

Quercetin Protects Against Chronic Aluminum-Induced Oxidative Stress and Ensuing Biochemical, Cholinergic, and Neurobehavioral Impairments in Rats

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Abstract In this study, we investigated the protective effect of chronic quercetin (a natural flavanoid) administration against Al-induced cognitive impairments, oxidative damage, and cholinergic dysfunction in male Wistar rats. Al lactate (10 mg/kg b.wt./day) was administered intragastrically to rats which were pre-treated with quercetin (10 mg/kg b.wt./day, intragastrically) for 12 weeks. At the end of 6 or 12 weeks of the study, several behavioral parameters were carried out to evaluate cognitive functions. Further after 12 weeks of exposure, various biochemical tests and H&E staining were performed to assess the extent of oxidative damage and neurodegeneration, respectively. Al levels were also estimated in HC and CS regions of rat brain. Chronic administration of quercetin caused significant improvement in the muscle coordination, cognition, anxiety, locomotion, and initial exploratory patterns in Al-treated rats. Quercetin supplementation to Al-treated animals also reduced oxidative stress, decreased ROS production, increased MnSOD activity and glutathione levels with decreased lipid peroxidation and protein oxidation. It increased AChE activity and ATP levels in HC and CS regions of rat brain compared to Al-treated rats. Quercetin administration ameliorates Al-induced neurodegenerative changes in Al-treated rats as seen by H&E staining. Further with the help of atomic absorption spectrophotometer, we

found that quercetin supplementation to Al-treated rats also decreases the accumulation of Al in the HC and CS regions of rat brain. Taken together the results of this study show that quercetin offers neuroprotection against Al-induced cognitive impairments, cholinergic dysfunction, and associated oxidative damage in rats.

Keywords Oxidative stress · Reactive oxygen species · Cholinergic · Quercetin · Cognitive · Neurobehavioral

Abbreviations

Al	Aluminum
ROS	Reactive oxygen species
MnSOD	Manganese superoxide dismutase
AChE	Acetylcholinesterase
MWM	Morris Water Maze
EPM	Elevated Plus Maze
HC	Hippocampus
CS	Corpus striatum
H&E	Hematoxylin and eosin

Introduction

Aluminum (Al) has been linked etiologically and epidemiologically to several neurological disorders including Alzheimer's disease (Solfrizzi et al. 2006; Santibanez et al. 2007), Parkinson's disease (Yasui et al. 1992), Guamian–Parkinsonian complex and Amyotrophic lateral sclerosis (Forbes et al. 1995). Exposure to high levels of Al leads to neurofibrillary degeneration and the concentration of Al has been found to be increased in degenerating neurons (Bharathi Shamasundar et al. 2006). Mounting evidence in the recent years has suggested Al to have severe toxic

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manifestations on the central nervous system. In the central nervous system, neurotransmission is coupled to learning and memory, and therefore changes in the neurotransmission would certainly affect the behavioral responses. It has been shown to result in changes in acetylcholinesterase (AChE) activity by many researchers (Zatta et al. 2002). Earlier studies from our laboratory have also shown that acute as well as subacute exposure to Al causes marked reduction in AChE activity and significant deterioration in cognitive functions in rats (Julka et al. 1995). There is a large body of evidence showing that cholinergic activity is necessary for acquisition and retrieval of several learning skills (Julka et al. 1995; Domingo 2006). Many workers have demonstrated that central cholinergic dysfunction is correlated with impaired performance on various cognitive tasks, whereas improved performance is often observed with elevated cholinergic function (Harder et al. 1998; Leanza et al. 1996; Vannucchi et al. 1997). Al-induced neurobehavioral deficits and morphological changes in rat brain were also reported by Colomina et al. (2002). They observed an increased percentage of perforated synapses in the Al-treated animals. In addition, various animal studies have also shown that Al exposure causes neuropathological, neurobehavioral, and neurochemical changes resulting in impaired learning ability (Julka et al. 1995; Colomina et al. 2002; Miu et al. 2003; Kaur and Gill 2006).

Mitochondria are considered to be the major source of reactive oxygen species (ROS) production and key contributors to neurodegenerative diseases (Leuner et al. 2007). Mitochondrial dysfunction has been implicated in many neurodegenerative diseases associated with increased levels of free radicals and oxidative damage (Williams et al. 1998). Primary function of mitochondria is oxidative metabolism and any defect in ATP generating capacity by toxic chemicals may lead to energy failure, cellular dysfunction, and eventually cell death as seen in many neurodegenerative diseases. Mitochondria are more prone to ROS-induced cellular injury (Liang and Godley 2003) as they lack protective proteins. Mitochondrial dysfunction may ensue mitochondrial damage including oxidation of mitochondrial DNA, proteins, and lipids leading to the opening of mitochondrial permeability transition pore, an event associated with neuronal cell death (Halliwell 1992). The generation of these ROS may induce the oxidative damage to mitochondrial proteins. Increased mitochondrial oxidative damage has also been observed in early pathological events leading to neurodegeneration (Beal 1996). Thus, Al might be implicated in the functional decline of mitochondria that may lead to ROS generation. This entire phenomenon may be associated with increased oxidative stress and compromised antioxidant defense system.

Enhanced ROS production and oxidative injury play a cardinal role in the onset and progression of neurodegenerative disorders. Our lab has already reported that chronic Al exposures increase ROS generation and is implicated in the impairment of mitochondrial functions in various regions of rat brain (Kumar et al. 2008b). It is widely believed that oxidative stress is a major causative factor for the progressive decline in motor and memory functions in humans and animals (Navarro et al. 2002).

Flavonoids are a class of dietary antioxidants that appear to have potential beneficial health effects. These are polyphenolic compounds widely distributed in fruits and vegetables. The average daily intake of flavonoids in the diet is about 23 mg of which quercetin is one of the most abundant representing more than 60 % of the average polyphenol ingestion (Goldberg et al. 1995; Sampson et al. 2002; Boots et al. 2008). Novel therapeutic neuroprotective strategies support the administration of ROS scavengers, transition metal chelators, or natural antioxidants and polyphenols in monotherapy or as part of an antioxidant cocktail formulation to regulate the progression of oxidative stress-related diseases (Slikker et al. 1999). Quercetin (3,5,7,3',4'-pentahydroxyflavone, a bioflavonoid), can be proposed as a therapeutic drug and can be used against aluminum-induced oxidative stress. Quercetin, frequently found in consumable foods including apples, berries, onions, tea, and brassica vegetables, has many beneficial effects on human health, including cardiovascular protection, anticancer activity, cataract prevention, antiviral activity, and anti-inflammatory effects (Nagata et al. 1999). Quercetin has been reported to possess free radical scavenging, Al chelating, and anti-inflammatory activities (Hanasaki et al. 1994). Moreover, several experimental investigations showed the potential of quercetin against cognitive deficit in various animal models (Kumar et al. 2008a; Liu et al. 2006; Pu et al. 2007; Singh et al. 2003; Tota et al. 2010; Yao et al. 2010). However, the effect of quercetin on oxidative status in animal models has not been analyzed thoroughly so far in context to Al neurotoxicity. Therefore, this study was designed to investigate the protective role of quercetin against neurobehavioral and cognitive impairments, cholinergic dysfunctions, and oxidative damage induced by chronic Al exposure.

Materials and Methods

Chemicals

Al L-lactate, quercetin (3,5,7,3',4'-pentahydroxyflavone), 5,5'-dithiobisnitrobenzoic acid (DTNB) and 2-thiobarbituric acid (TBA) were obtained from the Sigma Chemical

Co. (St. Louis, MO, USA). All other chemicals and solutions were of analytical grade unless otherwise specified.

Animals and Their Care

Male albino rats (Wistar strain) in the weight range of 120–150 g, at the beginning of the experiments were procured from the institute animal house. The animals were housed in polypropylene cages, and kept in well-ventilated rooms on a 12-h light–dark cycle. Animals were provided standard rat pellet diet (Ashirwad Industries, Chandigarh, India) and water ad libitum. Rats used in this study were maintained and treated in accordance with the guidelines established by the Ethical and Practical Principles of the Use of Laboratory Animals and all procedures used in this study complied with the Guide for the Care and Use of Laboratory Animals.

The animals were divided into eight sets of following four groups (6 animals in each group): Total number of animals used was 192.

Control group (C) In this group, animals received an equal volume of distilled water (vehicle) as administered to the animals in chronic Al dose group.

Chronic aluminum-treated group (Al) In this group, animals received Al, in the form of Al lactate (10 mg/kg b.wt./day) dissolved in distilled water, intragastrically for 12 weeks.

Chronic aluminum + quercetin-treated group (Al + Q) In this group, animals received Al, in the form of Al lactate (10 mg/kg b.wt./day) dissolved in distilled water and quercetin (10 mg/kg b.wt./day) and dissolved in 1 ml of 1 % methylcellulose intragastrically for 12 weeks.

Chronic quercetin-treated group (Q) In this group, animals received quercetin (10 mg/kg b.wt./day) dissolved in 1 ml of 1 % methylcellulose intragastrically for 12 weeks.

The formulation of quercetin and the corresponding vehicle was administered in the mornings and the formulation of Al in the form of Al lactate and its corresponding vehicle administered in the afternoon for a period 12 weeks. Behavioral studies were carried out as per plan, i.e., after 6 weeks and then after the last dose of treatment (Fig. 1). Each behavioral test was carried out in a separate set of six rats selected from each treatment group. For hematoxylin and eosin (H&E) staining, another set of animals was used (6 animals in each group), for neurochemical studies (another set of 6 animals in each group), and finally for measuring Al levels with the help of atomic absorption spectrophotometer (another set of 6 animals in each group) rats were killed by decapitation using sodium pentathol around 24 h after the last dose of treatment (12 weeks). The brains were removed, rinsed in ice-cold physiological saline (0.9 % NaCl). The hippocampus (HC) and corpus striatum (CS) regions were dissected as per the guidelines of Glowinski and Iversen (1966). Ethical

clearance for killing of animals was duly obtained from Institute's Animal Ethics Committee. Brain regions were processed immediately for the assay of parameters related to oxidative stress.

Neurobehavioral Studies

Motor Function Test

Rota rod test was carried out to evaluate the muscle strength and coordination movements of the animals (Dunham and Miya 1957). As a part of the test procedure, the animals were initially trained to maintain themselves on a rotating rod at 15 rpm for a period of more than 3 min. Subsequently, after a period of 24 h, the animals were again screened for their ability to remain on the rotating rod for three successive trials of 3 min each. In the event of their inability to remain on rotating rod for 3 min, the test was considered as positive, i.e., motor incoordination was said to have been produced.

Shuttle Avoidance Tests

Passive and active avoidance tasks were performed. A shuttle box (Techno, India) was used to assess the conditioned avoidance learning tasks (Piala et al. 1959) with slight modifications.

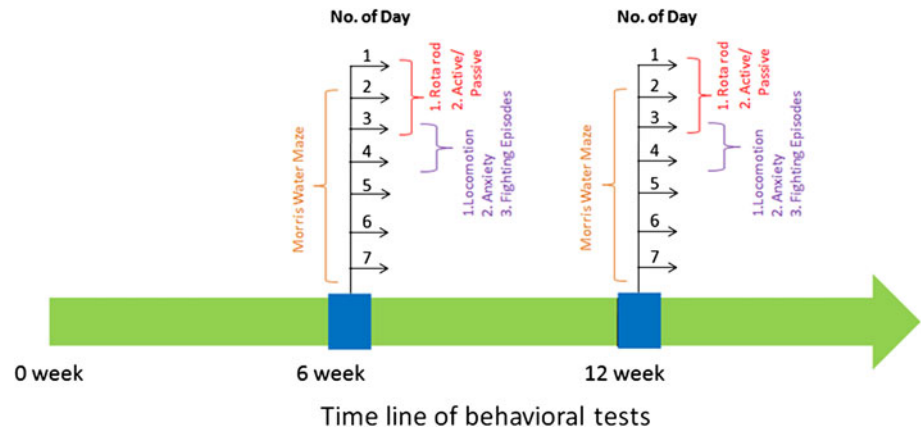
Passive Avoidance Test

The experiment was performed by the method of Piala et al. (1959) with slight modifications (Sharma et al. 2009). In brief, a shuttle box apparatus consisting of a dark and an illuminated chamber separated by a door was used in the study. The floor of dark chamber consisted of a metal grid wired to deliver shocks of controlled intensities and durations, whereas the floor of the illuminated chamber was properly insulated. On the first day, each animal was placed into the illuminated compartment and allowed to explore both chambers of the apparatus for 5 min. On the second day, each animal was placed into the illuminated chamber of the apparatus. As soon as the rat entered the dark chamber, 0.1 mA, 40 V was applied till the animal crossed the door and came back into illuminated chamber. After the shock, animal was removed and returned to their home cage. On day 3, each animal was placed into the illuminated chamber, and the latency to enter into the dark chamber was measured, which served as measure of retention of avoidance response.

Active Avoidance Test

The apparatus consisted of a shuttle box with two adjacent light and dark chambers connected through a passage of

Fig. 1 Schematic representation of all behavioral parameters performed after 6 and 12 weeks of AI and quercetin supplementation



sliding manual door. Scrambled electric foot shock of adjustable intensity was given to the animals through the grid floor in one chamber while the other served as a non-shock chamber. Rats received a conditioned stimulus in the form of a buzzer for 2 s followed by a buzzer and foot shock of 0.1 mA, 40 V for 5 s. The avoidance was considered positive if the rats jumped to a shock-free chamber to avoid the foot shock. Untrained rats from control and experimental groups were individually placed in one chamber and habituated for 5 min. On the final day, each animal was placed into the dark chamber of the apparatus, 0.1 mA, 40 V foot shock was applied for 5 s, and the latency to enter into the illuminated chamber (to prevent the shock) was measured, which served as measure of retention of avoidance response. After performing the task, animal was removed and returned to their home cage.

Aggressive Behavior

Aggressive behavior was tested in an aggressometer (Techno India, Lucknow). The apparatus consisted of a Persepex chamber ($8' \times 8' \times 7'$), which has a grid floor of stainless steel rods for the purpose of delivering the foot shock. Aggressive behavior was tested as per the method described by Tedeschi et al. (1959). Preliminary screening was done to exclude the non-responding animals. The rat pairs (one at a time) were placed in the test chamber, acclimatized for 3 min, and a foot shock of 100 V, 2 mA, 5 Hz was administered for 10 s. Following this, the numbers of fighting episodes in 1 min were counted. Only those pairs of rats which exhibited at least one fighting episode in 1 min were included in the study. The fighting episode was considered positive when the rats converged abruptly to close quarters, stood face to face with each other, and struck each other with their forelimbs.

Morris Water Maze (MWM) Test

This test was carried out by the method of Morris (1984) with slight modifications. The animal is trained to escape

from water by swimming to a hidden platform. It can find the platform, which is under the water and serves as a “rescue” from the stress situation, by using visual extra-maze cues. In the navigation tank, the place of the platform is the same on each day but the starting point of the animal varies. This method requires a long-term spatial memory and learning.

Acquisition Test

A water tank of 600 mm diameter was filled with water up to 8 cm from the top of the tank. A platform with an 8×8 cm top surface was placed in the middle of one quadrant about 24 cm from the side. The top surface of the platform was submerged about 1 cm below the surface of the water. All animals were given four training trials (acquisition) on days 1–4. On each training trial, the animal was placed into the water with its nose facing the side of the tank at one of four randomly selected locations corresponding to each quadrant of the maze, and then it was released. On fifth day, the number of entries and time spent in the quadrant containing the hidden platform were recorded.

Retrieval Test

On the sixth day, the platform was removed, and each animal was placed in the center of the water tank and allowed to swim for 90 s. The time spent in the target area (where the platform had been positioned on days 1–5) was recorded. The fifth day acquisition test is considered as measure of spatial reference memory and the retrieval test is considered as measure of the strength of spatial memory.

Open Field Test

Observations were recorded for three test sessions of 5 min each. Mean of test sessions total of control and treatment groups were compared for distance traveled (cm), total

time immobile (s), rearing (number), and ambulation (number). Open field tests were recorded automatically by Video Path Analyzer (ANY-maze™ software USA) comprising an open field chamber (50 × 50 × 35 cm) and a video camera fixed over the chamber. Behavioral testing was started 10 s after placing the rat over a black surface at the center of open field chamber.

Elevated Plus Maze (EPM) Test

The experiment was performed by the method as described before (Walf and Frye 2007) with slight modifications. The EPM is a widely used behavioral assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents and steroid hormones, and to define brain regions and mechanisms underlying anxiety-related behavior. In brief, rats or mice are placed at the junction of the four arms of the maze, facing an open arm, and entries/duration in each arm are recorded by a video-tracking system and observer simultaneously for 5 min. An increase in open arm activity (duration and/or entries) reflects anti-anxiety behavior. Defecation index was also calculated by measuring the number of pellets per minute.

Biochemical Markers of Oxidative Stress

Isolation of Mitochondria

Rat brain mitochondria were isolated using a recently described method of Brown et al. (2004). The dissected brains were placed in glass dounce homogenizer containing five volumes of isolation buffer with 1 mM EGTA, 215 mM mannitol, 75 mM sucrose, 0.1 % BSA, and 20 mM HEPES and pH adjusted to 7.2 with KOH. The tissue homogenate was spun at 1,300×g for 5 min in an Eppendorf microcentrifuge at 4 °C. The resulting pellet was resuspended in 0.5 ml of isolation buffer and spun again at 13,000×g for 10 min in order to pellet the mitochondria. The pellet was washed in the appropriate buffer and containing EGTA, centrifuged at 10,000×g for 10 min, and resuspended in the same buffer at a concentration of 10 mg/ml. The integrity of the mitochondria was checked by assessing respiratory control ratio (data not shown).

ROS Generation

Mitochondrial ROS generation was assessed as described previously (Wasilewski and Wojtczak 2005) with modifications. In brief, mitochondria were added to respiration buffer containing 5 mM pyruvate, 2.5 mM malate, and 10 μM of dichlorodihydrofluorescein diacetate (H2DCFDA). Fluorescence was quantified after 20 min incubation using a Cary

Eclipse fluorimeter (Varian, Palo Alto, USA) (excitation 488 nm, emission 525 nm) and related to total protein content. In control, carbonylcyanide-4-(trifluoromethoxy)-phenylhydrazone (FCCP) was added to inhibit membrane potential-dependent ROS production.

ATP Levels

ATP was determined luminometrically using ATP Bioluminescence assay kit (Sigma, St. Louis, MO, USA) according to the provided protocol. Mitochondrial samples were assayed for ATP content using the ATP dependence of the light emitting luciferase-catalyzed oxidation of luciferin. ATP concentration was calculated according to a standard curve and related to protein content assessed using the method of Lowry et al. (1951).

AChE Assay

AChE activity was assayed in the HC and CS homogenates by the method of Ellman et al. (1961) wherein the hydrolysis of acetylthiocholine to thiocholine and acetate is measured. The thiocholine reacts with 5,5'-dithiobisnitrobenzoic acid (DTNB) to give a mixed disulfide and 5-mercapto-2-nitrobenzoic acid, a yellow compound which was measured spectrophotometrically at 412 nm. The reaction mixture in a volume of 3.0 ml contained 0.1 M phosphate buffer (pH 7.5), 0.66 mM DTNB, 0.013 % triton X-100, and 100 μl of brain homogenate. The reaction was started by the addition of 0.83 mM acetylthiocholine and the rate of change in absorbance was followed at 412 nm using Perkin Elmer Lambda 20 UV VIS Spectrophotometer (Norwalk, CT, USA) for 2 min. Enzyme activity was calculated using the molar extinction coefficient of 5-mercapto-2-nitrobenzoic acid ($13.6 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$) and the results expressed as nmol product formed/min/mg protein.

Mitochondrial Manganese Superoxide Dismutase (MnSOD) Activity

MnSOD activity in total solubilized mitochondrial extracts was measured by the cytochrome *c* reduction method in the presence of 1 mM potassium cyanide to inhibit both Cu–Zn SOD and extracellular SOD as suggested by MacMillan-Crow et al. (1996). The amount of enzyme required to produce 50 % inhibition was taken as 1 U and results were expressed as U/mg protein.

The reaction mixture consisted of 1.9 ml of 50 mM sodium carbonate buffer (pH 10), 0.75 ml of 96 μM nitrobluetetrazolium (NBT in sodium carbonate buffer), and 0.15 ml of 0.6 % triton X-100 in sodium carbonate buffer in presence of 1 mM potassium cyanide. Initial absorbance (*X*) at 560 nm was taken for 3 min, and then

0.1 ml of mitochondrial sample from brain homogenate was added to this reaction mixture. Again the absorbance at 560 nm (Y) was noted for 3 min. Rate of inhibition of the reaction by the enzyme was calculated as $(X - Y \times 100/Y)$.

Glutathione Reductase Activity

Glutathione reductase activity was determined as previously described (Worthington and Rosemeyer 1974). The reaction mixture consisted of 0.5 ml of 100 mM phosphate buffer (pH 7.6), 0.2 ml double-distilled water (DDW), 0.05 ml of 60 mM EDTA, 0.05 ml of 2.0 mM NADPH, and 0.05 ml of 60 mM GSSG. The reaction was started by the addition of 0.1 ml mitochondrial sample from brain homogenate. The change in absorbance was observed for 5 min at 30 s interval at 340 nm. Enzyme activity was calculated on the basis of molar extinction coefficient for NADPH at 340 nm ($6.22 \times 10^{-6} \text{ M}^{-1} \text{ cm}^{-1}$). Specific activity of the enzyme was expressed as μmole of NADPH utilized/min/mg protein.

Glutathione Peroxidase Activity

Glutathione peroxidase activity was determined by the method of Necheles et al. (1969). The reaction mixture consisted of 0.4 ml of 0.4 M phosphate buffer (pH 7.0), 0.1 ml of 10 mM sodium azide, 0.2 ml of 8 mM GSH (freshly prepared), 0.1 ml mitochondrial sample from brain homogenate, 0.1 ml 2.5 mM H_2O_2 , and 1.1 ml DDW. This was incubated at 37 °C for 3 min. After incubation, 0.5 ml TCA 10 % was added to it. The reaction mixture was then centrifuged at 3,000 rpm for 15 min. After centrifugation, 0.5 ml supernatant was taken and 3.0 ml of 0.3 M disodium hydrogen phosphate was added to it. This was followed by the addition of 1.0 ml DTNB (2 mg/50 ml of 1 % sodium citrate, freshly prepared). Absorbance was recorded at 412 nm within 5 min of addition of DTNB. Appropriate blank and standard 0.02 mg/ml (freshly prepared) were also taken. The results were expressed as μg of GSH consumed/min/mg protein.

Lipid Peroxidation

Lipid peroxidation was assayed by the method of Wills (1966). A mitochondrial sample of 0.5 ml was diluted to 1.0 ml using Tris-HCl buffer (0.1 M, pH 7.4). The reaction mixture was incubated at 37 °C for 2 h with constant shaking. At the end of the incubation, 1.0 ml of TCA (10 %, w/v) was added and then after thorough mixing the reaction mixture was centrifuged at 500 rpm for 10 min. To 1.5 ml supernatant, 1.5 ml TBA (0.67 % w/v) was added and color developed by placing the tubes at 100 °C for 10 min in a boiling water bath. Samples were cooled and diluted with 1.0 ml DDW. The results were expressed as nmol TBARS/mg protein.

Protein Oxidation

Quantitation of protein carbonyl content as an index of protein oxidation in mitochondrial preparation was determined after derivatization with 2,4-dinitrophenyl hydrazine (DNPH) using protein carbonyl assay kit (Cayman, Chemicals, USA). The amount of protein carbonyl was calculated on the basis of molar extinction coefficient of DNPH ($0.022 \mu\text{M}^{-1} \text{ cm}^{-1}$). The results were expressed as nmol carbonyl/mg protein.

H&E Staining

After killing the rat, brain regions (HC and CS) were isolated and washed with normal saline followed by 50 ml of 4 % paraformaldehyde in phosphate-buffered saline, post-fixed in 10 % formalin for 5 days, and then paraffin-embedded blocks were made. Paraffin sections stained with hematoxylin and eosin were examined by light microscopy.

AI Estimation

AI analysis was carried out in the various brain regions by wet acid digestion method of Zumkley et al. (1979). Levels of AI in HC and CS regions of rat brain were estimated using atomic absorption spectrophotometer.

Protein Determination

Mitochondrial protein was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Statistical Analysis

All values are expressed as mean \pm SEM of 6 animals in each group. One-way ANOVA, with Holm-Sidak test were used for analysis of the data and values with $P < 0.001$, $P < 0.01$, $P < 0.05$ were considered statistically significant. All the calculations were carried out by Sigma Stat computer software program 3.5 (TE subsystems, Inc., Germany).

Results

Effect on the Body Weight of Rats

Chronic AI exposure for 12 weeks caused significant reduction (50.44 %) of gain in body weight in AI-treated animals compared to controls, whereas in AI + Q group there was marginal reduction (12.21 %) of gain in body weight as compared to AI-treated rats after 12 weeks of exposure, not much difference was observed in food intake (data not shown). No significant change in the body weight

Table 1 Effect of chronic Al and quercetin supplementation on the body weight of rats

	Initial body weight (g)	Final body weight (g)	Gain in body weight (%)
Control	102 ± 10.47	330 ± 18.39	100
Aluminum	105 ± 12.34	220 ± 19.27	50.44***
Al + Q	104 ± 11.39	235 ± 20.37	12.21 ^{##}
Quercetin	107 ± 13.87	340 ± 21.27	102.2 ^{NS}

The animals were divided into four groups ($n = 6$): Control, Al (10 mg/kg b.wt./day), Al + Q and Quercetin (10 mg/kg b.wt./day) for 12 weeks

NS non-significant difference

The values are mean ± SEM of 6 animals *** $P < 0.001$ significantly different from control, ^{##} $P < 0.01$ significantly different from Al

was observed in rats treated with quercetin alone compared with control rats (Table 1).

Neurobehavioral Studies

Behavioral studies were carried out as per plan, i.e., after 6 weeks and then after the last dose of treatment, i.e., after 12 weeks (Fig. 1).

Effect on Rota Rod Performance

The rota rod test revealed a marked impairment in the muscle strength and coordination of Al-treated animals. The muscular coordination was significantly decreased after 6 weeks ($P < 0.01$) and 12 weeks ($P < 0.001$) in the Al-treated rats as compared to the control rats. The average retention time was reduced by 18.9 and 55.2 % in Al-treated rats after 6 or 12 weeks of exposure, respectively, as compared to the controls. The Al-treated animals showed neuromuscular incoordination and seemed confused during training period as compared to the control animals. None of the Al-treated animals could maintain themselves on the rotating rod for the full quota of the cut-off time (180 s). However, 6-week chronic quercetin administration to Al-treated rats did not show any protection but 12 weeks of quercetin supplementation to Al-treated rats resulted in significant increase in the retention time (42.8 %). There is a significant decrease in rota rod performance in Al + Q-treated group as compared to control after 6 weeks ($P < 0.01$) and 12 weeks ($P < 0.05$) of exposure. No significant change on the rota rod performance was observed in rats treated with quercetin alone compared with control rats (Fig. 2).

Effect on Passive Avoidance Tests

The passive avoidance response decreased significantly in terms of latency time in Al-treated rats in comparison to

control animals after 6 or 12 weeks of exposure (Fig. 3a). But when the animals were given Al + Q, they showed increased latency time in comparison to Al group. There was a significant decrease (53.6 %) after 6 weeks and (66.2 %) after 12 weeks of exposure in the time taken by the Al-treated animals to remember not to enter the chamber where they would experience a foot pad shock as compared to the control animals, whereas the animals receiving quercetin along with Al showed about 23.8 and 41.5 % higher latency time as compared to Al-treated rats. There is a significant decrease in latency time in Al + Q-treated rats as compared to control rats after 6 weeks ($P < 0.01$) and 12 weeks ($P < 0.05$) of exposure. No significant change in passive avoidance tests was observed in rats treated with quercetin as compared to controls (3a). Thus, quercetin supplementation to Al-treated animals demonstrated significant improvement in passive avoidance task as compared to Al-treated animals.

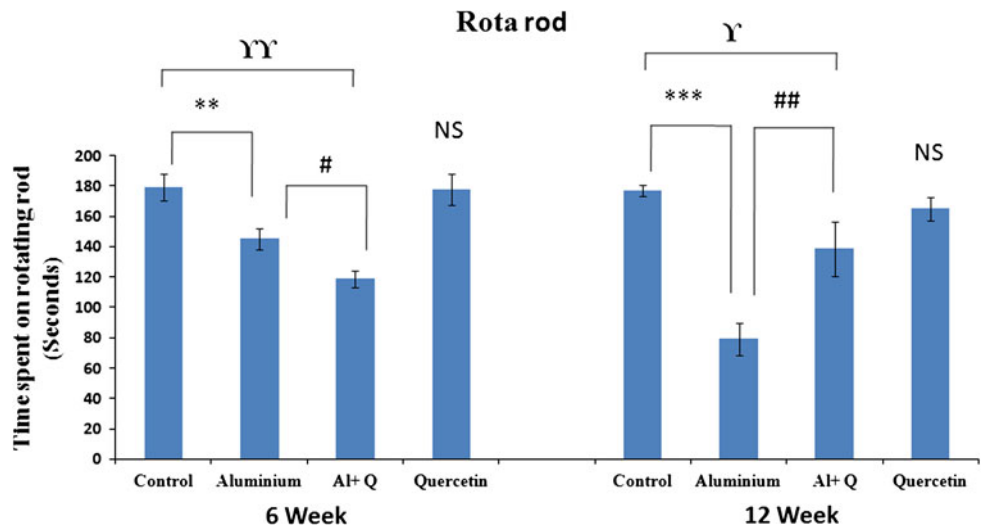
Effect on Active Avoidance Tests

The active avoidance response increased significantly in terms of latency time in Al-treated rats in comparison to control animals after 6 or 12 weeks of exposure (Fig. 3b), while the animals receiving Al along with quercetin showed decreased latency time as compared to Al-treated rats. The latency period of the Al group animals was about 76.9 % higher after 6 weeks and 78.5 % higher after 12 weeks than controls, whereas for animals in Al + Q-treated rats it was about 16.8 and 50.1 % less than Al-treated group after 6 or 12 weeks of exposure, respectively. Finally, this result proves that quercetin restores Al-induced impairments in short-term memory after 6 or 12 weeks of exposure. The latency time was significantly increased after 6 weeks ($P < 0.01$) and 12 weeks ($P < 0.05$) in the Al + Q-treated rats as compared to the control rats. No significant change on active avoidance tests was observed in rats treated with quercetin as compared to controls (3b).

Effect on Aggressive Behavior

Animals in the Al-treated group exhibited marked aggression, evidenced by significantly higher number of fighting episodes observed in them compared to the animals in the control group and there was a significant decreased number of fighting episodes in the Al + Q group as compared to the Al alone group (Fig. 4). There is a significant increase in aggressive behavior in Al + Q-treated rats as compared to control rats after 6 weeks ($P < 0.05$) and 12 weeks ($P < 0.05$) of exposure. No significant change in the aggressive behavior was observed in rats treated with quercetin alone compared with control rats.

Fig. 2 Effect of chronic Al and quercetin supplementation on the neuromuscular coordination in rat using rota rod test. The values are mean \pm SEM of 6 animals *** $P < 0.001$, ** $P < 0.01$ significantly different from control; ## $P < 0.01$, # $P < 0.05$ significantly different from Al group; $^{\gamma\gamma}P < 0.01$, $^{\gamma}P < 0.05$ significantly different from control group; and *NS* non-significant difference

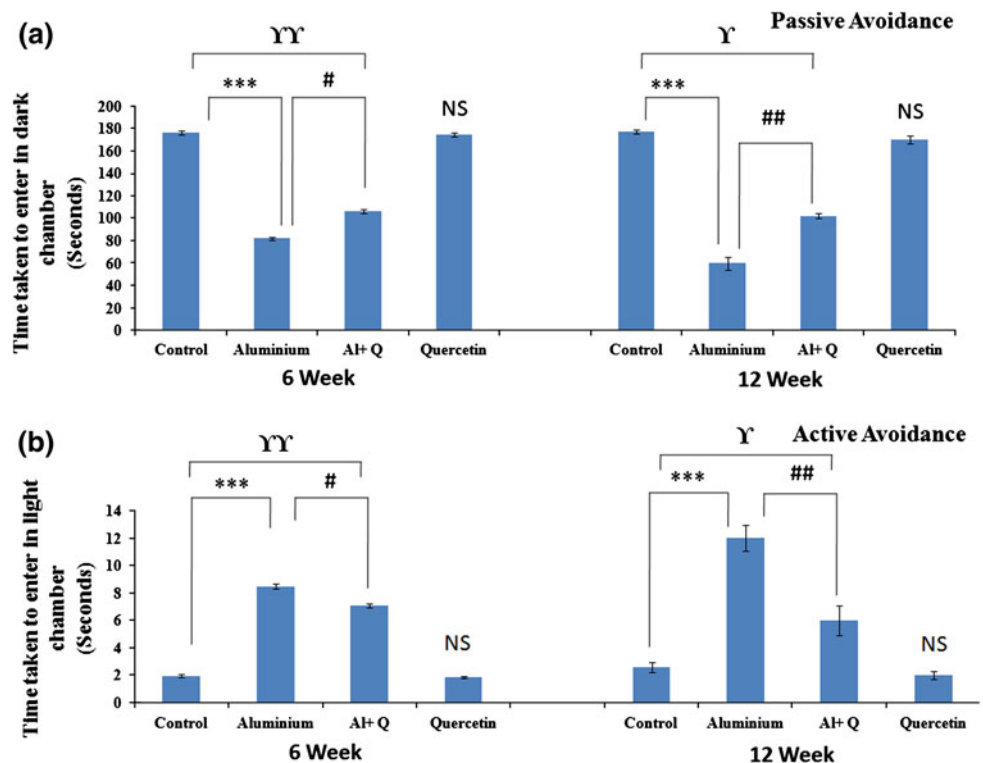


Effect on Spatial Memory (Acquisition Test)

MWM was carried out to test memory function. The time spent (s), number of entries, and overall average speed (m/s) in the quadrant containing hidden platform in the water maze beginning 24 h after the last quercetin exposure are illustrated in Fig. 5. The results indicate that the time spent, number of entries, and overall average speed in the quadrant containing hidden platform in the water maze for Al-exposed rats were comparatively less, when compared to control. At the same time in Al + Q group, the time spent, number of

entries, and overall average speed in the quadrant containing hidden platform were comparatively higher than that of Al alone after 6 or 12 weeks of exposure. On the final day, the time spent, number of entries, and overall average speed in quadrant containing hidden platform for Al-treated animals are 5.4 ± 0.18 , 3.9 ± 0.61 ; 4.7 ± 0.19 , 3.7 ± 0.56 ; and 0.20 ± 0.003 , 0.16 ± 0.02 , respectively, and for Al + Q-treated animals are 6.2 ± 0.07 , 7.5 ± 0.87 ; 6.1 ± 0.27 , 5.2 ± 0.48 ; and 0.22 ± 0.006 , 0.3 ± 0.12 , respectively, after 6 and 12 weeks of exposure. The time spent, number of entries, and overall average speed in the quadrant containing

Fig. 3 Effect of chronic Al and quercetin supplementation on **a** passive avoidance (time taken to enter in dark chamber) and **b** active avoidance (time taken to enter in light chamber) using shuttle avoidance test. The values are mean \pm SEM of 6 animals *** $P < 0.001$ significantly different from control; ## $P < 0.01$, # $P < 0.05$ significantly different from Al group; $^{\gamma\gamma}P < 0.01$, $^{\gamma}P < 0.05$ significantly different from control group; and *NS* non-significant difference



hidden platform in the water maze by Al + Q-exposed rats was comparatively less, when compared to control. No significant change in acquisition test was observed in rats treated with quercetin as compared to controls. The plot curve shows the path followed by animals of different groups after 6 or 12 weeks of treatment, which clearly shows that animals in the Al group are confused and take more time to locate the hidden platform (Fig. 6). Thus, animals from Al dose groups demonstrated significant impairments in performance of the task as compared to controls, whereas quercetin administration to Al-treated animals resulted in improvement in the performance of the task as compared to Al-treated animals.

Effect on Spatial Memory (Retrieval Test)

The effect of quercetin on the time spent, number of entries, and overall average speed in the quadrant are presented in Fig. 7, where the platform had been located during the first 5 days of testing. After 24 h of acquisition test, time spent, number of entries, and overall average speed in the case of Al-treated animals were significantly reduced when compared to control. The Al + Q-treated rats showed superior performance of retrieval test compared to Al alone (Fig. 7). On the final day, the time spent, number of entries, and overall average speed in quadrant for Al group animals are 6.8 ± 0.33 , 12.8 ± 0.36 ; 4.6 ± 0.17 , 5.5 ± 0.43 ; and 0.14 ± 0.002 , 0.13 ± 0.001 , respectively, and for Al + Q-treated animals were 8.1 ± 0.36 , 19.4 ± 0.4 ; 6.2 ± 0.2 , 7.2 ± 0.31 ; and 0.15 ± 0.002 , 0.15 ± 0.002 , respectively, after 6 or 12 weeks of exposure. We also calculated the number of entries in the area where earlier platform was placed. We found significant decrease in the number of entries in Al-treated animals (1 ± 0.31 , 0.83 ± 0.31) as compared to control (Fig. 7d, $P < 0.01$). On the other hand,

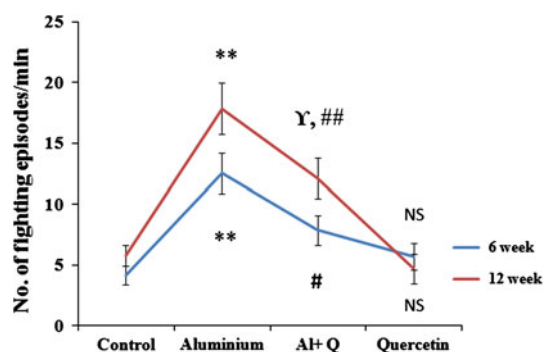


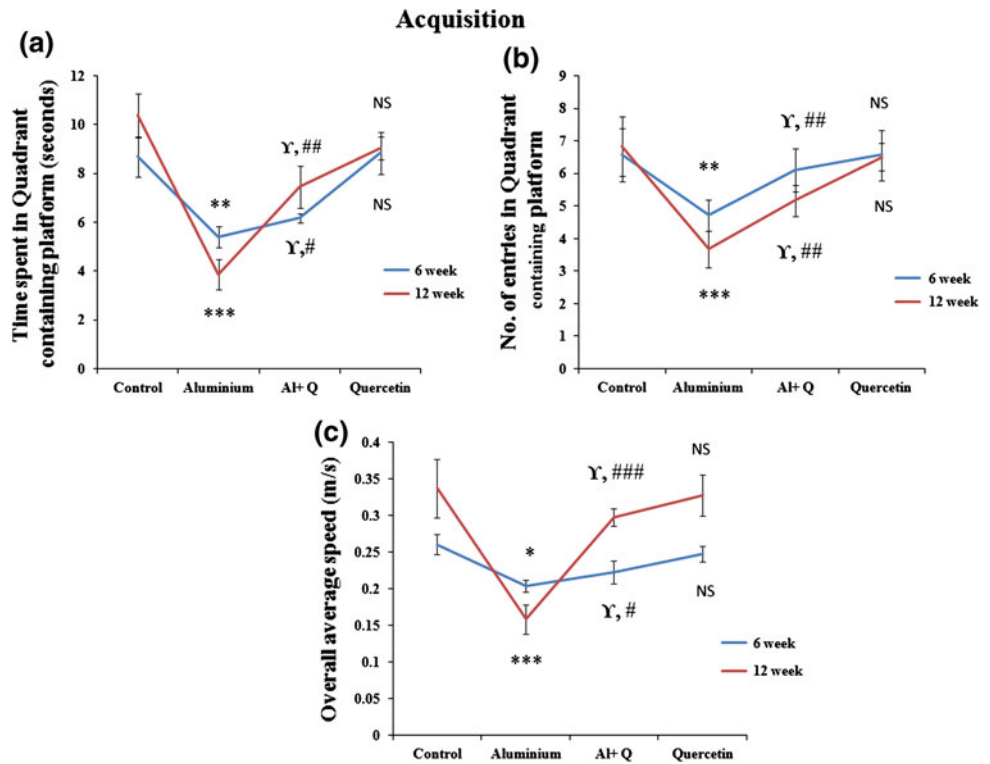
Fig. 4 Effect of chronic Al and quercetin supplementation on number of fighting episodes per minute. The values are mean \pm SEM of 6 animals $**P < 0.01$ significantly different from control; $##P < 0.01$, $#P < 0.05$ significantly different from Al group; $^?P < 0.05$ significantly different from control group; and *NS* non-significant difference

there was significant increase in the number of entries in Al + Q-treated animals (3 ± 31 , 3 ± 0.73) as compared to Al-treated animals ($P < 0.01$) after 6 or 12 weeks of exposure, respectively. The Al group animals showed decrease in their ability to recognize the area where platform was placed as compared to control animals. At the same time, Al + Q group showed improvement in their ability to recognize the area where platform was placed as compared to Al-treated animals. There is a significant decrease in the time spent, number of entries, overall average speed in the quadrant ($P < 0.05$) and also found decrease in number of entries in the area where earlier platform was placed ($P < 0.05$) in Al + Q-treated rats as compared to control rats after 6 or 12 weeks of exposure. The plot curve shows the path followed by different group animals after treatment, which clearly shows that Al-treated animals were more confused and took more time to recognize the position where hidden platform was placed earlier (Fig. 8) as compared to control. At the same time, Al + Q-treated animals were less confused and took less time to recognize the position where earlier hidden platform was placed as compared to Al-treated animals. No significant change in retrieval test was observed in rats treated with quercetin as compared to controls. Thus, quercetin supplementation to Al-treated rats demonstrated significant improvement in performance of the task as compared to Al-treated animals.

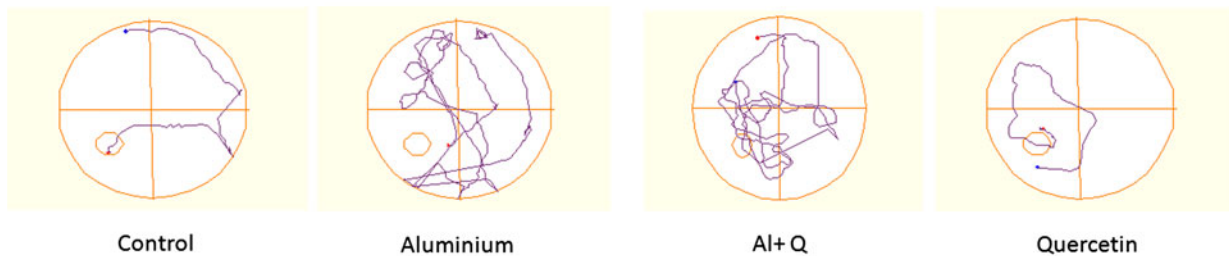
Effect on Spontaneous Locomotor Activity

Locomotor activity refers to the movement from one location to another. In rodents, one of the most important components of exploration, a prominent activity of the rat's repertoire of spontaneous activity, is locomotion. Hence, we studied the open field behavioral functions after chronic Al exposure. Rats were tested for behavioral activity after 6 and 12 weeks of Al and quercetin administration. Figure 9 illustrates the effect of chronic Al administration to rats on the locomotor activity in the open field. Six weeks after chronic Al administration, rats exhibited a significant decrease in all the components of the spontaneous locomotor activity which was more significant at 12 weeks of Al exposure. In this context, there was an appreciable decrease in the total distance traveled and total immobile time after 6 and 12 weeks of exposure and the alterations were more pronounced at 12 weeks post-exposure when compared to control. The distance traveled (cm) was significantly ($P < 0.001$) decreased and the rest time was increased in the Al group as compared to the control group, whereas there was a significant restoration in distance traveled and rest time in the Al + Q-treated rats as compared to the Al-treated rats alone (Fig. 9a, b). Rearing and ambulation were also observed by actophotometer. Total number of ambulations as well as rearing movements was also decreased significantly in the

Fig. 5 Effect of chronic Al and quercetin administration on **a** the time spent in the quadrant containing platform (acquisition), **b** number of entries in the quadrant containing platform (acquisition), and **c** overall average speed, in the quadrant containing platform (acquisition). The values are mean \pm SEM of 6 animals $***P < 0.001$, $**P < 0.01$, $*P < 0.05$ significantly different from control; $###P < 0.001$, $##P < 0.01$, $#P < 0.05$ significantly different from Al group; $^?P < 0.05$ significantly different from control group; and *NS* non-significant difference



(a) Acquisition Test



(b) Retrieval Test

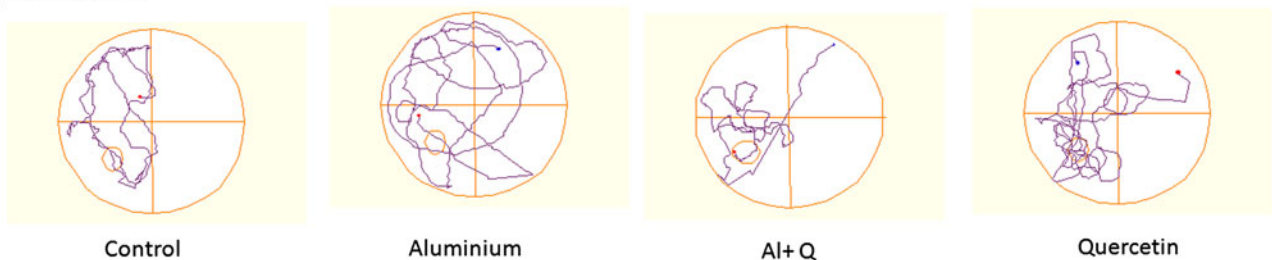


Fig. 6 The acquisition and retrieval plot curve shows the path followed by different dose groups after 6 weeks of Al and quercetin administration

Al-treated animals compared to the animals in the control group, whereas there was significant recovery in the rearings and ambulation activity of the Al-exposed animals due to quercetin treatment (Al + Q) as compared to the Al-treated rats (Fig. 9c, d). However, no significant effects on total

distance traveled, total time immobile, number of rearing, and ambulations were observed in control rats compared to quercetin-treated rats alone (Fig. 9). A significant increase was observed in the total distance traveled, total time immobile, number of rearing, and ambulations in

Fig. 7 Effect of chronic Al and quercetin administration on **a** the time spent in the quadrant without containing platform (retrieval), **b** number of entries in the quadrant without containing platform (retrieval), **c** overall average speed in the quadrant without containing platform (retrieval) and **d** the number of entries in area where earlier platform was placed. The values are mean \pm SEM of 6 animals *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ significantly different from control; #### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$ significantly different from Al group; $^{\gamma}P < 0.05$ significantly different from control group; and NS non-significant difference

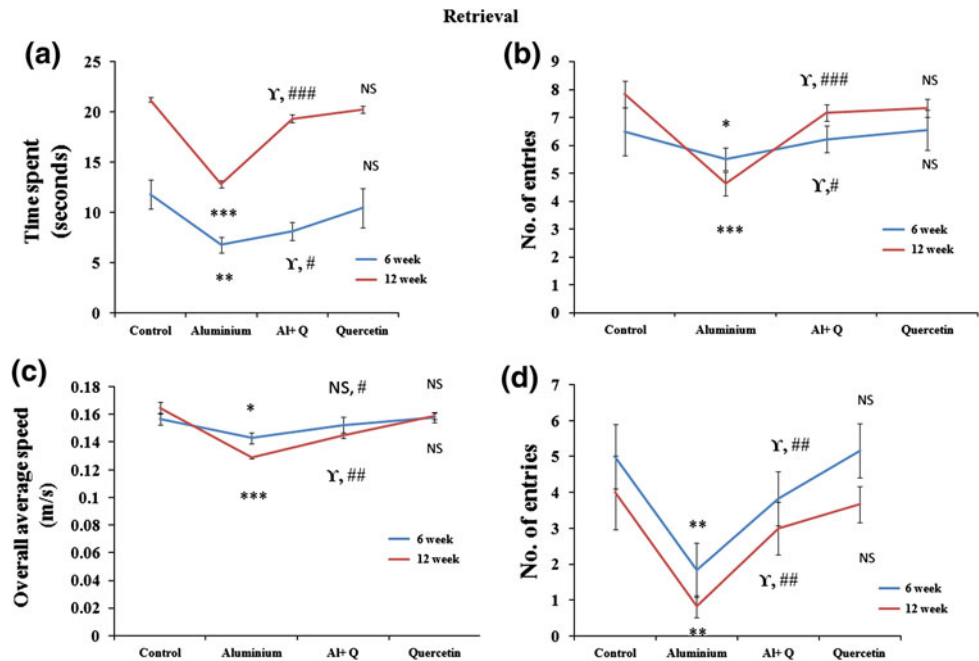
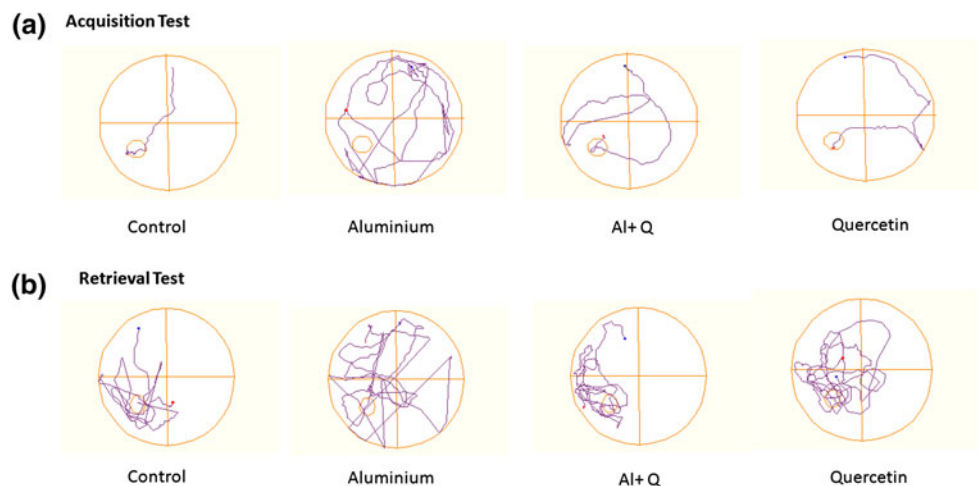


Fig. 8 The acquisition and retrieval plot curve shows the path followed by different dose groups after 12 weeks of Al and quercetin administration



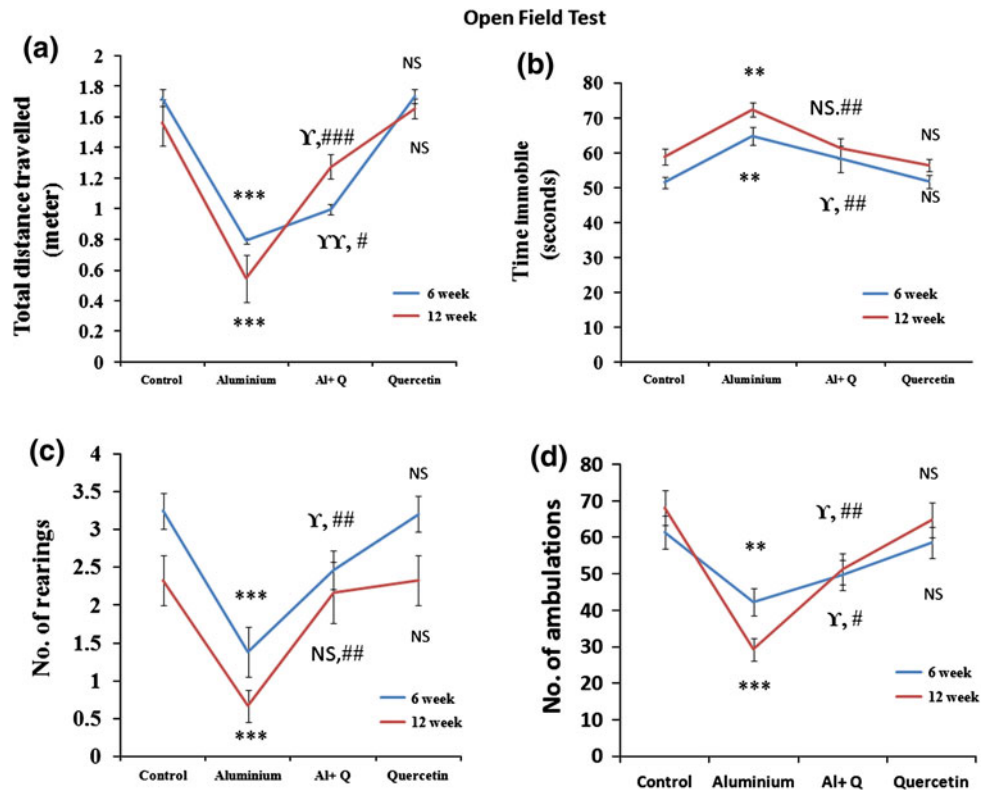
Al + Q-treated rats in open field test as compared to control rats after 6 and 12 weeks of exposure. Collectively, these results generated from the behavioral tests demonstrate that quercetin protected against Al-mediated impairments in locomotor activities.

Effect on EPM Test

The elevated plus maze is a widely used behavioral assay for rodents and it has been validated to assess the anti-anxiety effects of pharmacological agents and steroid hormones, and to define brain regions and mechanisms underlying anxiety-related behavior. An increase in the open arm activity (duration and/or entries) reflects anti-anxiety behavior. Al-treated animals spent more time

($P < 0.001$) as well as more entries ($P < 0.01$) in the closed arm of the EPM than that of control. Administration of quercetin along with aluminum to rats increased the time spent ($P < 0.01$) and number of entries ($P < 0.05$) after 6 weeks and ($P < 0.01$) after 12 weeks in the open arm of the EPM (Fig. 10). It means in the combined treatment group there is attenuation of Al-induced impairment in the behavior with less anxiety level compared to Al-treated animals, brought toward normal and the animals were observed to be less stressed as compared to the Al-treated group. There is a significant decrease in open arm activity in Al + Q-treated rats as compared to control rats after 6 weeks ($P < 0.05$) and 12 weeks ($P < 0.05$) of exposure. No significant change was observed in EPM test in rats treated with quercetin as compared to controls.

Fig. 9 Effect of chronic Al and quercetin supplementation on **a** total distance traveled, **b** total time immobile, **c** number of rearings and **d** number of ambulations by rats in open field test. The values are mean \pm SEM of 6 animals *** $P < 0.001$, ** $P < 0.01$ significantly different from control; ### $P < 0.001$, ## $P < 0.01$, # $P < 0.05$ significantly different from Al group; $^{??}P < 0.01$, $^?P < 0.05$ significantly different from control group; and *NS* non-significant difference



Effect on Defecation Index

Furthermore, defecation index was also counted by counting the number of pellets per minute. The number of pellets per minute was significantly ($P < 0.01$) increased in the Al-treated rats as compared to the control rats but it was significantly lesser ($P < 0.01$) in Al + Q group as compared to Al-treated rats (Fig. 11). There is significant increase in the number of pellets in Al + Q-treated rats as compared to control ($P < 0.05$) after 6 and 12 weeks of exposure, and there is no significant difference observed in quercetin alone treated rats as compared to control.

Neurochemical Studies

Effect on Mitochondrial ROS Production

Intracellular ROS were assessed using the dye DCF-DA. DCF-DA is deesterified to the ionized free acid (DCFH), which then reacts with ROS to form the fluorescent 2,7-dichlorofluorescein (DCF). Animals were pre-treated with quercetin 8 h prior to Al administration as described in “Materials and Methods” section. Table 2 shows the effect of quercetin on the levels of ROS in HC and CS regions of rat brain mitochondria. The ROS levels were significantly increased by 72.73 % in HC and 63.16 % in CS ($P < 0.001$)

in the Al-treated group as compared to the control group. However, there was significant decrease in ROS levels by 36.36 % in HC ($P < 0.001$) and 36.84 % in CS ($P < 0.01$) in Al + Q-treated rats as compared to Al-treated rats alone. The ROS levels were significantly increased by 57.14 % in HC and 41.67 % in CS ($P < 0.05$) in the Al + Q-treated group as compared to the control group. These results indicate that quercetin pre-treatment is capable of inhibiting ROS generation at the time points evaluated compared to Al group. No significant change in the ROS levels was observed in HC and CS brain regions in rats treated with quercetin as compared to rats in the control group (Table 2).

Effect on ATP Levels

The ATP levels in brain mitochondria were estimated following chronic Al and quercetin supplementation. As evident from Table 2, the ATP levels declined significantly in both the regions (HC and CS) of Al-treated rats. The ATP levels decreased by 66 % in HC and 56.7 % in CS of Al-treated group as compared to control ($P < 0.001$). The ATP levels were significantly increased by 48 % in HC and 35 % in CS of Al + Q-treated rats as compared to Al-treated rats ($P < 0.001$). However, ATP levels were significantly decreased by 34.78 % in HC and 33.26 % in CS of Al + Q-treated rats as compared to control ($P < 0.001$). No significant change in ATP levels was

Fig. 10 Effect of chronic Al and quercetin supplementation on **a** the time spent in the open and **b** closed arm, and the number of entries in the **c** open and **d** closed arm by rats (anxiety test) in EPM test. The values are mean \pm SEM of 6 animals *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ significantly different from control; ## $P < 0.01$, # $P < 0.05$ significantly different from Al group; $^{\gamma}P < 0.05$ significantly different from control group; and NS non-significant difference

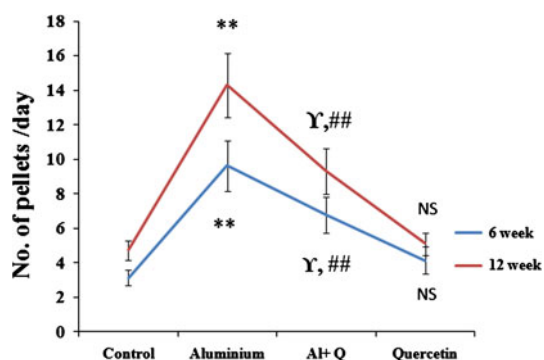
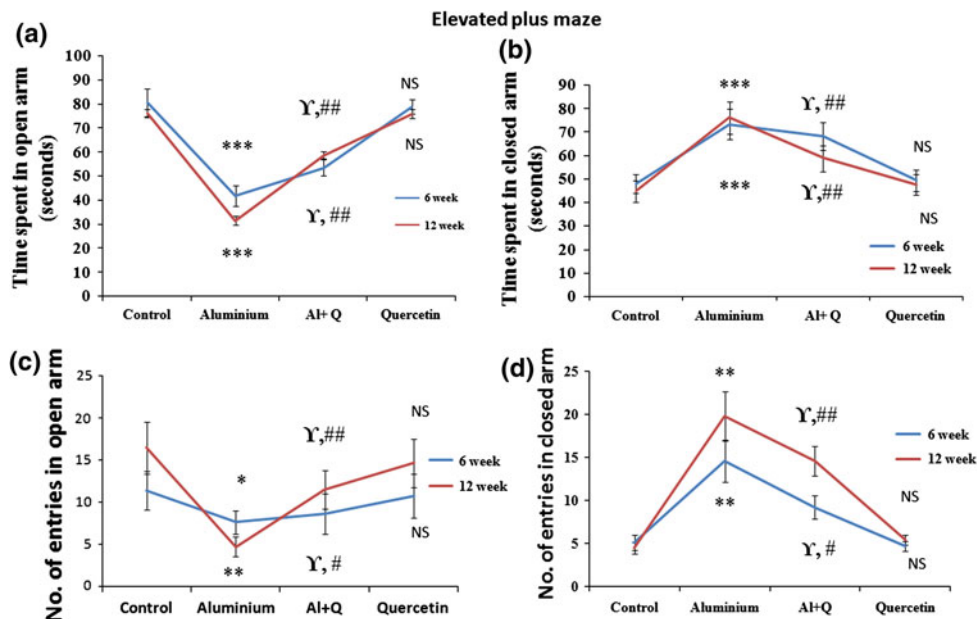


Fig. 11 Effect of chronic Al and quercetin supplementation on number of defecation pellets during EPM test subjected to Al treatment. The values are mean \pm SEM of 6 animals *** $P < 0.001$ significantly different from control, ## $P < 0.01$ significantly different from Al group, $^{\gamma}P < 0.05$ significantly different from control group; and NS non-significant difference

observed in the two brain regions in rats treated with quercetin as compared to rats in the control group (Table 2).

Effect on AChE Activity

AChE is a marker of extensive loss of cholinergic neurons in the brain. Al treatment caused a significant decrease in the AChE activity both in the HC (46.2 %) and CS (44.7 %) as compared to normal controls ($P < 0.001$, Table 2). In the combined treatment group, the enzyme activity was found to be significantly increased by 33.3 % in HC and 29.5 % in CS ($P < 0.01$) as compared to Al treatment group. However, AChE activity was significantly

decreased in Al + Q group by 19.3 % in HC and 21.68 % in CS as compared to control ($P < 0.05$). Thus, quercetin administration to Al-treated animals enhances the AChE activity as compared to Al-treated animals. No significant change in AChE activity was observed in any of the brain regions in rats treated with quercetin as compared to rats in the control group (Table 2).

Effect on MnSOD Assay, Mitochondrial Glutathione Reductase and Glutathione Peroxidase Contents

Higher levels of ROS production lead to faster rate of mitochondrial damage. In lieu of this, we next examined the effect of Al and quercetin administration on brain mitochondrial antioxidants viz., MnSOD, glutathione reductase, and glutathione peroxidase. We observed a significant decrease in MnSOD activity and mitochondrial glutathione levels in Al-treated rat brains compared to control group. There was a 60.55 % decrease in MnSOD activity, 65.13 % decrease in glutathione reductase, and 64.54 % decrease in glutathione peroxidase in HC region of Al-treated group as compared to control group ($P < 0.001$, Table 3). However, quercetin administration to Al-treated rats resulted in restoration of MnSOD activity (49.69 %), an increase in glutathione reductase (24.6 %) and glutathione peroxidase (48.1 %) in HC as compared to Al-treated group ($P < 0.01$, Table 3). Similarly, in CS region of rat brain mitochondria, we found a 43.87 % decrease in MnSOD activity, 59.25 % decrease in glutathione reductase, and 58.43 % decrease in glutathione peroxidase in Al-exposed group as compared to control group ($P < 0.001$). Quercetin administration to Al-treated

Table 2 Effect of chronic Al and quercetin supplementation on the levels of ROS, ATP levels and AChE activity in HC and CS of rat brain mitochondria

	Hippocampus				Corpus striatum			
	Control	Aluminum	Al + Q	Quercetin	Control	Aluminum	Al + Q	Quercetin
	ROS (DCF Fluorescence)	6 ± 0.985	22 ± 1.89***	14 ± 1.34 ^{†,###}	5 ± 0.678 ^{NS}	7 ± 0.98	19 ± 1.35***	12 ± 1.27 ^{†,###}
ATP levels(nmol/mg protein)	12.317 ± 1.155	4.183 ± 0.259***	8.033 ± 0.296 ^{†,###}	11.917 ± 0.743 ^{NS}	14.533 ± 0.556	6.3 ± 0.267***	9.7 ± 0.477 ^{†,###}	13.35 ± 0.272 ^{NS}
Acetylcholinesterase activity (nmol product formed/min/mg protein)	171 ± 4.76	92 ± 3.23***	138 ± 3.87 ^{†,###}	165 ± 4.12 ^{NS}	143 ± 4.09	79 ± 2.56***	112 ± 3.12 ^{†,###}	138 ± 3.87 ^{NS}

The animals were divided into various groups ($n = 6$) and received Vehicle, Al (10 mg/kg b.wt./day), Al + Q and quercetin (10 mg/kg b.wt./day) for 12 weeks. Mitochondria were isolated from the brains of control and treated animals and subjected to biochemical analysis

NS non-significant difference

The values are mean ± SEM of 6 animals *** $P < 0.001$ significantly different from control and ### $P < 0.001$ significantly different from Al group, ^{††} $P < 0.01$ significantly different from Al group, [†] $P < 0.05$ significantly different from control group

rats resulted in restoration of MnSOD activity (29.13 %), an increase in glutathione reductase (32.7 %) and glutathione peroxidase (37.82 %) as compared to Al-treated group ($P < 0.01$, Table 3). However, there was a significant decrease in the MnSOD activity and glutathione levels in Al + Q group as compared to control group ($P < 0.01$). No significant change in the activity of MnSOD and glutathione levels was observed in any of the brain regions of the rats treated with quercetin as compared to controls (Table 3).

Biochemical Markers of Oxidative Stress

As ROS are capable of damaging mitochondrial membrane lipids and proteins, we studied malondialdehyde (MDA) and protein carbonyl formation as an index of oxidative stress in vehicle, Al, Al + Q, and quercetin-treated rat brains.

Effect on Lipid Peroxidation

The results of this study demonstrate significant increase in lipid peroxidation in Al-treated group (1167.17 ± 34.67 nmol MDA/mg protein in HC and 1149.76 ± 43.26 nmol MDA/mg protein in CS) as compared to control rat brain mitochondria, whereas significant decrease in lipid peroxidation in HC (895.18 ± 31.45 nmol MDA/mg protein) and CS (691.68 ± 29.98 nmol MDA/mg protein) was seen in Al + Q-treated rats as compared to Al-treated rats (Table 4). However, there was a significant increase in lipid peroxidation in Al + Q-treated group as compared to control group in both the regions ($P < 0.01$). Hence, quercetin administration to Al treatment markedly attenuates Al-induced lipid peroxidation in mitochondria isolated from HC and CS regions of rat brain. No significant change in the lipid peroxidation was observed in any of the brain regions of rats treated with quercetin as compared to controls (Table 4).

Effect on Protein Oxidation

As the mitochondrial ROS were found to be raised in Al-treated rats, these ROS are also capable of damaging mitochondrial proteins. Therefore, we studied protein carbonyl formation in rats treated with vehicle, Al, Al + Q, and quercetin. The protein carbonyl formation was found to be increased significantly in Al-treated rat brains compared to control ($P < 0.001$, Table 4). However, protein carbonyl formation was found to be significantly reduced in Al + Q-treated rats compared to Al-treated rats in both the regions. There was also a significant increase in protein carbonyl content in Al + Q-treated group as compared to control group in HC and CS regions of rat brain after 12 weeks of exposure ($P < 0.01$). No significant change in the protein

Table 3 Effect of chronic Al and quercetin supplementation on MnSOD, glutathione reductase and glutathione peroxidase in HC and CS of rat brain mitochondria

	Hippocampus				Corpus striatum			
	Control	Aluminum	Al + Q	Quercetin	Control	Aluminum	Al + Q	Quercetin
	MnSOD (U/mg protein)	32.85 ± 3.76	12.96 ± 1.34***	25.76 ± 2.58 [?] ##	31.87 ± 3.74 ^{NS}	29.86 ± 3.23	16.76 ± 1.65***	23.65 ± 2.54 [?] ##
Glutathione reductase (NADPH utilized/ min/mg protein)	52.74 ± 5.45	18.39 ± 3.26***	24.39 ± 3.98 [?] ##	48.72 ± 5.21 ^{NS}	34.26 ± 4.65	13.96 ± 3.18***	20.74 ± 3.54 [?] ##	37.72 ± 4.56 ^{NS}
Glutathione peroxidase (GSH consumed/ min/mg protein)	6.9 ± 1.34	2.45 ± .056***	4.72 ± .098 [?] ##	6.64 ± 1.2 ^{NS}	7.12 ± 1.41	2.96 ± .054***	4.76 ± .087 [?] ##	6.92 ± 1.2 ^{NS}

The enzymatic activity of mitochondrial MnSOD, glutathione reductase and glutathione peroxidase were measured spectrophotometrically as described in materials and methods
NS non-significant difference

The values are mean ± SEM of 6 animals *** $P < 0.001$ significantly different from control, ## $P < 0.01$ significantly different from Al group, [?] $P < 0.05$ significantly different from control group

oxidation was observed in any of the brain regions of rats treated with quercetin as compared to controls (Table 4).

Effect of H&E Staining

Al-treated HC and CS sections showed marked cell distortion with high level of degeneration in the cells as compared to control. Neurons were morphologically damaged (swollen cytoplasm or shrunken, darkened nuclei) after Al exposure. The extent of Al-induced neuronal damage was significantly decreased at the HC and CS in the rats treated with Al + Q. Morphologic changes, most notably cell swelling, were also prevented by quercetin. There was also a significant level of degeneration in the Al + Q-treated cells as compared to control group in HC and CS regions of rat brain after 12 weeks of exposure. No sign of degeneration was observed in the H&E staining in the brain regions (HC and CS) of rats treated with quercetin as compared to controls. These results suggest that quercetin contributes significantly to the protective effects of neuronal cells from oxidative stress-induced neurodegeneration (Fig. 12).

Effect on Al Levels

As evident from Table 5, the Al levels increased significantly in both the regions (HC and CS) of Al-treated rats. The Al levels increased by 62.24 % in HC ($P < 0.001$) and 46.83 % in CS ($P < 0.01$) of Al-treated group as compared to control. However, the Al levels were significantly decreased by 50 % in HC and 32.9 % in CS of Al + Q-treated rats as compared to Al-treated rats ($P < 0.01$). Moreover, Al levels were significantly increased by 24.5 % in HC, 20.8 % in CS of Al + Q-treated rats as compared to control ($P < 0.05$). Thus, quercetin supplementation to Al-treated animals prevents the accumulation of Al levels considerably as compared to Al-treated animals. No significant change in Al levels was observed in any of the brain regions in rats treated with quercetin as compared to rats in the control group (Table 5).

Discussion

Enhanced oxidative stress has been suggested to be an important mechanism in Al-induced neurotoxicity. In this study, we have shown that chronic Al administration resulted in oxidative damage by increasing ROS production and by decreasing antioxidant defense (MnSOD and glutathione levels) in HC and CS regions of rat brain. It also caused a significant decrease in the AChE activity and ATP levels. Chronic Al lactate exposure results in poor performances in several behavioral and cognitive task paradigms. Chronic administration of quercetin to Al-treated rats significantly

Table 4 Effect of chronic Al and quercetin supplementation on lipid peroxidation and protein oxidation in HC and CS of rat brain mitochondria

	Hippocampus				Corpus striatum			
	Control	Aluminum	Al + Q	Quercetin	Control	Aluminum	Al + Q	Quercetin
Lipid peroxidation (nmol TBARS/mg protein)	525.28 ± 28.78	1167.17 ± 34.67***	895.18 ± 31.45 ^γ ##	595.16 ± 27.89 ^{NS}	539.3 ± 31.45	1149.76 ± 43.26***	691.68 ± 29.98 ^γ ###	582.92 ± 27.89 ^{NS}
Protein oxidation (nmol carbonyl/mg protein)	5.02 ± .057	12.65 ± 1.29***	7.89 ± 1.12 ^γ ##	4.21 ± .046 ^{NS}	4.43 ± .054	11.06 ± 1.45***	6.98 ± .098 ^γ ##	4.56 ± .043 ^{NS}

NS non-significant difference

The values are mean ± SEM of 6 animals *** $P < 0.001$ significantly different from control, ### $P < 0.001$ significantly different from Al group, # $P < 0.01$ significantly different from Al group, ^γ $P < 0.05$ significantly different from control group

improved learning and memory retention in all the tasks, attenuated oxidative damage, increased AChE activity, and ATP levels. This study demonstrates that quercetin offers neuroprotection against Al-induced cognitive dysfunction, cholinergic impairment, and oxidative damage.

The effects of flavonoid-rich diet on cognitive function have been linked to the ability of flavonoids to interact with the cellular and molecular frameworks involved in learning and memory (Havsteen 2002). Flavonoids also have known antioxidant abilities, effectively protecting neurons against neurotoxins and enhancing neuronal function (Ward et al. 2002; Vafeiadou et al. 2007). To check the potential of quercetin against Al neurotoxicity in rats, first of all we performed the neurobehavioral studies. The importance of neurobehavioral studies in risk assessment lies in the fact that behavior can be regarded as the net output of the sensory, motor, and cognitive functions occurring in the nervous system and can serve as potentially sensitive end points of chemically induced neurotoxicity (Evans 1995). It has also been suggested that in some cases, behavioral changes may be more sensitive than neurochemical alterations, as indicators of neurotoxicity and may be observed early during exposure (Tilson 1993), especially to Al (Baydar et al. 2003).

In the current study, muscle coordination was seen to be significantly altered after Al exposure and the rats did not learn the task as effectively as normal animals. This is in concurrence with our previous lab findings (Kaur et al. 2006) in which it has been shown that Al exposure causes neuromuscular incoordination in male Wistar rats. Therefore, Al affects the motor functions and leads to decreased motor activities. Diminished motor activities and grip strength have also been seen after Al exposure to mice (Hu et al. 2005). Chronic administration of quercetin to Al-treated rats resulted in improved muscle coordination as seen in rota rod test.

Al-treated rats also showed decrease in active and passive avoidance tests. This is in line with our previous lab findings (Julka et al. 1995) and in other studies also (Gonda and Lehotzky 1996; Connor et al. 1989; Miu et al. 2003) in which they have shown impaired performance in active and passive avoidance tasks after Al treatment. Quercetin administration to Al-treated rats significantly improved the active and passive avoidance performances in this study which is consistent with earlier reports in which quercetin-fed mice showed significantly improved learning and memory ability in step-through passive avoidance tests compared with D-gal-treated mice (Lu et al. 2006). In another study, single intraperitoneal injection of 50 mg/kg of quercetin at 1 h before the scopolamine treatment prevented scopolamine-induced memory deficits in an inhibitory avoidance paradigm (Richetti et al. 2011). In yet another study, chronic administration of quercetin significantly reversed the

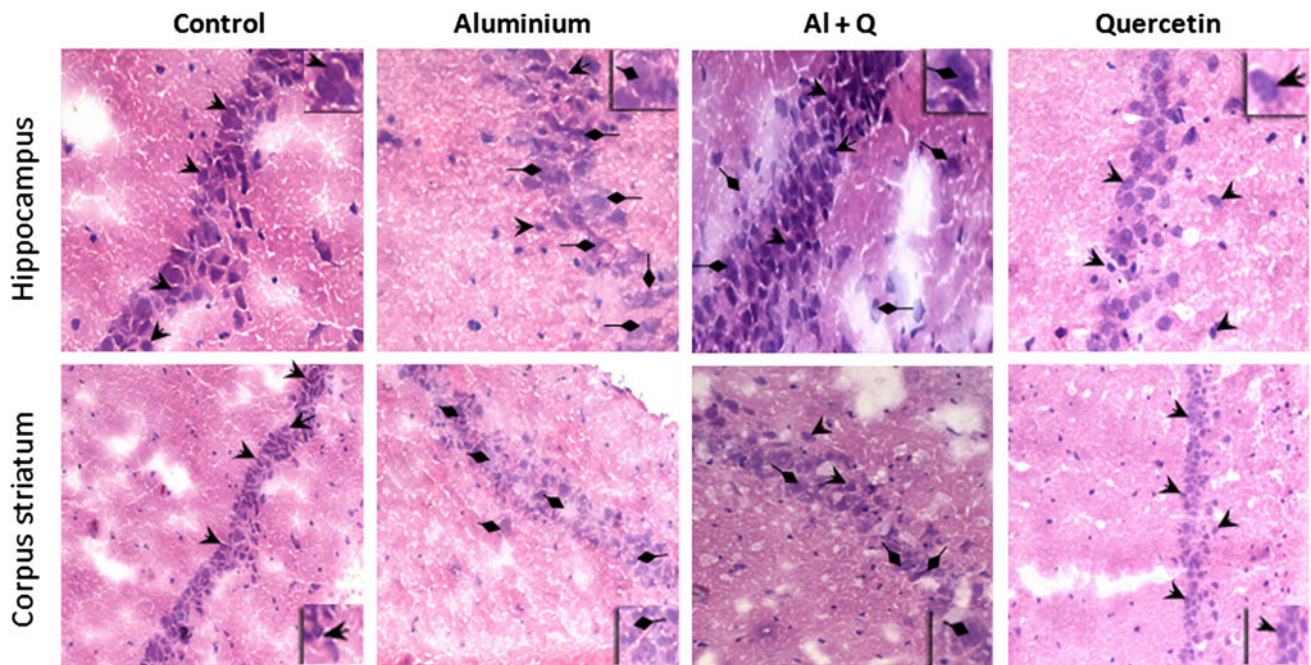


Fig. 12 Representative H&E images in the HC and CS regions of rat brain after 12 weeks of Al and quercetin administration and the photographs were taken ($\times 200$). Arrow heads represent normal cell and diamond heads represent distorted cell. All insets are of scale bar 50 μm

age-related or chronic ethanol-induced step-down passive avoidance task (Singh et al. 2003).

We have also found impairment in water maze and open field tasks in Al-treated rats as compared to control. These rats presented with impairments in both acquisition and retention of the water maze task. Moreover, impairment in maze tasks performance after high Al injection was also reported in prenatally exposed adult mice (Santucci et al. 1994; Alleva et al. 1998). Roig et al. (2006) suggested that a prolonged oral Al exposure produces behavioral changes in rats. Chronic administration of quercetin along with Al significantly improved the poor retention of memory in MWM and also shows more interest in initial exploratory and habituation pattern in open field task paradigms. Our results are similar to the earlier study in which quercetin-fed mice showed higher activity upon induction by new environmental stimuli and higher novelty-seeking behavior in the open field tasks, and significantly improved learning and memory ability in MWM tests compared with D-gal-treated mice (Lu et al. 2006). Quercetin supplementation to Al-exposed animals proved beneficial and the animals performed a better learning task as shown by an increase in the retention trial time and the same has also been reported by others (Yao et al. 2010; Bhutada et al. 2010). In another set of experiment, quercetin (40 mg/kg, p.o., twice daily) treatment during training trials (31–35 days) markedly decreased escape latency and increased time spent in target quadrant during MWM task (Bhutada et al. 2010). Treatment of quercetin (5 mg/kg i.p. for 14 days) was found to improve the

performance of learning and memory of ischemic rats in the MWM (Yao et al. 2010). Recently, it has been reported that quercetin exhibited protective effect against memory impairments induced by D-galactose and repeated cerebral ischemia in animals (Pu et al. 2007; Lu et al. 2006).

Behavior assessment in the EPM is a model of anxiety for rodents. In this study, animals spent more time as well as more number of entries in the closed arm and also showed increased defecation index thus depicting an increased anxiety level in Al-exposed animals. The defecation index is also an indicator of potential anxiety (Suarez-Fernandez et al. 1999). Therefore, our study shows that Al disrupts the cognitive behavior of animals and increases anxiety levels. Quercetin administration to Al-treated rats spent more time and entries in the open arm thus depicting increased anti-anxiety levels. Treatment with quercetin (5–20 mg/kg, p.o., twice daily, 30 days) in streptozotocin-induced diabetic rats prevented the changes in performance in morris water and EPM tasks. Quercetin administration was able to reduce the anxiety levels as well as improve the cognitive function (Lu et al. 2006; Bhutada et al. 2010). Recently, evidences have also indicated the anxiolytic and antidepressant-like effects of quercetin in various animal models (Lu et al. 2006; Kaur et al. 2007; Kumar and Goyal 2008; Kawabata et al. 2010; Aguirre-Hernandez et al. 2010).

In this study, a decrease in AChE activity was observed after Al exposure in both HC and CS regions of rat brain. This finding is in conformity with previous studies in our lab

Table 5 Effect of chronic Al and quercetin supplementation on Al levels in HC and CS of rat brain

	Hippocampus				Corpus striatum			
	Control	Aluminum	Al + Q	Quercetin	Control	Aluminum	Al + Q	Quercetin
Aluminum concentration ($\mu\text{g/g}$ of tissue)	0.37 \pm 0.03	0.98 \pm 0.04 ^{***}	0.49 \pm 0.08 ^{7##}	0.4 \pm 0.02 ^{NS}	0.42 \pm 0.04	0.79 \pm 0.06 ^{**}	0.53 \pm 0.05 ^{7##}	0.38 \pm 0.03 ^{NS}

NS non-significant difference

The values are mean \pm SEM of 6 animals *** $P < 0.001$ significantly different from control, ** $P < 0.01$ significantly different from control, # $P < 0.01$ significantly different from Al group, ⁷⁷ $P < 0.01$ significantly different from control group, ⁷ $P < 0.05$ significantly different from control group

showing decrease in AChE activity following Al treatment (Julka et al. 1995; Kaur and Gill 2006; Kumar et al. 2008b). Al is a pro-oxidant and indirectly results in the production of free radicals leading to oxidative damage (Cucarella et al. 1998) and this is also confirmed in this study (Table 2) and thus may be responsible for the decreased AChE enzyme activity. As discussed above, Al causes disturbances in cholinergic neurotransmission, which may be linked to altered memory in Al-exposed animals. The cholinergic system is involved in many physiological processes, including synaptic plasticity and learning and memory (Flood et al. 1981; Power et al. 2003; Weinberger 2006). Cholinergic agonists can facilitate memory, whereas cholinergic antagonists can impair memory (Mattson 2004). From the above quoted references, we can say that Al can affect the enzyme activity and thus interfere with cholinergic neurotransmission, affecting learning, memory, cognition, and motor functions. The restoration of AChE activity by quercetin in Al-treated rats can also be attributed to metal-chelating properties of quercetin. Protein–metal interaction have been suggested to be a reason for deleterious effects of Al on AChE activity (Julka et al. 1995; Gulya et al. 1990). Here in this study, it is possible that quercetin may have chelated Al which remains no longer available for protein–metal interaction (Slikker et al. 1999; Hanasaki et al. 1994). Therefore, quercetin has been shown to improve the AChE activity, which ultimately is responsible for the improvement in learning, memory, cognition, and motor functions.

Al-treated sections showed significant degeneration of neurons. Whereas we observed that quercetin supplementation decreased the neuronal damage and scavenged the free radicals induced by Al and protects Al-induced neurodegeneration and oxidative stress which is in continuation with the recent findings (Kelsey et al. 2010; Bavithra et al. 2012; Selvakumar et al. 2012). It has been shown that quercetin scavenges ROS and reduces oxidative damage (Cai et al. 1997). Various reports suggest that quercetin passes through the blood–brain barrier and influences the neuronal cells directly. A higher concentration of quercetin metabolites appear in the brain after several hours of administration of quercetin (Day et al. 2001; Paulke et al. 2006). In this study, pre-treatment with quercetin was found to reverse the oxidative stress in the combined treatment group animals after 12 weeks of exposure. Quercetin inhibits lipid peroxidation, protein oxidation and restores levels of MnSOD, glutathione peroxidase, and glutathione reductase hence there is significant elevation in the antioxidant enzymes in the Al + Q-treated rats. Quercetin significantly increased SOD activity and decreased MDA level (Connor et al. 1989). Interestingly, Tota et al. (2010) reported that quercetin (5–10 mg/kg) dose dependently restored the MDA and reduced glutathione levels in brain homogenates of intracerebral STZ administered mice. Similar results were observed by Kumar et al.

(2007) in colchicine-induced memory dysfunction in rats at higher doses (20–40 mg/kg). In one of the studies, quercetin (10–50 mg/kg) also reversed cognitive dysfunction and decreased thiobarbituric acid reactive substances levels, and decline in forebrain total glutathione, SOD, and catalase levels by chronic ethanol administration in mice (Singh et al. 2003). Therefore, in this study, quercetin might have protected Al-induced cognitive dysfunction by reducing oxidative stress.

This proves two important actions of quercetin in oxidative stress, i.e., quenching of ROS and enhancing the cellular antioxidant defense system. The antioxidant efficacy of quercetin is reported to be due to its higher diffusion into the membranes (Moridani et al. 2003) allowing it to scavenge oxygen radicals at several sites throughout the lipid bilayer and its pentahydroxy flavone structure allowing it to chelate metal ions via the ortho-dihydroxy phenolic structure, thereby scavenging lipid alkoxyl and peroxy radicals (Lien et al. 1999; Cao et al. 1997). For checking the Al chelating efficacy of quercetin in this study, we checked the Al levels with the help of atomic absorption spectrophotometer in HC and CS regions of rat brain. The Al levels significantly increased in HC and CS regions of Al-treated group as compared to control. However, Al levels significantly decreased in case of Al + Q-treated rats as compared to Al-treated rats in both the brain regions. These results clearly show that Al can accumulate to significant levels in the HC and CS regions of rat brain after Al exposure. Moreover, pre-treatment of quercetin has two beneficial effects, it decreases oxidative stress and it can chelate Al, thereby preventing it from reaching the cited brain regions. Previous studies from our lab have already shown that significant levels of aluminum are present in the brain regions especially HC when rats are fed with aluminum lactate at same dose, i.e., 10 mg/kg b.wt. (Julka et al. 1996; Kaur et al. 2006). Quercetin treatment caused the decrease in lipid hydroperoxides levels, as it can neutralize the ROS by directly reacting with $O_2^{\cdot-}$, NO, and peroxynitrite (Muthukumaran et al. 2008; Bongiovanni et al. 2007). All these observations suggest that quercetin metabolites found in brain could attenuate oxidative stress not only through radical scavenging but also through non-radical scavenging activities (Williamson et al. 2005). In any case, it should be important that the quercetin metabolites interact with the target sites (probably hippocampus and/or striatum) to attenuate oxidative stress induced by forced swimming stress (Ishisaka et al. 2011). The observation that Al + Q-treated animals had increased total antioxidants and decreased lipid hydroperoxides and protein oxidation support the antioxidant properties of this flavonoid, and demonstrate the protective effect of quercetin on oxidative stress parameters and maintain antioxidant levels. Quercetin also chelate Al from Al-treated brain regions because of its ortho-dihydroxy phenolic structure (Lien et al. 1999; Cao et al.

1997). In this study, the results showed a significant decrease in ATP level in Al-treated rats suggesting a disturbed energy metabolism, an observation supported by previous findings also (Kumar et al. 2008b). However, quercetin along with Al increased the ATP level depleted by Al toxicity. This increase in ATP may be because of quercetin-enhanced markers of mitochondrial biogenesis like mitochondrial DNA and cytochrome *c* concentration in mice as reported recently by Davis et al. (2009). There were no significant changes in the behavioral as well as oxidative stress parameters in any of brain regions of rats treated with quercetin alone as compared to controls.

Conclusion

This study clearly demonstrates that quercetin has a neuro-protective effect against Al-induced behavioral and biochemical changes and further warrants need for molecular studies to elucidate the mechanisms underlying the protective effects of quercetin. On the second hand, its widespread use as dietary supplement should be encouraged to ward off age-associated memory disorders like Alzheimer's disease and Parkinson's disease because of its antioxidant and metal-chelating properties. Pharmacological agents like flavonoid quercetin, capable of scavenging free radicals, maintaining antioxidant homeostasis, and/or inhibiting lipid hydroperoxides, protein oxidation and thereby protecting neurons from oxidative injuries may provide useful therapeutic potentials for the prevention or treatment for the neurodegenerative disorders caused by Al-induced neurotoxicity. Neuroprotective action of quercetin may be beneficial for the prevention and treatment of Al-induced oxidative damage in the brain.

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Conflict of interest The authors declare that there are no conflicts of interest

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