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Protective actions of quercetin in manganese-induced neurotoxicity; behavioral, neurostructural, and neurochemical evaluations

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Abstract

BACKGROUND AND AIM: Excessive exposure to manganese (Mn) alters neuronal structure and functions. This leads to manganism, a neurological syndrome similar but not identical to Parkinson's disease. Therapeutic interventions for this Parkinsonian syndrome have been largely unsuccessful due to little knowledge on mechanism of manganese induced brain damage. Oxidative stress is implicated as one mechanism of Mn induced neurotoxicity. Here, we investigated the effects of quercetin, a potent antioxidant obtained from fruits and vegetables on Mn induced neurotoxicity.
METHODOLOGY: Adult male rats were exposed to either Mn only or Mn co-administered with 5 or 10 mg/kg of quercetin, while Control rats were treated with normal saline. All treatments were via intraperitoneal injections for 5 weeks. After treatments, behavioral assessments were performed. Following which, rats were sacrificed, brain excised for microanatomical analysis and biochemical quantification of oxidative stress markers.
RESULTS: Cognitive and locomotor associated behaviors were significantly impaired in Mn only treatment, however quercetin co-administration attenuated only cognitive behavior. Mn treatment induced degenerative changes in brain neurons, accompanied by astrogliosis (abnormal increase in astrocyte population). Co-administration with quercetin reduced these microstructural deficits. Additionally, quercetin co-treatment reduced oxidative stress imposed on brain tissues in Mn only treatment.
CONCLUSION: These results suggest that the dietary antioxidant quercetin may attenuate structural and behavioral anomalies associated with Mn overexposure, probably due to its ability to resist oxidative stress.

Keywords:
Manganese; quercetin; brain; behavior; oxidative stress

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INTRODUCTION

Globally, overexposures to heavy metals such as manganese (Mn) are increasingly of health concern. Mn, an important trace metal is regularly found within the environment. Mn exist in all tissues and is required for the maintenance and regulation of various biochemical and cellular functions (Sidoryk-Wegrzynowicz and Aschner 2013). Nonetheless, in humans, too much accumulation of Mn in the brain from overexposure is associated with neurological deficits. Overexposure to Mn is an environmental risk factor for Parkinson's disease (PD) and results in a parkinsonian syndrome known as manganism. This syndrome is characterized by variety of psychiatric, cognitive and motor disturbances that are akin to those inherent to Parkinson's disease (PD) (Crossgrove and Zheng, 2004; Erikson *et al*., 2005; Cersosimo and Koller, 2006; Sidoryk-Wegrzynowicz and Aschner 2013).
Neurons and astrocytes are key targets for Mn upon entering the brain. Hence astrocytes serve as main homeostatic regulator and storage site for Mn in the brain (Milatovic *et al*., 2009).

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However, amplified accumulation of Mn in astrocytes may modify glutamate release and elicit excitatory neurotoxicity (Erikson and Aschner, 2003). Mn favorably accumulates in mitochondria, where it upsets oxidative phosphorylation and intensifies the generation of reactive oxygen species (ROS). Increased striatal concentrations of ascorbic acid and glutathione (GSH), antioxidants that when increased, signal the presence of elevated burden from ROS, as well as other markers of oxidative stress, have been previously reported (Milatovic *et al*., 2009). Oxidative stress is implicated as one of the mechanisms of neurotoxicity by Mn (Karki *et al*., 2013; Avila *et al*., 2012).

Quercetin is a classic flavones-type flavonoid; ubiquitously distributed in fruits and vegetables and comprises the dominant part of flavonoids from daily foods. Quercetin, is a potent antioxidant, known to prevent oxidative injury and cell death by chelating metal ions, scavenging oxygen radicals and defending against lipid peroxidation (Ebokaiwe *et al*., 2016). Previous study has shown the protective effect of dietary quercetin against oxidative toxicity induced by lead, cadmium, atrazine on several organs in rat, including liver, renal, testis (Ebokaiwe and Farombi, 2015).

Given that oxidative stress is one of the major mechanisms characterizing Mn induced neurotoxicity, it is hypothesized that quercetin may attenuate toxic effects of Mn on brain structures and functions. In view of the foregoing, the present study investigated the effects of quercetin on behavior and neuronal histology following Mn-induced neurotoxicity in rat models.

Materials and Methods

Animal care and treatments

Twenty-four male adult albino strain Wistar rats (150-200 g) were used for this study. They were bred at the Animal Holdings of Faculty of Basic Medical Sciences of the University. Animals were housed in clean plastic cages in a clean environment of 12 hours day/light cycle, at room temperature. Animals in all groups were allowed access to standard laboratory rat pellets and water *ad libitum*. All experimental protocols were in strict accordance with the guidelines for animal research, as detailed in the NIH Guidelines for the Care and Use of Laboratory Animals (NIH, 2011) and conformed to local institutional guidelines for the protection of animal welfare during the experiments.

The rats were randomly assigned into 4 groups of 6 animals and treated as follows;

Group 1: Control rats received normal saline

Group 2: received 10 mg/kg manganese chloride ($MnCl_2$) as $MnCl_2$ tetrahydrate ($MnCl_2.4H_2O$, Sigma-Aldrich, US).

Group 3: received $MnCl_2$ and 5 mg/kg of quercetin (Sigma-Aldrich, US).

Group 4: received $MnCl_2$ and 10 mg/kg of quercetin.

All administrations were via daily intraperitoneal injections for 5 weeks. Doses used for rat models of Mn toxicity were generated based on previous studies that have shown significant increase in Mn accumulation in brain tissues and biochemical alterations in rats (Bouabid *et al*., 2014; Cordova *et al*., 2012; Marreilha dos Santos *et al*., 2011). Additionally, the selection of the dose, route and duration was based on previous studies (Chan *et al*., 2014; Dong *et al*., 2014; Nassiri-Asl *et al*., 2013; Bhutada *et al*., 2010), which have shown the effectiveness of intraperitoneally administered quercetin in protecting against damage in major organs.

Neurobehavioral studies

Rats were kept over-night in behavior testing room for acclimatization before administration. Behavioral studies were carried out 1 hour after last administration in quiet room between the hours of 10 am and 3 pm. The apparatuses were cleaned with 5% ethanol before testing a new animal to eliminate possible bias due to odours left by previous animal. The tests were recorded using a digital camera and later scored by trained blind observers.

Y-maze test: The tests were performed as previously described (Ijomone *et al*., 2015; Kim *et al*., 2008) using protocols adapted from Mori *et al*. (2001). Y-maze task assesses short-term spatial memory as a measure of cognitive functions using spontaneous alternation behaviors of rats. In this test, rats are placed on a start arm of a Y-shaped maze and allowed to move freely for 8 min. Hind paws of the rats have to be completely within an arm to be considered as rats having entered the arm. Entering all 3 arms in the overlapping triplet sets is defined as spontaneous alternation. The percentage of spontaneous alternation is calculated as {spontaneous alternation / (total number of arm entries – 2)} × 100.

Open-field test (OFT): The tests were carried out as previously described (Ijomone *et al*., 2014; Brown *et al*., 1999) in an apparatus consisting of a box (72x72x36 cm) with the floor divided into 18x18 square units. In this test, animals are placed in the centre of the box and allowed to move freely for 5 min. In the present study, we obtained the following parameters; locomotion frequency (number of crossings from one square to the other), rearing frequency (number of times the animals stood on their hind paws), rearing against the wall (no of times the animals stood on their hind paws against the wall), hinding (calculated by adding the rearing frequency to rearing against the wall).

Histology and histomorphometry

Following behavioural tests, rats were sacrificed and brains were harvested and fixed using neutral buffered formalin. The tissues were processed with the routine tissue processing procedure and stained with haematoxylin and eosin using established protocols (Bancroft and Gamble 2008).

Furthermore, separate sections were stained with phosphotungstic acid hematoxylin (PTAH) – a specific technique for demonstrating astrocytes– as described by Drury and Wallington, (1980). Briefly, Mallory's PTAH prepared with 0.1 g hematoxylin and 2 g phosphotungstic acid was dissolved in 100 ml of distilled water and allowed to naturally ripen. Deparaffinized sections were treated with 4% iron alum for about 60 minutes, rinsed in water, and transferred to Mallory PTAH for about 18 hours. Sections were blot dry with filter paper, differentiated rapidly in absolute alcohol, cleaned, air dried and mounted.

A digital microscope (OMAX microscopes, Irvine, CA, USA) was utilized to obtain photomicrographs of the region of the hippocampus and striatum were obtained using landmarks from the rat brain atlas (Paxinos and Watson 2007). Normal appearance (intact) and degenerating neuronal cells were identified and counted at x400 magnification using the cell counter tool on Image Analysis and Processing for Java (Image J) program. The percentage of intact neurons was calculated as number of intact neurons divided by total number of neurons and then multiplied by 100. Astrocytes were also identified and counted in PTAH stained sections.

Biochemical assays

Brains samples from the Control and treated rats were separately homogenized in eight volumes of 50 mM of Tris-HCl buffer (pH 7.4) containing 1.15% potassium chloride, and the homogenates were centrifuged at 10,000×*g* for 15 minutes at 4 ºC. Supernatant; post mitochondria fraction was collected for enzyme assays.

Determination of catalase (CAT) activity: CAT activity was assayed by the method of Claiborne (1995).

Determination of superoxide dismutase (SOD) activity: Superoxide dismutase activity was determined by measuring the inhibition of autoxidation of epinephrine (at pH 10.2) at 30°C by the method of Misra and Fridovich (1972).

Determination of glutathione peroxidase (GSH-Px) activity: Activity of GSH-Px was determined by the method of Rotruck *et al*. (1973).

Determination of glutathione S-transferase (GST) activity: GST activity was determined by the method of Habig *et al*. (1974) using CDNB as a substrate.

Determination of reduced glutathione (GSH) level: GSH was determined according to Jollow *et al*. (1974).

Determination of lipid peroxidation (LPO) level: LPO was quantified as malondialdehyde (MDA), according to the method described by Farombi *et al*. (2000).

Determination of Myeloperoxidase (MPO) activity: An aliquote of the post-mitochondrial supernatant of the brain homogenate was allowed to react with a solution of 1.2 mM tetramethylbenzidine and 100mM H_2O_2 in 43mM NaH_2PO_4 (pH 5.4) according to Eiserich at al., (1998).

Determination of Nitric oxide (NO) level (nitrite): Nitric oxide measured as nitrite was determined by using Griess reagent, according to the method of Moshage *et al*. (1995) where nitrite, stable end product of NO radical, is mostly used as an indicator for the production of NO.

Statistical analysis

Data were expressed as mean±SEM. Data comparisons were performed using One-way ANOVA, followed by Student Newman-Keuls (SNK) for post hoc. GraphPad Prism (Version 5.03, GraphPad Software, USA.) was the statistical package used for data analysis. Statistical significance was set at $P<0.05$.

RESULTS

Y-maze and Open field tests

Percentage of spontaneous alternation on the Y-maze was significantly decreased following $MnCl_2$ administration compared to the Control. Co-administration with quercetin at 10 mg/kg significantly attenuated $MnCl_2$ effects on Y-maze spontaneous alternation but not at 5 mg/kg [$F_{3,16}$=6.06; P=0.0059] (Fig. 1). Locomotion frequency on the OFT was significantly reduced following $MnCl_2$ administration compared to the Control, an effect that was not improved significantly with quercetin administration [$F_{3,20}$=5.16; P=0.0084]. On the other hand, hinding was significantly lowered following $MnCl_2$ treatment compared to the Control, however no effect was observed following quercetin co-treatment [$F_{3,20}$=3.57; P=0.0323] (Fig. 2).

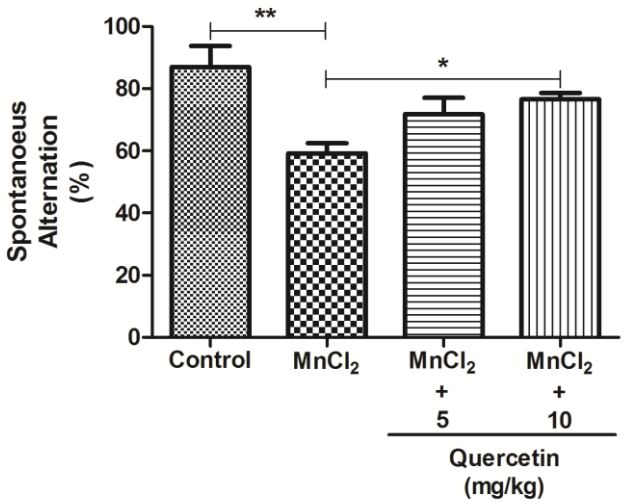

Fig. 1. Effect of Mn and Quercetin co-administered with Mn on short term memory on the Y-maze task. Values are expressed as mean + SEM. *P<0.05, **P<0.01. One-way ANOVA followed by SNK for post-tests.

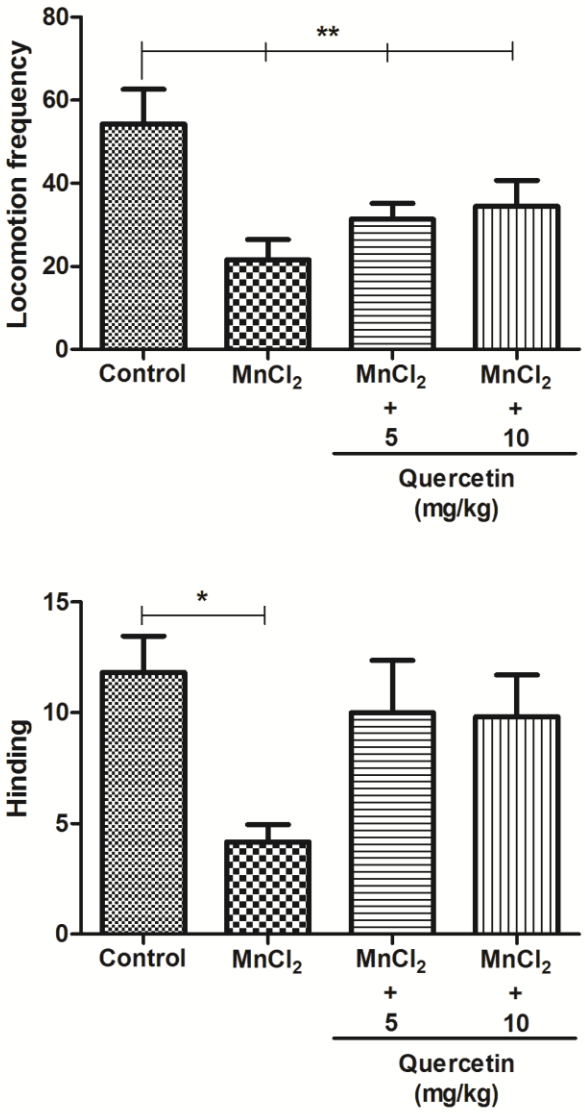

Fig. 2. Effect of Mn and Quercetin co-administered with Mn on locomotor and exploratory activities on the OFT. Values are expressed as mean + SEM. *P<0.05, **P<0.01. One-way ANOVA followed by SNK for post-tests.

Histology and histomorphometry

Histologically, intact neurons in the hippocampal pyramidal layer are characterized by their large pyramidal shape, with large nuclei and prominent nucleoli. On the other hand, striatal neurons are predominantly medium-sized with prominent nuclei and nucleoli. Treatment with $MnCl_2$ showed increases in neurons with degenerating features in both hippocampus and striatum. Neurodegenerating features in routinely stained brain sections include; prominent eosinophilic cytoplasm, pyknotic nuclei, neuron swelling and/or vacuolation within the cytoplasm (Garman, 2011). Image J cell count analysis revealed significant decrease in percentage of intact neurons following $MnCl_2$ treatment compared to the Control, in both hippocampus [$F_{3,12}$=15.76; P=0.0002] and striatum [$F_{3,12}$=205.4; P<0.0001]. However, neurodegeneration was worse in the striatum with over 50% reduction in intact neurons. Co-administration with quercetin significantly improved percentage of intact neurons in both brain regions, with 10 mg/kg quercetin treatment better improved compared to 5 mg/kg treatment (Fig. 3).

PTAH staining for astrocytes showed that $MnCl_2$ induced astrocytosis in hippocampus and striatum. Astrocytosis or astrogliosis is an abnormal increase in astrocyte population. $MnCl_2$ treatment significantly increased number of astrocytes compared to the Control in both the hippocampus [$F_{3,12}$=14.29; P=0.0003] and striatum [$F_{3,12}$=14.48; P=0.0003]. Co-administration with quercetin at 10 mg/kg attenuated the observed astrocytosis in these brain regions, however 5 mg/kg quercetin co-treatment showed no such attenuating effect (Fig. 4).

Biochemical assays of markers of oxidative stress

There was significant decrease in activities of SOD [$F_{3,20}$=43.99; P<0.0001], CAT [$F_{3,20}$=131.5; P<0.0001], GST [$F_{3,20}$=67.62; P<0.0001], and GSH-Px [$F_{3,20}$=52.65; P<0.0001] following $MnCl_2$ administration compared to the Control. Quercetin co-administration attenuated the effects of $MnCl_2$ on these oxidative stress markers, with 10 mg/kg quercetin treatment better improving the activities of these markers compared to 5 mg/kg quercetin (Fig. 5).

Levels of GSH [$F_{3,20}$=19.15; P<0.0001] were significantly lowered following $MnCl_2$ administration compared to the Control, with quercetin co-treatment also attenuating this effect. On the other hand, levels of LPO [$F_{3,20}$=26.16; P<0.0001] and NO [$F_{3,20}$=38.12; P<0.0001], and MPO [$F_{3,20}$=34.18; P<0.0001], activity were significantly increased with $MnCl_2$ administration, while quercetin co-treatment at both 5 and 10 mg/kg reduced these effects (Fig. 6).

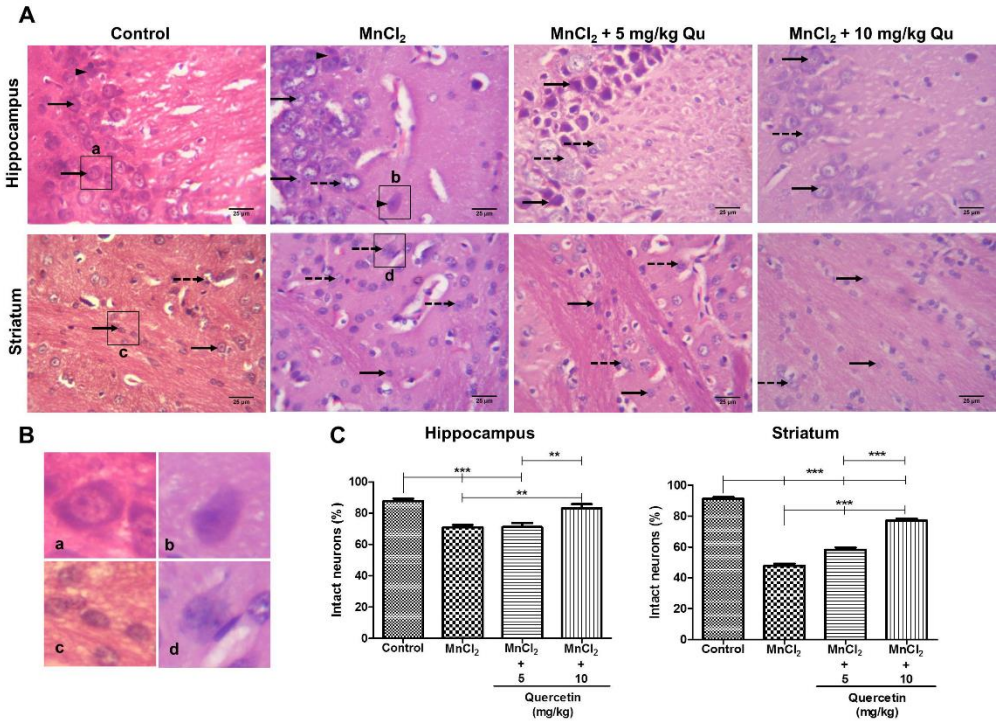

Fig. 3. (**A**) – Photomicrographs of hippocampus (CA3) and striatum (CPu) of control and treated rats. H&E x 400, Scale bars – 25 µm. Arrows – intact neurons; arrow heads – prominent eosinophilic cytoplasm; dashed arrows – neuron swelling and/or vacuolation within the cytoplasm. Boxes a, b, c, and d, are enlarged to show details of histological changes in (**B**). (**C**) – Image J analysis of H&E stained sections. Values are expressed as mean + SEM. ***P*<0.01, ****P*<0.001. One-way ANOVA followed by SNK for post-tests.

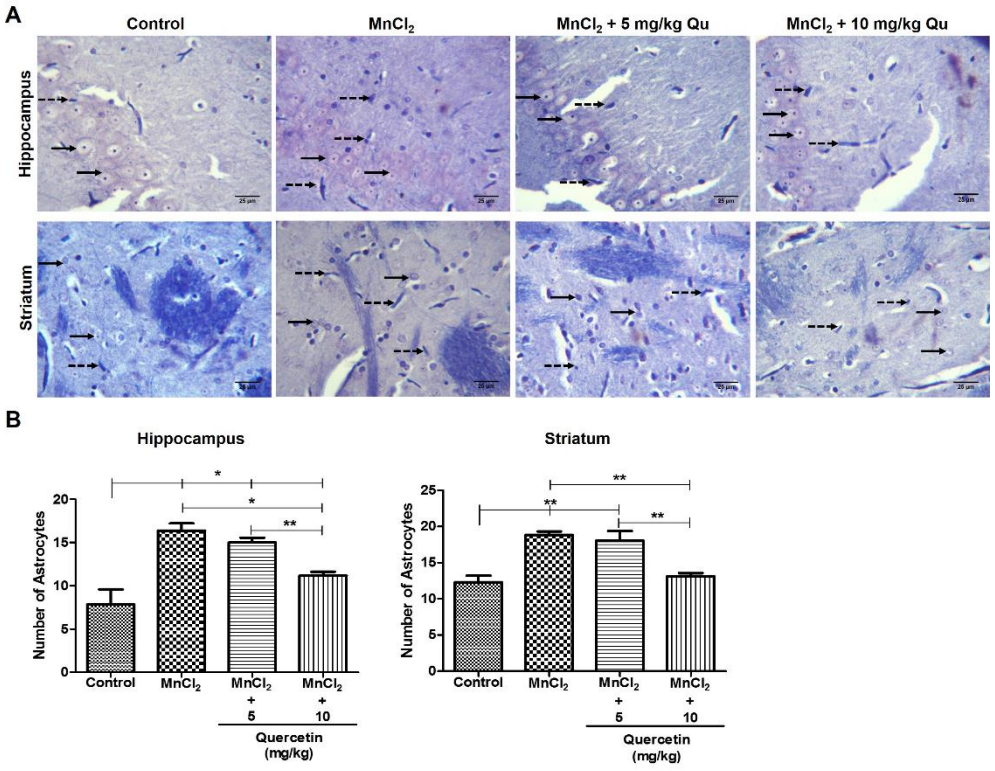

Fig. 4. (**A**) – Photomicrographs of PTAH stained sections of the hippocampus (CA3) and striatum (CPu) of control and treated rats. PTAH x 400, Scale bars – 25 µm. PTAH technique particularly marks astrocytes as purplish to bluish cells (dashed arrows) on brain sections. Neurons in the sections of the hippocampus and striatum are clearly identified (arrows). (**B**)– Image J analysis of PTAH stained sections. Number of astrocytes where identified and counted. Values are expressed as mean + SEM. **P*<0.05, ***P*<0.01. One-way ANOVA followed by SNK for post-tests.

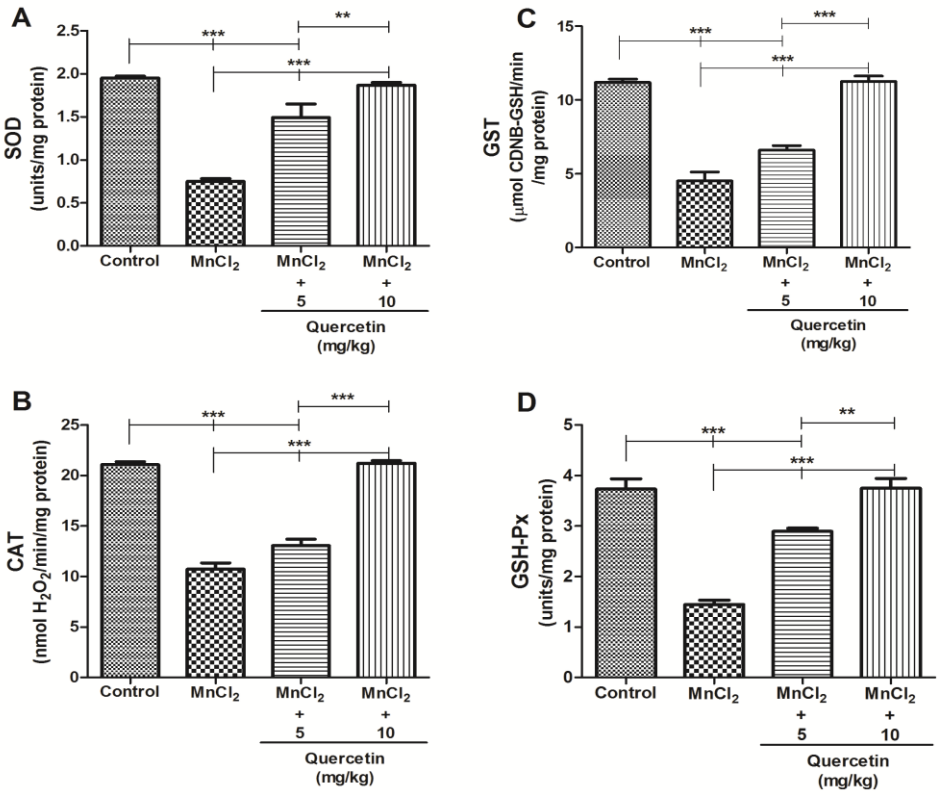

Fig. 5. Effect of Mn and Quercetin co-administered with Mn on activities of SOD (**A**), CAT (**B**), GST (**C**), and GSH-Px (**D**). Values are expressed as mean + SEM. ***P*<0.01, ****P*<0.001. One-way ANOVA followed by SNK for post-tests.

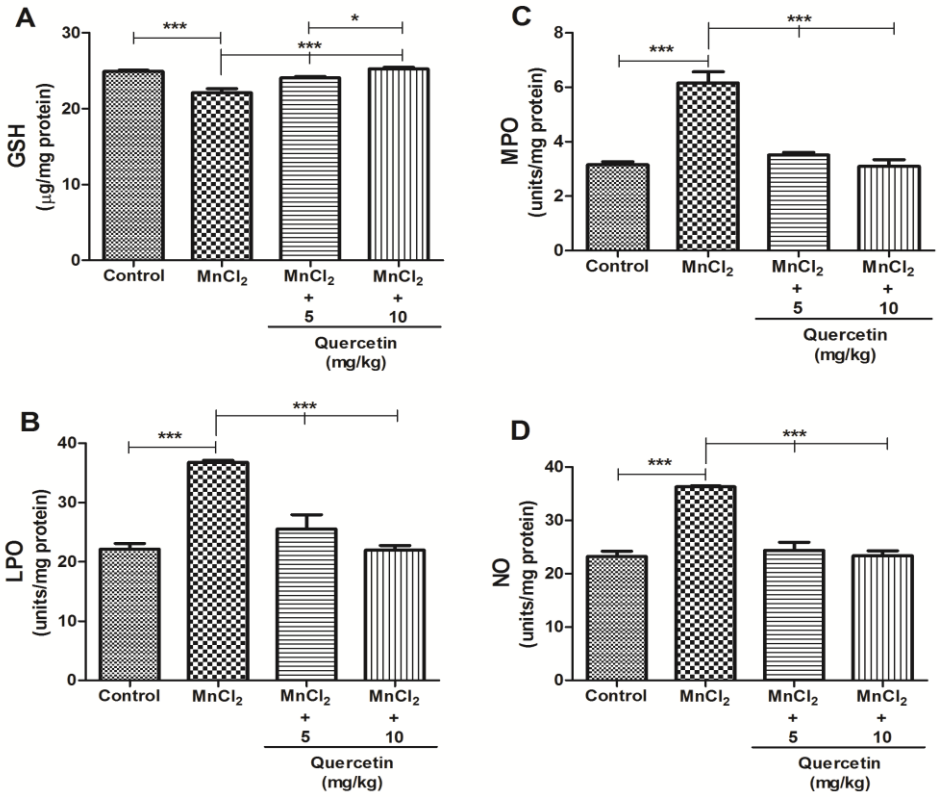

Fig. 6. Effect of Mn and Quercetin co-administered with Mn on GSH (**A**), LPO (**B**), MPO (**C**), and NO (**D**). Values are expressed as mean + SEM. **P*<0.05, ****P*<0.001. One-way ANOVA followed by SNK for post-tests.

DISCUSSION

Overexposure to Mn induces parkinsonian-like syndrome that includes behavioral deficits such as motor un-coordination and cognitive impairment, as well as degenerative changes to certain brain regions (Liu *et al.*, 2006a). In the present study, the protective effect of quercetin on Mn-induced neuronal dysfunction in rat was revealed.

In the present study, behavioral analysis of performance on the Y-maze revealed that quercetin co-administration with Mn exposure at 10 mg/kg significantly attenuated memory deficits caused by Mn exposure. Spontaneous alternation is a classic indicator of short-term memory on the Y-maze (Ijomone *et al.*, 2015). In support of our present observation on short-term memory, several studies have shown cognitive enhancing effects of quercetin in experimental rodent models where memory impairment was induced as well as in rodent models with no induced memory deficits. Quercetin attenuates detrimental effects on spatial learning and memory on the Morris water maze following cognitive impairment induced by chronic stress (Mohammadi *et al.*, 2014), in 6-OHDA rat model of Parkinson disease (Sriraksa *et al.*, 2012), and in D-galactose-induced aged mice (Liu *et al.*, 2006b). Quercetin enhances memory retrieval of kindled rats in the passive avoidance test (Nassiri-Asl *et al.*, 2013), and ameliorate cognitive dysfunction in diabetic rats (Bhutada *et al.*, 2010). Additionally, quercetin showed cognitive enhancing effects when administered to normal rats via intranasal route (Tong-Un *et al.*, 2010).

The primary interest in the OFT is movement, which is greatly influenced by locomotor output and exploratory drive. Locomotion frequency and rearing are indications of animals' overall locomotor and exploratory activities respectively (Ijomone *et al.*, 2014). Previous studies have shown that quercetin improves overall locomotor activities in several models of motor deficits. Quercetin was shown to improve motor coordination, balance and gait in MPTP-induced mouse model of Parkinson's disease (Lv *et al.*, 2012) and 3-NP-induced model of Huntington's disease (Sandhir and Mehrotra, 2013). However, behavioral analysis on the OFT in the present study showed that though quercetin administration had no effect on reduced locomotion following Mn administration, it prevented reduction in exploratory activity induced by Mn.

Here, we have examined microstructural changes to two regions – hippocampus and striatum – of the brain due to their high susceptibility to Mn deposition (Daoust *et al.*, 2014; Robinson *et al.*, 2013; Liu *et al.*, 2006a; Erikson *et al.*, 2005). Histological analyses in the present study suggest that Mn exposure imposes degeneration in the hippocampal and striatal neurons with percentage of neurodegeneration worse in the striatum. A previous study showed that mouse primary hippocampal neurons are sensitive to Mn toxicity (Daoust *et al.*, 2014). Another study on mouse striatum showed that Mn treatment resulted in neuronal injury that was evident by the presence of condensed, pyknotic neurons and presence of dying neurons (Liu *et al.* 2006a). It was possible that compromised behaviors observed in the present study following Mn administration, was due to the increased degeneration of neurons in these brain regions. The hippocampus is well recognized for its role in cognitive processes of memory and learning (Squire, 2009; Moser *et al.,* 2008). The striatum on the other hand, is associated with central control of overall control of locomotor functions (Voytek and Knight, 2010). It is also likely that the greater neuronal damage imposed on the striatum by Mn exposure may have resulted in the inability of quercetin co-administration to attenuate Mn-impaired locomotor related deficits. This is in contrast to attenuating effect of quercetin treatment on Mn-impaired cognitive abilities, probably due to the less damage on hippocampal neurons.

The present study also showed that Mn induced astrocytosis in both the hippocampus and striatum. Several studies have revealed that astrocytosis is indicative of neurotoxicity and consequent neuronal injuries (Jahanshahi *et al.*, 2013; Granado *et al.*, 2010; Granado *et al.*, 2011; Adori et al., 2006; Aguirre et al., 1999), as we have also observed in the present study following Mn treatment. Liu *et al.* (2006a) observed that increase in astrocyte-derived NO together with astrocytosis is involved in neurodegeneration imposed on the striatum following Mn treatment. Our present study revealed that quercetin at 10 mg/kg attenuated abnormal astrocyte proliferation and this may be a contributing mechanism in protecting against neuronal damage.

Previous studies have shown that exposure to Mn significantly induced oxidative stress in the brain, thus eliciting various pathological conditions associated with neurodegenerative diseases (Milatovic *et al.*, 2009). Our findings were consistent; having yielded the same observations in the brain of rats exposed to Mn. Oxidative stress and formation of free radicals are the major factors of many neurodegenerative disorders (Ebokaiwe and Farombi, 2015). Among the prominent antioxidant enzymes; SOD is at the front line, it helps to dismutate superoxide radicals to less harmful product like H_2O_2 which also can be a potential oxidant. CAT and GSH-Px acts in tandem to convert the generated H_2O_2 to water and less harmful products (Ebokaiwe *et al.*, 2013) whereas GST, a phase-II xenobiotic detoxifying enzyme, helps in the excretion of the toxic products of oxidative stress from the system (Ebokaiwe and Farombi, 2015). However, when there is a surge in production of superoxide radicals, the integrity of these enzymes are compromised which could be the reason for the significant decrease in the activities of SOD, CAT, GSH-Px enzymes and GST, a phase-II xenobiotic detoxifying enzyme, following Mn administration. Induction of oxidative stress by Mn exposure was further confirmed by significant elevation in the levels of LPO; which destroy and compromise the integrity of the

membrane, MPO; which generates hypochlorous acid that damages nearby tissues and NO that can react with many other free radicals i.e. superoxide radical, generating peroxynitrite radical causing oxidative changes to numerous tissues (Sally *et al*., 2015) these were followed by a concomitant decrease in GSH; an important antioxidant that functions as a direct reactive free radical scavenger being utilized by GSH-Px enzyme.

Taken together, the current results support earlier studies (Milatovic *et al*., 2009; Chtourou *et al*., 2011) that Mn accumulation leads to generation of free radicals, and alterations in antioxidant enzymes, consequently resulting in oxidative stress. Studies have also shown that inflammatory responses and apoptotic changes are involved in Mn induced neurotoxicity, with oxidative stress acting as triggers (Milatovic *et al*., 2009; Milatovic and Aschner 2009). The pathways involved in Mn induced oxidative stress, inflammation and apoptosis are interrelated (Tarale *et al*., 2016; Li *et al*., 2010).

Quercetin can effectively prevent oxidative damage to DNA or to cell membrane (Kandaswamy *et al*., 2012). One mechanism has to do with quercetin stabilizing lipid membranes and protecting lipid peroxidation caused by environmental toxicants including Mn exposure through the free radical-scavenging mechanism, thereby protecting tissues. This is evidenced in the current study by observations made in brain of rats co-treated with quercetin. This finding also corroborates earlier studies (Ebokaiwe and Farombi 2015). Furthermore, quercetin has also shown anti-apoptotic and anti-inflammatory effects (Kanter *et al*., 2016; Lei *et al*., 2015; Endale *et al*., 2013; Cho *et al*., 2003). Taken together, it is likely that in addition to anti-oxidative stress mediated inhibition of inflammatory and apoptotic response by quercetin upon Mn exposure, quercetin may also have direct influence on these processes in exhibiting its neuroprotective effects.

In conclusion, the present study suggests that quercetin attenuates Mn-induced behavioral deficits, neuronal injuries and astrocytosis via its potency to resist increased oxidative stress. These results indicate that the dietary antioxidant quercetin from food or feed consumed by human and animals may improve impaired health as a result of environmental or occupation Mn exposure.

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Conflict of interests

The authors declare no conflict of interest.

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