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Original article

# Manganese neurotoxicity and protective effects of resveratrol and quercetin in preclinical research

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ABSTRACT

*Background:* Exposure to Mn results in a neurological syndrome known as manganism.
*Methods:* We examined how 4-week Mn exposure (20 mg/kg $MnCl_2$ *po*, 5 days/week) induces neurotoxic effects in rats. Oxidized-to-reduced glutathione ratio (GSSG/GSH), malondialdehyde (MDA), superoxide dismutase (SOD) activity, catalase (CAT) activity, vitamin E content and caspase-3 activity were measured in several rat brain structures. Further, we examined protective effects of the polyphenols: resveratrol (R) or quercetin (QCT) against Mn-induced neurotoxicity.
*Results:* After exposure to Mn, we found a rise in GSSG/GSH ratio and a reduction in SOD activity in the rat striatum (STR), while in the nucleus accumbens (NAC) decreases in *alpha*-tocopherol content and in SOD activity were noted. In the frontal cortex (FCX), an enhancement in GSSG/GSH ratio and a reduction in SOD and CAT activities were observed. In the cerebellum (CER), a significant increase in the caspase-3 activity paralleled a rise in the GSSG/GSH ratio and a diminution of SOD activity. In the rat hippocampus (HIP), Mn evoked an enhancement in GSSG/GSH ratio. There were no changes in the MDA levels. Pretreatment with R and QCT protected against the Mn-induced (i) enhancement in GSSG/GSH ratio in the STR, (ii) decreases in the NAC *alpha*-tocopherol content and (iii) reduction in SOD activity in FCX, NAC and CER.
*Conclusion:* Repeated Mn administration induces toxic effects in several rat brain structures and treatment with R and QCT may be a potential therapeutic strategy to attenuate the metal neurotoxicity.
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## Introduction

Manganese (Mn) is a trace nutrient required for normal growth, development, and cellular homeostasis [1]. Mn acts as a cofactor of several enzymes important for neuronal and glial functions including neurotransmitter synthesis and metabolism [2]. The non-physiological applications of Mn include the production of steel, batteries, potassium permanganate, electronics, agriculture, fuel additive methylcyclopentadienyl Mn tricarbonyl, fungicides and contrast agents for MRI studies [3].

The excessive occupational and environmental exposure to Mn results in a neurological syndrome known as *manganism*. Manganism is associated with motor disturbances, cognitive and neuropsychiatric deficits as well as sleep dysfunctions [4]. In humans and animals the disorder mimics Parkinson's disease, and the globus pallidus is the primary brain target of Mn accumulation and toxicity [5]. However, many symptoms of manganism are linked with pathologies found in other basal ganglia (the striatum (STR) [6], subthalamic nucleus [7]) as well as in the cerebral cortex and the hypothalamus [8,9].

Several mechanisms have been suggested to induce Mn neurotoxicity [10,11]. The most widely examined mechanistic hypothesis suggests that the excess Mn causes an imbalance in the mitochondrial electron transport that leads to mitochondrial oxidative stress and excitotoxic cell death [12]. In fact, the *in vivo* and *in vitro* studies showed that several radical scavengers exert protective effects against manganism. Recent preclinical studies point to the potential beneficial anti-manganism therapy with natural antioxidants, including the sylimarin [12], anthocyanin [13], and lycopene [14].

There is no preclinical and clinical data on the effects of natural polyphenolic antioxidants, among which resveratrol (R) and quercetin (QCT) are probably the most representative, on Mn toxicity. In the present study, we examined how repeated Mn

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exposure that mimics occupational conditions (one dose a day, 5 days a week) for 4 weeks affects reactive oxygen species (ROS) production and neuronal cell death in rats. The wide range of markers of the pro-/antioxidant balance (oxidized-to-reduced glutathione ratio (GSSG/GSH), lipid peroxidation, superoxide dismutase (SOD) activity, catalase (CAT) activity, vitamin E content) and apoptosis (caspase 3-activity) were measured in several rat brain structures (the frontal cortex (FCX), hippocampus (HIP), STR, nucleus accumbens (NAC) and cerebellum (CER)). Further, we examined whether R or QCT exerts protective effects against Mn-induced neurotoxicity. In addition, we evaluated the effect of a combination of Mn + ethanol (Et) on brain neurotoxicity as the 10% Et solution was employed as the medium for *po* administration of the used polyphenols. Et improves the absorption of polyphenols from the gastrointestinal tract (in beer or wines), while by itself it may also increase oxidative effects in the nervous system [15–17].

## Materials and methods

### Animals

Male adult Wistar rats (180–190 g at the beginning of the experiment) were used in the experiment. The animals were housed two per cage in standard plastic rodent cages (40 cm × 25 cm × 20 cm) in a colony room maintained at 21 ± 1 °C and 40–50% humidity under a 12-h light-dark cycle (lights on at 6:00 a.m.) Rodent chow and water were available *ad libitum*. Number of animals/group = 8.

### Drugs

Mn ($MnCl_2 \times 4H_2O$), QCT and R were purchased from Sigma-Aldrich (USA). Mn was dissolved in saline and administrated intraperitonealy (*ip*) at a dose of 20 mg/kg calculated as the anhydrous salt. The dose of Mn was chosen based on the literature data showing that this dose is significantly accumulated and alters biochemical markers in the rat brain tissues [18–20]. To assess the effect of polyphenols on prooxidative effect of Mn, we used two doses of R (10 and 20 mg/kg) given per os (*po*), of which the higher one is 1000 times the amount consumed by 70-kg person taking 1.4 g of R/d [21]. In our 28-day experiment, this dose neither affected hematologic and biochemical variables nor produced a damage to organs in rats. The used doses of QCT (13 or 26 mg/kg) were equimolar to those of R. The polyphenols were suspended in 10% Et and given 5 min before Mn administration. All the drugs were given in a volume of 2 ml/kg.

### Treatment schedules

The first set of experiments included treatment of separate groups of animals with vehicle (saline) + vehicle, Et + vehicle, vehicle + Mn and Et + Mn. In the second set of experiments, the following drug combinations were given to rats: vehicle + vehicle, vehicle + Mn, R(10) + Mn, R(20) + Mn, QCT(13) + Mn and QCT (26) + Mn. The drugs were administered during five consecutive days/week, for 4 weeks; the used model mimics exposures to drugs in occupational settings.

### Brain tissue preparation

On the next day after the 4-week drug administration, the rats were sacrificed by decapitation. The brain structures were isolated and rinsed in ice-cold physiological saline solution, immediately frozen on dry ice and stored at −80 °C until use. Before biochemical analysis tissue samples were weighed and homogenized in ice-cold double distilled water (1:20).

### Biochemical assays

#### Protein concentrations

In all homogenates, determination of the total protein was performed according to the procedure of Lowry et al. [22] that has been adapted to the microplates, using bovine serum albumin (BSA) as the standard.

#### MDA concentration

The level of malondialdehyde (MDA) was measured by HPLC method after derivatization with 2,4-dinitrophenylhydrazine (DNPH) [23]. Spectrophotometric detection was carried out at the wavelength $\lambda = 310$ nm. Data are expressed as nmol/mg protein.

#### GSSG/GSH ratio level

The contents of GSH and GSSG were determined according to the HPLC method [24]. GSH level was measured after pre-column derivatization with o-phthalaldehyde (OPA).

The concentration of GSSG was determined after a blockage of the thiol groups of GSH by N-ethylmaleimide (NEM). Next, the disulfide bounds of GSSG were reduced by DL-dithiothreitol (DTT) solution and then samples were derivatized with OPA for GSH (derived from GSSG; molar ratio 2:1) determination.

The levels of GSSG and GSH, expressed as nmol/mg protein were used to calculate the GSSG/GSH ratio.

#### Alpha-*tocopherol content*

The tissue *alpha*-tocopherol contents were determined in homogenates after saponification and extraction with hexane by the HPLC method [25]. Fluorometric detection was performed at the wavelengths $\lambda_{ex} = 298$ nm and $\lambda_{em} = 325$ nm. The level of *alpha*-tocopherol was expressed as nmol/mg protein.

#### SOD activity

The total SOD activity was determined based on the inhibition of epinephrine oxidation by the enzyme [26]. Original method was adopted to microscale using Infinite M200 Pro 96-well plate spectrophotometer (Tecan, USA). In the reaction mixture containing homogenate samples, carbonate buffer and epinephrine solution, absorbance was monitored continuously at $\lambda = 480$ nm for 4 min. The SOD activity was expressed as U/mg protein.

#### CAT activity

The CAT activity was determined by measuring hydrogen peroxide decomposition, as described previously [27]. The CAT activity was determined as mmol of $H_2O_2$ consumed/s/mg protein and expressed as U/mg protein.

#### Caspase-3 activity

The activity of caspase-3 was measured by the Caspase 3 Fluorometric Assay Kit (Sigma Aldrich, USA). This commercial test was performed according to their manufacturer's instructions.

#### Statistical analysis

Data are presented as the mean ± SEM. An one-way or two-way analyses of variance (ANOVA) and *post hoc* Dunnett's and Newman-Keuls tests, respectively, were used. Additionally, to detect changes between vehicle + vehicle and vehicle + Mn, and Et + vehicle and Et + Mn, an unpaired Student's *t*-test was also employed. Statistically significant differences between groups are revealed for $p < 0.05$.

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