by measuring the inhibition of pyrogallol activity as described by Marklund and Marklund (1974). One unit (U) corresponded to the enzyme activity required to inhibit the half of pyrogallol oxidation. SOD activity was expressed as U/mg protein.

Glutathione peroxidase activity (GPx) was measured according to Flohe and Gunzler (1984). The enzyme activity was expressed as nmoles of GSH oxidized/min/mg protein.

Non-enzymatic antioxidants

Acid ascorbic (AA) content was determined spectrophotometrically by dinitrophenyl-hydrazine method described by Jacques-Silva et al. (2001). Briefly, the ascorbic acid in the homogenate was oxidized by Cu^{2+} to form dihydro-ascorbic acid, which reacts with acidic 4-dinitrophenyl hydrazine to form a red hydrazone. Final color development was achieved with 65% sulfuric acid which was measured at 540 nm. The calibration curve was prepared using ascorbic acid as standard. Results were expressed as μmol/g tissue.

Glutathione (GSH) in tissue was determined by the method of Ellman (1959) modified by Jollow et al. (1974) based on the development of a yellow colour when 5,5-dithiobis-2-nitrobenzoic acid (DTNB) was added to compounds containing sulfhydryl groups. 500 μl of tissue homogenate in phosphate buffer were added to 3 ml of 4% sulfosalicylic acid. The mixture was centrifuged at 1600×*g* for 15 min. 500 μl of supernatant were taken and added to Ellman's reagent. The absorbance was measured at 412 nm after 10 min. total GSH content was expressed as μmol/g tissue.

Nitric oxide production was determined based on the Griess reaction (Green et al. 1982). Briefly, 100 μl of deproteinized sample was incubated with 100 μl of the Griess reagent at room temperature for 10 min. Absorbance was measured at 550 nm using a microplate reader. Nitrite concentration was determined from a standard nitrite curve generated using $NaNO_2$. The results were expressed as μM/mg protein.

Lipid peroxidation assay

Lipid peroxidation in the brain tissue was estimated colorimetrically by measuring thiobarbituric acid reactive substances (TBARS) which were expressed in terms of malondialdehyde content according to Draper and Hadley (1990) method. Briefly, 1 ml of cold 5% trichloroacetic acid was added to 500 μl of pre-treated cerebral cortex supernatants and centrifuged at 4000×*g* for 10 min. 500 μl of the supernatants were transferred to a Pyrex tube and incubated with 1 ml of thiobarbituric acid reagent (TBA, 0.67%) on a boiling water bath for 15 min. The tubes containing the mixture were allowed to cool on running tap water for 5 min. The resulting pink-stained TBARS were determined in a spectrophotometer at 532 nm. The MDA values were calculated using 1,1,3,3-tetraethoxypropane as the standard and expressed as nmoles of MDA/g of tissue.

Protein oxidation assays

Protein carbonyl contents were detected by the reaction with 2,4-dinitrophenylhydrazine (DNPH) method as reported by Evans et al. (1999). Briefly, the DNPH reaction proteins were precipitated with an equal volume of 20% (w/v) trichloroacetic acid and washed three times with 2 ml of an ethanol/ethyl acetate mixture (1:1). Finally, the precipitates were dissolved in 6 M guanidine HCl solution. The absorbance was measured at 370 nm, using the molar extinction coefficient of DNPH, $e = 22000\ M^{-1}\ cm^{-1}$ and the results were expressed as nmol of carbonyl/mg protein.

Non-protein thiol (NpSH) levels were determined by the method of Sedlak and Lindsay (1968). A 500 μl aliquot of supernatant was mixed with 10% trichloroacetic acid (V/V). After centrifugation, the protein pellet was discarded and free –SH groups were determined in a clear supernatant. A 100 μl aliquot of supernatant was added to 850 μl of 1 M potassium phosphate buffer (pH 7.4) and 50 μl of DTNB (10 mM). The colorimetric reaction was measured at 412 nm. Reduced glutathione was used as standard. NpSH levels were expressed as μmol NpSH/g tissue.

Advanced protein oxidation products (AOPP) assay was performed by the modification of Witko-Sarsat method (Witko et al. 1992). Briefly, samples were prepared in the following way: 200 μl of the homogenate supernatant fraction was diluted (1:5 v/v) in PBS, 10 μl of 1.16 M potassium iodide were then added to each tube, followed 2 min later by 20 μl acetic acid. AOPP concentrations were expressed as micromoles per liter of chloramine-T equivalents.

Determination of acetylcholinesterase activity

AChE activity was determined after the hydrolysis of acetylthiocholine according to the method of Ellman et al. (1961), modified by Tsakiris et al. (2000). The incubation mixture (1 ml) contained 50 mM Tris–HCl, pH 8, 240 mM sucrose and 120 mM NaCl. The protein concentration of the incubation mixture was 80–100 μg/ml. The reaction was initiated after addition of 0.03 ml of DTNB and 0.05 ml of acetylthiocholine iodide, which was used as substrate. The

final concentrations of DTNB and substrate were 0.125 and 0.5 mM, respectively. The increase of absorbance (ΔOD) was determined spectrophotometrically at 412 nm.

Quantitative protein determination

Protein concentration in cerebral cortex homogenates were measured by Bradford method (Pierce, BCA Protein Assay Kit, USA) using bovine serum albumin as standard.

Histological studies

Cereberal cortex tissue, extracted from the control and treated rats was fixed in 10% buffered formalin and was processed for paraffin sectioning. Sections of about 5 μm thickness were stained with hematoxylin and eosin to examine under light microscope.

Statistical analysis

The data were analyzed using the statistical package program Stat view 5 Soft Ware for Windows (SAS Institute, Berkeley, CA). Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by Fisher's protected least significant difference (PLSD) test as a post hoc test for comparison between groups [treated groups (Mn, Mn + SIL, SIL) vs. (controls)] and [Mn + SIL] vs. [(Mn, SIL)]. All values were expressed as mean ± SD. Differences were considered significant if $P < 0.05$.

Results

Lipid peroxidation product, nitrite formation and protein oxidation on cerebral cortex

The lipid peroxidation product (MDA), nitrite formation (NO), protein carbonyls (PCO) and advanced oxidation protein products (AOPP) in the cerebral cortex are shown in Table 1. A significant increase in MDA ($F = 74.91$, $P < 0.0001$), NO ($F = 53.\ 11$, $P < 0.0001$), PCO ($F = 10.37$, $P < 0.006$) and AOPP ($F = 20.58$, $P < 0.0001$) levels were observed in the cerebral cortex of Mn-treated group compared with the control group. Co-administration of Silymarin at 100 mg/kg significantly decreased ($P < 0.01$) the level of lipid peroxidation, nitrite formation and protein oxidation products compared with the Mn-treated group. Further, there were no alterations in the lipid peroxidation, nitrite and protein oxidation levels due to silymarin treatment as compared to control group.

Antioxidant enzyme activities in cerebral cortex

The effects of manganese treatment on the activity of SOD, CAT and GPx in cerebral cortex are shown in Fig. 1. SOD, CAT and GPx activities in cortex were significantly decreased ($F = 47.08$, $P < 0.0001$; $F = 13.47$, $P < 0.0001$; $F = 12.48$, $P < 0.0002$, respectively) upon Mn treatment. Co-treatment with silymarin at 100 mg/kg ameliorated the decreasing activities of these enzymes obtained in the group treated only with Mn.

Non-enzymatic antioxidant levels in cerebral cortex

Table 2 reported the changes of some non-enzymatic antioxidant parameters in the cortex.

Mn treatment led to a significant decrease in GSH ($F = 32.04$, $P < 0.0001$), ascorbic acid ($F = 8.82$, $P < 0.001$), Non-protein thiol ($F = 18.95$, $P < 0.0001$) levels in the cerebral cortex compared with control group. These effects were significantly mitigated by 100 mg/kg silymarin co-treatment compared with Mn-treated group. These biochemical variables did not differ noticeably between control and silymarin treated groups.

Acetylcholine esterase (AchE) activity

Figure 2 demonstrated the activity of AChE in the cortex of control and treated groups.

Acetylcholine esterase activity in the cerebral cortex was significantly inhibited ($F = 18.68$, $P < 0.0001$) in Mn-treated group compared to control group. While it reached control values by co-administration of silymarin.

Hydrogen peroxide production in vitro

Figure 3 showed the effects of $MnCl_2$ and silymarin on H_2O_2 production in Neuro2a Cells. To check if the combination of $MnCl_2$ and silymarin had any

Table 1 Effects of different treatments on oxidative stress parameters: lipid peroxidation (MDA), protein carbonyl content (PCO), advanced oxidation protein product (AOPP) and nitrite (NO_2^-) formation in cerebral cortex of controls (C) and rats treated with Manganese chloride (Mn), silymarin (SIL) or their combination (Mn + SIL)

Groups	Control	Mn	Mn + SIL	SIL
MDA (nmol/g tissue)	217.70 ± 7.08	276.70 ± 5.36***	239.60 ± 5.21**,###	233.60 ± 7.77*
AOPP (μmol/l chloramine-T equivalents)	23.58 ± 1.13	33.34 ± 2.17***	23.59 ± 2.53###	28.65 ± 2.98*
PCO (nmol/mg protein)	1.95 ± 0.40	2.62 ± 0.14**	1.96 ± 0.09##	2.02 ± 0.08
NO_2 (μmol/mg protein)	2.20 ± 0.16	4.28 ± 0.13***	2.58 ± 0.35###	2.48 ± 0.30

Values are expressed as means ± SD of six rats in each group. Mn; Mn + SIL and SIL groups vs. control group: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Mn + SIL group vs. Mn group: # $P < 0.05$; ## $P < 0.01$; ### $P < 0.001$

benefits, cells were treated with $MnCl_2$ (200 and 800 μM) and varying doses of silymarin (10, 50 and 100 μM) for 24 h. The levels of H_2O_2 generated in medium of cells were significantly ($P < 0.05$) increased by 159 and 241% compared to controls after exposure to $MnCl_2$ (200 and 800 μM) respectively, and were significantly ($P < 0.05$) deceased by (27%, 41% and 20%) cells co-culture with silymarin (10, 50 and 100 μM) and $MnCl_2$ at dose (200 μM) and by (40%, 49% and 25%) at dose (800 μM) compared with $MnCl_2$ alone (200 and 800 μM) respectively.

Histological examination of cerebral cortex

Figure 4 illustrates the histopathological assessments of cortex brain tissue in experimental rats. Histopathological examination of the cerebral cortex tissue revealed that manganese treatment caused abnormal cellular arrangement with few pyknotic nucleus, vacuolated spaces and haemorrhage. However, co-administration of silymarin at 100 mg/kg bw prevented these changes and maintained normal architecture with less number of pyknotic nuclei and showed almost normal architecture similar to that of the untreated control. There were no histological alterations in the cerebral cortex of positive controls treated with silymarin alone when compared to negative controls.

Discussion

Human exposure to Mn is of growing concern given its ubiquitous nature and prevalence both in the environment and occupational settings. A recent study suggests that high levels of Mn in drinking water (>300 mg/l) are associated with reduced intellectual function (Wasserman et al. 2006) and induced neurological disorders similar to Parkinson diseases (Aschner 1997; Lander et al. 1999). Recent studies suggest that oxidative stress may play a key role in manganese-induced neurotoxicity (Aschner 1997; Galvani et al. 1995). Therefore the brain is very susceptible to oxidative stress due to its high oxygen consumption, its high iron and lipid contents, especially polyunsaturated fatty acids (PUFA), and the low activity of antioxidant defenses, a fact that makes this tissue more vulnerable to increased levels of oxygen reactive species (Halliwell and Gutteridge 2007). In fact, oxidative stress has been implicated in the pathophysiology of common neurodegenerative disorders, such as Parkinson's disease, Alzheimer's disease, as well as in epileptic seizures and demyelination (Bogdanov et al. 2001; Behl and Moosmann 2002; Berg and Youdim 2006).

In the present study, exposure rats to manganese through drinking water resulted in a significant increase in lipid peroxidation, nitrite formation and protein oxidation as indicated by the significant increase in MDA content, NO_2^- levels, protein carbonyls and AOPP levels suggesting that Mn activated the formation of free radicals in brain tissue. Our results corroborated with previous findings which demonstrated that Mn exposure stimulated the generation of reactive oxygen species (ROS) (HaMai et al. 2001; Gunter et al. 2006) and enhanced quinones and oxidative species (Migheli et al. 1999; Shen and Dryhurst 1998). Moreover, Milatovic et al. (2009) demonstrated recently that Mn increased F_2-isoprostanes formation (lipid peroxidation products) and activated the depletion of ATP in neuronal culture. Although the specific molecular targets that by which Mn-induced oxidative stress are not known, it has been reported that Mn could interact directly with low

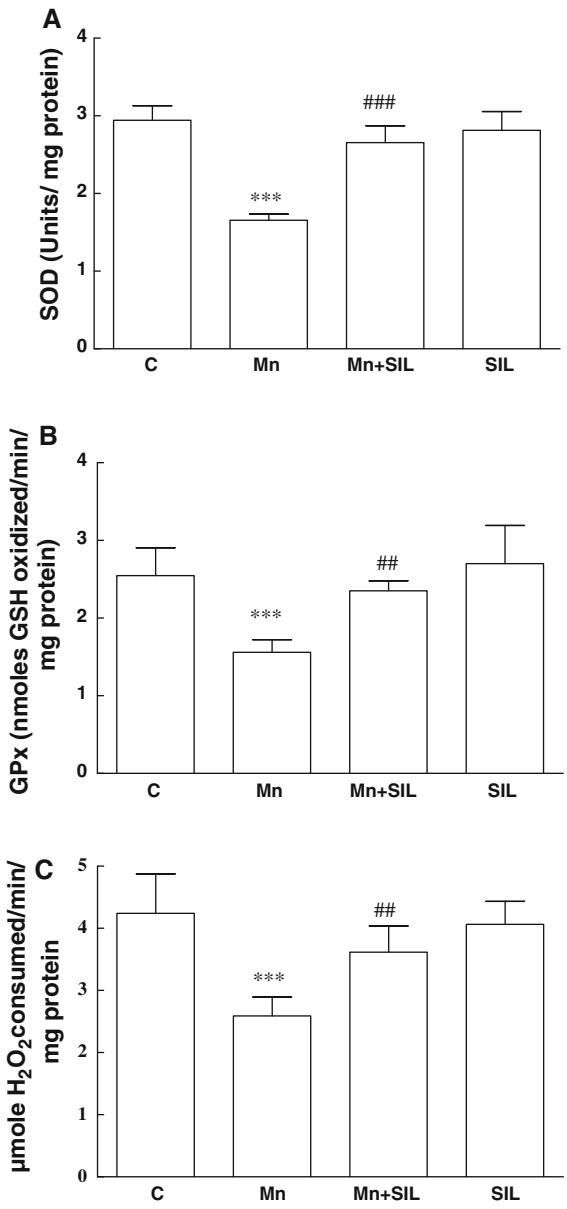

Fig. 1 Antioxidant enzyme activities (CAT, GPx and SOD) in cerebral cortex of controls (C) and rats treated with Manganese chloride (Mn), silymarin (SIL) or their combination (Mn + SIL). **a** Catalase (CAT). **b** Superoxide dismutase (SOD). **c** Glutathione peroxidase (GPx). Values are expressed as means ± SD of six rats in each group. Mn; Mn + SIL and SIL groups vs. control group: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Mn + SIL group vs. Mn group: ### $P < 0.001$, ## $P < 0.01$

molecular thiols oxidizing them to disulfides. In fact, reduced cysteinyl residue from proteins could also react with the manganese, which might cause the loss of enzyme catalytic activities (Prabhakaran et al. 2009). The decrease of glutathione, non protein thiols and ascorbic acid contents in cerebral cortex of rats observed in our study supports these findings. Glial cells are also known to protect neurons against oxidative stress and cell death by releasing GSH extracellularly and keeping it in the reduced form (Sagara et al. 1993; Stone et al. 1999). Considering that GSH is the major naturally occurring non-enzymatic antioxidant in the brain (Lissi et al. 1995; Halliwell and Gutteridge 1999; Evelson et al. 2001) this may be related to our results showing that the manganese causes a depletion of sulfhydryl groups, reducing the non-enzymatic antioxidant defenses in cerebral cortex of rats. It is therefore presumed that GSH levels were reduced intracellularly due either to the excess of free radical formation, including NO or to its derivative peroxynitrite forming nitrosoglutathione or by regenerating the nitrosyl groups in order to limit NO deleterious effects (Stamler and Toone 2002; Rodríguez-Martín et al. 2002).

Another explanation of Mn toxicity can also be related to the capacity of this metal to bind transferrin (iron binding protein in blood) and thus affects the binding of Fe to proteins. So the concentration of free intracellular Fe increases and facilitates the Fenton reaction leading to the peroxidation of membrane lipids. Co-administration of silymarin at dose of 100 mg/kg bw reduces significantly lipid peroxidation, nitrite formation and protein oxidation in brain tissue of animals exposed to manganese. We suggest that silymarin scavenges free radical generation by Mn. These effects may reflect the ability of silymarin (i) to enhance the scavenging and inactivation of H_2O_2 and hydroxyl radicals; (ii) to chelate with redox metals including Fe^{2+} which catalyzes the formation of free radicals via the Fenton reactions; (iii) and to achieve lipid peroxidation by induction of enzymatic and non-enzymatic antioxidants, such as GSH, SOD and CAT (Miquel et al. 2002). Accordingly, the protection afforded by silymarin against Mn-induced ROS (e.g. H_2O_2) generation is likely attributable to its antioxidant effects. To confirm this hypothesis, we have also investigated in vitro the neuroprotective properties of silymarin on N2a cells exposed to $MnCl_2$. In this context, cloned neuroblastoma cell lines, including mouse neuroblastoma cell lines (e.g. Neuro2a and N1E-115) and human neuroblastoma cell lines (e.g. SH-SY5 and SK-N-AS) were the

Table 2 Non-enzymatic antioxidant status in cerebral cortex of controls (C) and rats treated with Manganese chloride (Mn), silymarin (SIL) or their combination (Mn + SIL)

Groups	Control	Mn	Mn + SIL	SIL
GSH (μmol/g tissue)	3.21 ± 0.06	2.75 ± 0.08***	3.17 ± 0.15$^{\#\#\#}$	3.29 ± 0.02
NpSH (nmol/mg protein)	72.52 ± 5.35	59.11 ± 3.71*	77.35 ± 6.77$^{\#\#}$	90.15 ± 9.23
AA (μmol/g tissue)	5.95 ± 0.37	4.22 ± 0.57***	5.69 ± 0.29$^{\#\#}$	6.06 ± 1.0

Values are expressed as means ± SD of six rats in each group. Mn; Mn + SIL and SIL groups vs. control group: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. Mn + SIL group vs. Mn group: $^{\#}$ $P < 0.05$; $^{\#\#}$ $P < 0.01$; $^{\#\#\#}$ $P < 0.001$

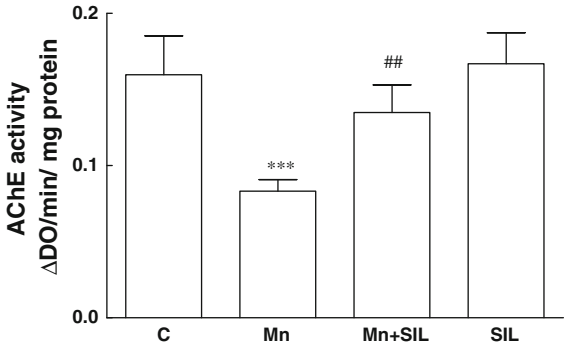

Fig. 2 Acetylcholinesterase (AChE) activity in cerebral cortex of controls (C) and rats treated with Manganese chloride (Mn), silymarin (SIL) or their combination (Mn + SIL). Values are expressed as means ± SD of six rats in each group. Mn; Mn + SIL and SIL groups vs. control group: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Mn + SIL group vs. Mn group: $^{\#}$ $P < 0.05$, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$

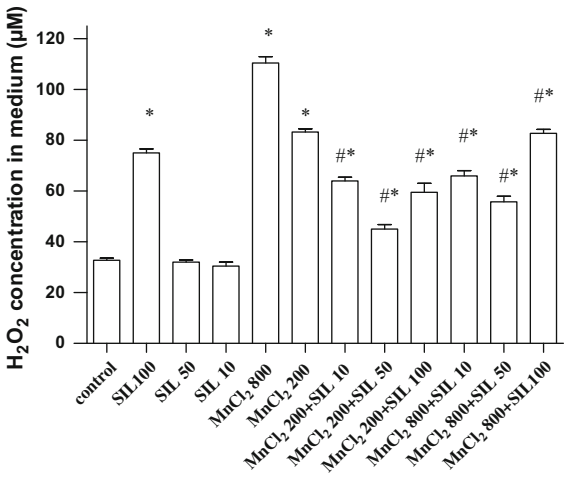

Fig. 3 Effect of SIL on H_2O_2 production in the medium culture of Neuro2a cells exposed to $MnCl_2$. The cells were exposed to $MnCl_2$ or co-exposed to $MnCl_2$ (200 and 800 μM) and SIL (10, 50 and 100 μM) for 24 h. Data represent the mean ± SD from four independent experiments. * $P < 0.05$ vs. control, and $^{\#}$ $P < 0.05$ vs. $MnCl_2$ exposed cells

established in vitro models that have widely used to investigate the neurotoxicity of xenobiotics. In the present study, exposure of N2a cells line to Mn Cl_2 (200 and 800 μM) increased significantly H_2O_2 production in extracellular medium indicating the role of ROS generation as primary mechanism for Mn-induced toxicity. The ability of SIL to exert great effect on Mn-induced cellular injury is consistent with its increased potency in reducing ROS (e.g. H_2O_2) generation. Our results are consistent with those of Fu et al. (2009) who have found that silymarin, flavonolignans isolated from *S. marianum*, has an antioxidative activity and free radical scavenging properties in vitro, which can scavenge various oxidizing radicals such as $OH^{\bullet}$, $NO_2^{\bullet}$, $O_2^{\bullet}$, $RNS^{\bullet}$. Furthermore, Nencini et al. (2007) demonstrate the efficacy of silymarin in restoring GSH content in brain against acetaminophen-induced neuronal damages. Under physiological conditions, SOD is an important intracellular antioxidant which catalyses the conversion of the superoxide anion radical to molecular oxygen and hydrogen peroxide H_2O_2 and thus protects against superoxide-induced damage (Hunt et al. 1990). The present study demonstrates that Mn modifies the activity of the antioxidant enzymes by reducing SOD, CAT and GPx. It should be emphasized that these enzymes represent the first barrier against reactive species and are essential to cell survival (Remacle et al. 1992; Matés et al. 1999; Halliwell 2001). The failure in the antioxidant system corroborated with previous studies which demonstrated that superoxide anion, produced in the mitochondrial transport chain, may catalyze the oxidation of Mn^{2+} to Mn^{3+}. Thus it led to increase oxidant capacity of this metal (Gunter et al. 2006). It is well known that SOD and CAT own sequential functions in ROS removing, by $O_2^{\bullet}$ dismutation, followed by H_2O_2 conversion to H_2O and O_2, respectively. The decrease in the activity of SOD can be attributed to

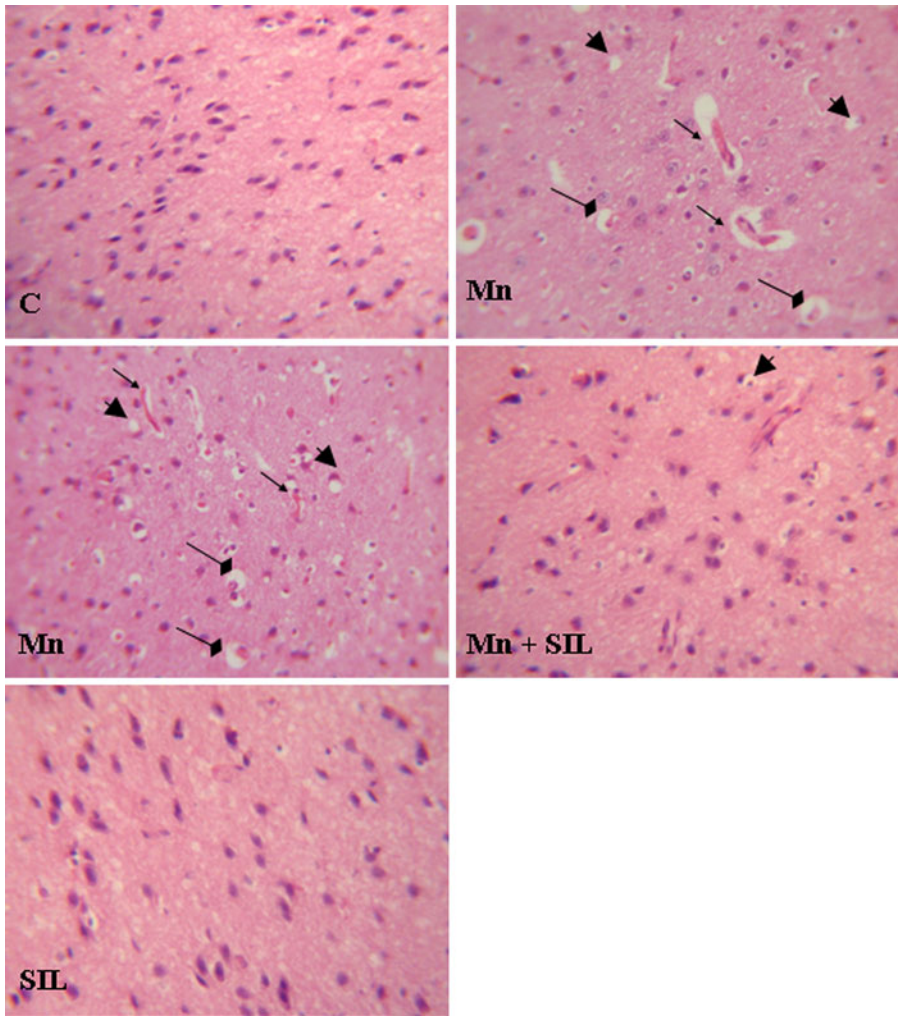

Fig. 4 Photographs showing histopathological changes in cerebral cortex in different groups, Control group(C), Mn-treated group (Mn), silymarin treated group (SIL), manganese chloride + silymarin treated group (Mn + SIL) (hematoxylin and eosin staining, 400×). (↘) Haemorrhage; (↘) vacuolated cytoplasm; (↘) pyknotic nuclei (PN)

the enhanced superoxide production during manganese metabolism. The superoxide radical also inhibits the activity of catalase (Gupta et al. 2005). Silymarin significantly prevents the alterations in the activities of SOD, CAT and GPx in cerebral cortex probably likely attributable to its antioxidant effects.

Acetylcholine, a neurotransmitter associated with learning and memory, is degraded by the enzyme acetylcholinesterase, which achieves its physiological action. In addition to their role in cholinergic transmission, cholinesterases may also play a role during morphogenesis and neurodegenerative diseases (Reyes et al. 1997; Layer et al. 1987). In the present study, the exposure of rats to Mn significantly decreases AchE activity in the cerebral cortex suggesting the ability of Mn^{2+} to interfere with the calcium action as a regulator of cell function leading to the inhibition of AchE in cholinergic systems. Co-administration of silymarin, a natural antioxidant, to Mn-treated rats improves AchE activity. It seems that the increase of free radicals, observed in the present experiment, may inhibit the acetyl cholinesterase activity. Our results corroborate with previous findings (Tsakiris et al. 2000) which demonstrated that AChE activity in rat brain was inhibited by free radicals. Furthermore, Histopathological examination of the cerebral cortex tissue reveals that manganese treatment causes abnormal cellular arrangement with

few pyknotic nuclei, vacuolated spaces and haemorrhage. However, co-treatment with silymarin prevents these changes and also maintains normal architecture with less number of pyknotic nuclei.

In conclusion, the findings of the present study suggest that manganese administration induces the oxidative damage in the cerebral cortex objectified by an increase of lipid peroxidation, protein oxidation, nitrite formation and depletion of enzymatic and non-enzymatic antioxidant. There is a highly reduced capacity to scavenge free radicals produced in the cerebral cortex in response to Mn-neurotoxicity. Silymarin co-administration leads to a significant attenuation in all these parameters. Acting as an antioxidant, silymarin alleviates the oxidative damage in the cerebral cortex. However further investigations are needed to elucidate the precise mechanism of silymarin protection against Mn-neurotoxicity.

Acknowledgments The authors are indebted to Miss Dalenda Kchaou for their assistance in histolological techniques. The present work was supported by the grants of DGRST (Appui a la Recherche Universitaire de base, ARUB 99/UR/08-73), Tunisia.

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