Prevention of cadmium-induced neurotoxicity in rats by essential nutrients present in nuts

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Cadmium, a heavy metal with no physiological function in the human body, is considered a bio-hazard. It is also considered to be a potent neurotoxin. The primary sources of cadmium exposure are diet and cigarette smoke. It has been postulated that nutritional deficiencies can increase the risk of cadmium toxicity. Nuts provide essential nutrients which are necessary for the maintenance of brain health in humans. The present study was designed to investigate the possible protective effects of almond and walnut supplementation on cadmium-induced neurotoxicity. Cadmium was orally administered at a dose of 50 mg/kg weekly with or without the supplementation of almond and walnut in rats. Intensities of depression- and anxiety-related behaviors were assessed by the forced swim test and light/dark transition test, respectively. Memory function was also evaluated by the elevated plus maze, Morris water maze and novel object recognition task. After four weeks of treatment it was observed that cadmium administration significantly induced depressogenic and anxiogenic behaviors. Memory function was also impaired by cadmium administration. Cadmium-treated rats exhibited reduced noradrenalin, dopamine and serotonin levels in the brain, whereas the levels of their respective metabolites were significantly increased. The dietary supplementation of almond and walnut at a dose of 400 mg/kg/day significantly attenuated cadmium-induced depression, anxiety and memory impairments. Neurochemical aberrations also normalized following supplementation with these nuts in rats. The present study demonstrates that long-term supplementation with almond and walnut provides essential nutrients which may overcome nutritional deficiencies and thereby reduce heavy-metal intoxication.

Key words: almond, behavioral despair, biogenic amines, cadmium intoxication, memory functions, walnut

INTRODUCTION

Heavy metals, such as mercury, lead and cadmium, are widely distributed in nature; however, they have no nutritional importance. Cadmium is an extensively distributed toxicant because of its excessive use in modern lifestyles (Andrade et al., 2017). For example, this heavy metal is used in the manufacture of household appliances, batteries and paints. The improper disposal of these accessories causes contamination of the environment with cadmium (Järup, 2003). Fuel combustion by automobiles and cigarette smoke are also major sources of cadmium exposure that almost all humans come across in daily life (Richter et al., 2017). The toxic effect of cadmium is due to its long half-life, causing its accumulation in living tissues for long periods of time. Cadmium is also regarded as a neurotoxicant as it can easily cross the blood brain barrier (BBB) and impairs neuronal

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functions (Andrade et al., 2017), including changes in neurotransmitter synthesis, release and synaptic activity. Cadmium exposure has also been shown to alter the activity of acetylcholinesterase and sodium-potassium ATPase in various rat brain regions including cortex, hippocampus, cerebellum, striatum and hypothalamus (Wang et al., 2018). Abdel Moneim et al. (2014) observed reduced levels of dopamine and serotonin and increased lipid peroxidation in rats following intraperitoneal administration of cadmium for five days. These authors also reported an increased generation of reactive nitrogen species and significantly reduced levels of antioxidants in cadmium-treated animals. Cadmium has also been shown to alter neurotransmitter release by blocking calcium influx into nerve terminals. Cadmium was found to disrupt the binding of the calcium-calmodulin complex causing an impairment in calcium-induced signaling pathways and, thus, altered neuronal function (Sadiq et al., 2012). There is growing evidence linking cadmium exposure to the prevalence of neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease and psychiatric disorders. Intoxication with cadmium caused hyper-phosphorylation of tau protein through the activation of glycogen synthase kinase-3β (Ben et al., 2016). Hyper-phosphorylated tau protein is a major hallmark of neurodegenerative diseases such as Alzheimer's disease (Hussien et al., 2018). Elevated risk of neurodegenerative disease due to cadmium exposure may result from increased generation of free radicals, elevated lipid peroxidation and enhanced oxidative stress, leading to impaired neurogenesis, neuronal differentiation and axonogenesis and ultimately neuronal cell death (Chong et al., 2017).

Neuronal changes due to cadmium intoxication can manifest in the behavioral patterns of an affected individual. Olfactory abnormalities, reduced ability to concentrate, learning dysfunction and reduced IQ level have been reported in children exposed to cadmium toxicity (Wang and Du, 2013). Early cadmium exposure during the neonatal stage affects the more vulnerable CNS due to differences in the integrity of the BBB in neonates versus adults (Korpela et al., 1986). Bao et al. (2009) reported psychiatric problems such as withdrawal, social problems and attention deficits in Chinese children who showed a higher concentration of cadmium in their hair. Exposure to the metal along with nutritional deficiency augments the toxic effects in women during reproductive age (Wright and Baccarelli, 2007). Such exposure is precarious for the fetus and may lead to a course of diseases in adulthood, particularly neurodegenerative diseases (Allam et al., 2016).

It has been suggested that a nutritional deficiency of essential amino acids, fatty acids, vitamins, minerals and other beneficial polyphenolic anti-oxidants may potentiate the metal toxicity and supplementation of such nutrients may attenuate the metal toxicity (Flora et al., 2008). Nutritional intervention has been investigated by a number of researchers to reduce the deleterious effects of metal toxicity. Administration of melatonin for four weeks reduced oxidative stress, increased enzymatic and non-enzymatic antioxidant levels and attenuated histopathological changes induced by cadmium exposure in rat brain (Mukherjee et al., 2010). L-theanine, a major amino acid in green tea, ameliorated disturbed redox balance and hyper-phosphorylation of tau protein induced by cadmium exposure in male mice (Ben et al., 2016). Essential trace minerals such as iron, zinc and selenium have been reported to diminish cadmium absorption, enhance its chelation and stimulate the synthesis of metal detoxifying proteins, such as metallothionein (Bernhoft, 2013). These minerals also act as cofactors of antioxidant enzymes; therefore, they can reduce the cadmium-induced oxidative stress via antioxidant enzymatic activity (Koekkoek and van Zanten, 2016). Studies have shown that a deficiency of vitamin B1 and B6 increased susceptibility to cadmium toxicity (Prasad et al., 1982). Hence, supplementation with these vitamins is a suggested method for providing protection against cadmium-induced toxicity. The toxicity of cadmium has also been reduced in rats by pre-treatment with vitamin E, which reduced cadmium-induced lipid peroxidation and enhanced the antioxidant defense system (Paunović et al., 2017). Supplementation with a plant-based diet, at suitable levels, has been shown to protect against cadmium toxicity. For example, administration of a diet rich in soybean, tomato, garlic, ginger, onion, green tea, curry leaf and grapes has been shown to alleviate cadmium-induced toxicity in different animal models (reviewed in Zhai et al., 2015). This is due to the fact that plant-based foods contain a variety of micro-nutrients including vitamins, minerals and phytochemicals (Patel et al., 2017). Hence, the presence of essential micronutrients in a single food may provide augmenting effects to reduce cadmium toxicity.

Among natural sources of essential nutrients, nuts are considered to be the healthiest complete food. Almond and walnut are the most popular nuts, rich in macro- and micro-nutrients including minerals, vitamins and polyphenols (Gorji et al., 2017). These nuts are also considered an effective source of essential amino acids such as tryptophan, phenylalanine and tyrosine, which act as precursors for the synthesis of neurotransmitters in brain. The provision of these amino acids during neurotransmitter deficient conditions may improve neuronal function due to increased availability of precursor and increased synthesis of neu-

rotransmitters (Fernstrom and Fernstrom, 2007). The present study was designed to determine the protective effects of almonds and walnuts on the neurotoxic effects of cadmium by investigating altered brain neurotransmitter levels and resultant behavioral deficits.

METHODS

Animals

Locally bred albino Wistar rats weighing 180-200 g, were purchased from Dow University of Health Sciences, OJHA Campus, Karachi, Pakistan. Animals were housed individually with free access to standard rodent diet and tap water under a 12:12 h light/dark cycle (lights on at 7:00 am) at a controlled room temperature (22±2°C). The rodent diet (4.47 kcal/g) was comprised of wheat flour (400 g), gram flour (171 g), barley flour (171 g), corn flour (100 g), vegetable oil (50 g), milk powder (100 g), vitamin mixture (2.5 g) and iodized salt (NaCl; 5.5 g). All experiments were carried out in a balanced design to avoid the influence of order or time. The experimental procedures were approved by the institutional Board of Advanced Studies and Research (BASR/03009/Sc). Experiments were performed in strict accordance with Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH Publication no. 85-23, revised 1996) and in compliance with the recommendations of ARRIVE guidelines (Kilkenny et al., 2010).

Experimental protocol

The current study was conducted in two different sets of experiments to determine the effects of almonds and walnuts. In the first set of experiment, rats were divided into control-, almond-, cadmium- and almond+cadmium (Alm+Cad) treated groups. In the second set of experiments, control-, walnut-, cadmium- and walnut+cadmium (Wal+Cad) treated groups were used. The protocol in both experiments was similar and is outlined in Fig. 1. Almonds and walnuts were purchased from the local super market, peeled and finely crushed into a fine powder using a mortar and pestle. Suspensions of finely crushed almonds and walnuts were freshly prepared in deionized water and administered at a dose of 400 mg/kg/day to almond and walnut groups, respectively (Batool et al., 2016; Haider et al., 2018). Cadmium rats were intoxicated with cadmium by giving oral $CdCl_2$ at a dose of 50 mg/kg once per week. Alm+Cad and Wal+Cad groups were treated with almonds and walnuts respectively, along with the oral administration of cadmium. Control rats were administered with vehicle (deionized water). The treatment continued for 28 days, after which the rats were subjected to behavioral analysis by monitoring depressogenic and anxiogenic behaviors in the forced swim test (FST) and light/dark transition (LDT) test, respectively. Memory function was assessed using the elevated plus maze (EPM), Morris water maze (MWM) and novel object recognition (NOR) task. Administration of nuts was continued until the end of the behavioral analysis. Rats were decapitated after behavioral analysis to collect brain samples, which were stored at -20°C for analysis of biogenic amine levels.

Behavioral analysis

Forced swim test (FST)

Depressive behavior was tested in a glass tank (56 cm height and 30 cm width) using an established method for quantifying depression responses in rats
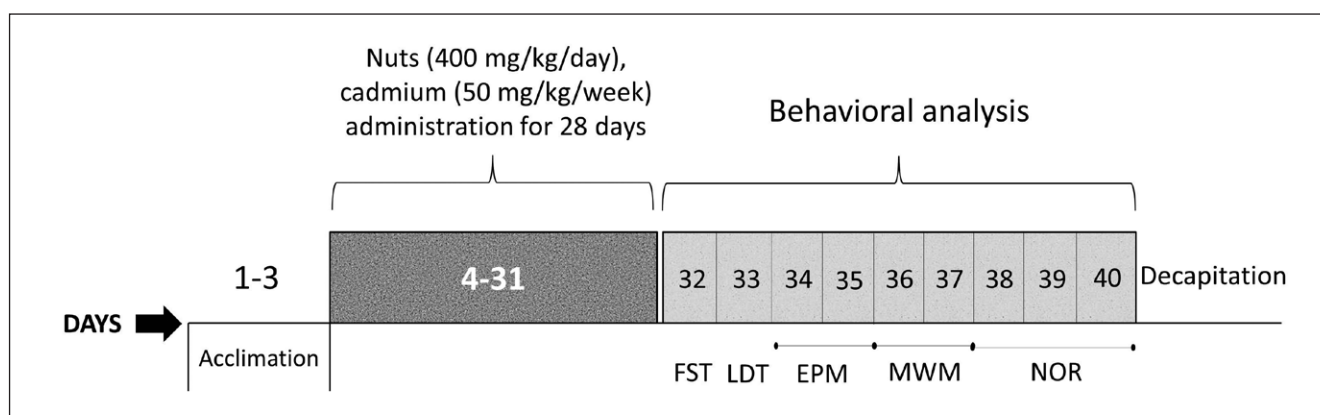

Fig. 1. Schematic representation of the experimental protocol followed in the current study. Forced swim test (FST); Light/dark transition test (LTD); Elevated plus maze (EPM); Morris water maze (MWM); Novel object recognition (NOR).

(Haider et al., 2015). The apparatus was filled with water to a height of 22-24 cm so that the rat was forced to swim but could not escape the surface of the water. The procedure induces behavioral despair in rats when they fail to escape and leads to an immobilization posture, which is considered an indicator of behavioral depression. The total cutoff time in this procedure was 300 sec during which the total immobilization time for each rat was recorded in seconds.

Light/dark transition (LDT) test

Anxiogenic behavior was evaluated by the LDT test. The apparatus consisted of a light and dark compartment. The light compartment was made up of transparent plastic whereas the black compartment was comprised of black plastic. Both compartments were the same size (26×26×26 cm) with a door (10×10 cm) connecting them. The rat was placed in the light area for 5 min during which latency to move into the dark box and time spent in the light box was monitored. The activity in this box was measured in an illuminated (360 lx) condition, using a 60 W white light bulb. A significant decrease in latency to move into dark compartment and decrease in time spent in the light box was taken as an index of anxiogenic behavior (Samad et al., 2007).

Elevated plus maze (EPM)

The EPM apparatus consisted of two closed arms and two open arms with the same dimensions (50×10 cm). The closed arm had 60 cm high walls. Closed and open arms were connected with each other via a central square (10×10 cm) giving the apparatus a plus sign shape. In the present study, the apparatus was used to assess aversive memory based on the idea that the rat feels fear from elevation in the open area. Upon repeated testing, the rat acquires information regarding the maze area and recalls the places where it feels safe (Haider et al., 2015). The test was comprised of two sessions. During the training session the rat was placed on one of the open arms facing away from the central square and trained to explore the maze for 90 sec. A test was conducted 24 h after training during which transfer latency, that is the time taken by the rat to enter the closed arm, was monitored. Significantly decreased transfer latency during the test session was considered an index of retention of memory.

Morris water maze (MWM)

Spatial memory was assessed by monitoring behavior in the MWM task. The apparatus consisted of a circular pool with a radius of 45 cm and a height of 37 cm.

The tank was filled with water (23±2°C) to a depth of 12 cm. Both the circular pool and platform were made of white painted metal. The escape platform had a flat metallic top with a surface diameter of 8 cm, and was placed 2 cm below the water level. The test is predicated on the rat finding the hidden platform under milky opaque water using spatial cues. This test was also comprised of both training and test sessions during which escape latency, i.e. the time to find the hidden platform, was recorded. Cutoff time for the training session was 2 min and test session was carried out 24 h after training. A significant decrease in escape latency during the testing period was considered an index of task learning.

Novel object recognition (NOR) task

Cognitive ability following the administration of nuts and cadmium was also assessed using a NOR task. In this test the ability to discriminate between new and old objects was measured (Batool et al., 2016). Briefly, the rat was trained and familiarized with two similar objects in a box with an area of 40×40×40 cm. During test session one of the old objects (A) was replaced by a new object (B) and time spent sniffing both objects was monitored during the cutoff time of 5 min. At the end of the experiment a discrimination index was determined using the formula [B-A/A+B]. A higher discrimination index indicated that the rat demonstrated a better cognitive ability in discriminating the new object from the old object.

Neurochemical analysis

For the determination of biogenic amines, homogenization of frozen brains was carried out in an extraction medium using an electrical homogenizer (Polytron; Kinematica). The neurochemical analysis was done to assess concentrations of noradrenaline (NA), dopamine (DA), 5-hydroxytryptamine (5-HT), and their metabolites dihydroxyphenyl acetic acid (DOPAC), homovanillic acid (HVA) and 5-hydroxyindoleacetic acid (5-HIAA) in the brain as described by Haider et al (2015). Reversed-phase High Performance Liquid Chromatography (HPLC) with an electrochemical detector (Schimadzu LEC 6A detector) was performed to detect levels of biogenic amines in brain samples. The EC detector was operated at a potential of +0.8 V. The stationary phase used for separation was a 5-μ Shim-pack ODS column with an internal diameter of 4.0 mm and a length of 150 mm. The mobile phase that passes through the column with a pump pressure of 2000–3000 psi contained octyl sodium sulfate (0.023%) in 0.1 M phosphate buffer at pH 2.9.

Statistical analysis

Data is represented as mean±SEM (n=6). Results of the EPM and MWM were analyzed by three-way ANOVA with repeated measure design followed by Bonferroni test, whereas the remaining data was analyzed by two-way ANOVA with Tukey's post-hoc test. P values <0.05 were considered significant.

RESULTS

Behavioral results

Depressogenic behavior

Depression-like behavior was assessed by using the FST with a measure of immobility time, struggling time and swimming time. Almond supplementation had a significant effect on immobility time ($F_{1,20}$=36.94, P<0.01), struggling time ($F_{1,20}$=47.40, P<0.01) and swimming time ($F_{1,20}$=31.02, P<0.01). Cadmium administration significantly affected immobility time ($F_{1,20}$=6.38, P<0.05) and swimming time ($F_{1,20}$=25.64, P<0.01). Furthermore, interaction of almond and cadmium also significantly affected immobility time ($F_{1,20}$=7.18, P<0.05), struggling time ($F_{1,20}$=6.57, P<0.05) and swimming time ($F_{1,20}$=20.76, P<0.01). Tukey's post-hoc test demonstrated a significant increase in struggling (P<0.01) and swimming (P<0.01) time following the almond supplementation compared to control animals (Fig. 2A). Cad-mium administration significantly increased immobility time (P<0.01) and decreased struggling time (P<0.01) compared to controls, whereas almond supplementation significantly decreased immobility time (P<0.01) and increased struggling time (P<0.01) when compared with cadmium-treated rats.

Fig. 2B shows the effects of walnut and cadmium administration on FST activity. Two-way ANOVA showed significant effects for walnut ($F_{1,20}$=119.88, P<0.01), cadmium ($F_{1,20}$=0.49, P<0.01) and walnut×cadmium ($F_{1,20}$=16.09, P<0.01) on immobility time. Struggling time was also significantly affected by the administration of walnut ($F_{1,20}$=93.43, P<0.01), cadmium ($F_{1,20}$=23.91, P<0.01) and walnut×cadmium ($F_{1,20}$=16.9, P<0.01). Moreover, walnut ($F_{1,20}$=65.0, P<0.01), cadmium ($F_{1,20}$=15.42, P<0.01) and the interaction of walnut × cadmium ($F_{1,20}$=5.17, P<0.05) had significant effects on swimming time. Post-hoc analysis by Tukey's test revealed that walnut supplementation resulted in anti-depressant effects as evident by significantly decreased immobility time (P<0.01) and increased struggling time (P<0.01) and swimming time (P<0.01) compared to controls. Cadmium administration resulted in depressogenic effects, observed as significantly increased immobility time (P<0.01) and decreased struggling time (P<0.01) when compared with control animals. Cadmium-induced depressive behaviors were significantly attenuated by pre-supplementation with walnut in the Wal+Cad group which exhibited significantly decreased immobility time (P<0.01) and increased struggling (P<0.01) and swimming (P<0.01) time compared to the rats treated with cadmium alone.
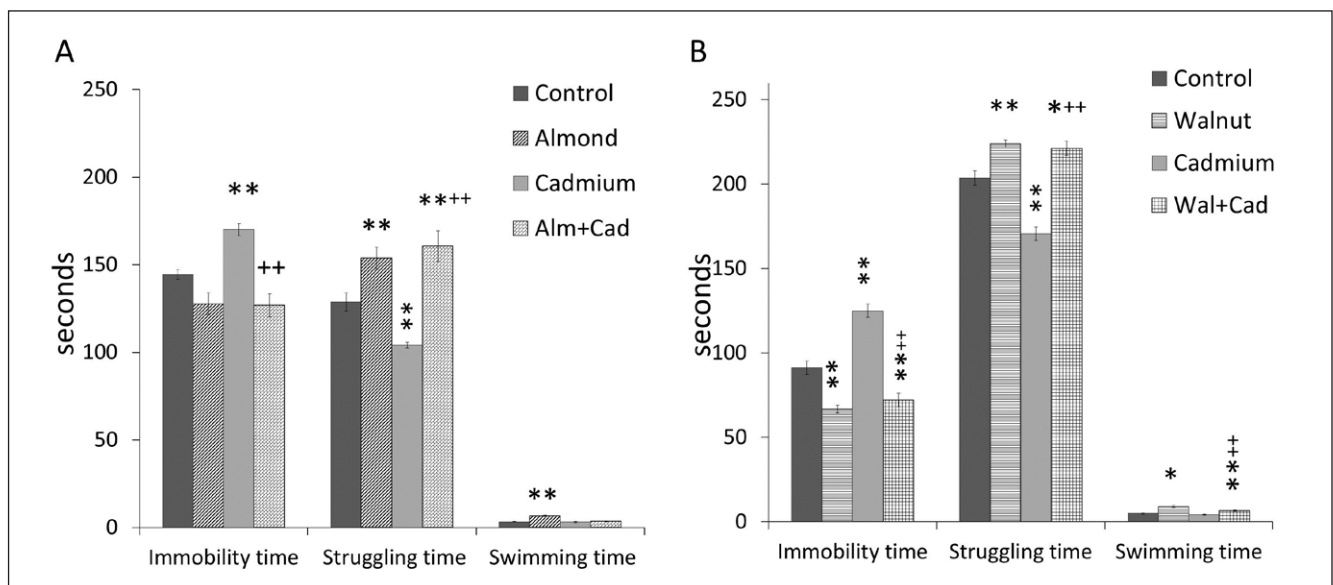

Fig. 2. Effects of almond (A) and walnut (B) supplementation following co-administration with cadmium were observed in the forced swim test in separate sets of rats. Values are mean±SEM (n=6). Data was analyzed by two-way ANOVA and Tukey's post-hoc test. **p<0.01 as compared to controls; ++p<0.01 with respect to cadmium-treated group.

Anxiogenic behavior

Effects of almond and cadmium were also evaluated in the LDT test (Fig. 3A). Latency to move into the dark box was significantly affected by almond ($F_{1,20}$=57.92, P<0.01) and cadmium ($F_{1,20}$=31.07, P<0.01) administration. Time spent in the light box was also significantly altered by the treatment of almond ($F_{1,20}$=30.40, P<0.01) and cadmium ($F_{1,20}$=17.47, P<0.01). Post-hoc testing indicated that supplementation of almond significantly increased latency to move (P<0.01) and time spent in light box (p<0.05) compared to control animals. Cadmium administration induced anxiogenic behavior as shown by significantly decreased latency (P<0.01) and time spent in the light box (P<0.01) when compared with control animals. The Alm+Cad group showed significantly increased latency (P<0.01) and time spent in light box (P<0.01) compared to cadmium-treated rats, indicating attenuation of cadmium-induced anxiety by almond supplementation.

Fig. 3B shows the effects of walnut and cadmium treatment on anxiogenic behavior. There was an effect of walnut supplementation on latency ($F_{1,20}$=67.91, P<0.01) and time spent in the light box ($F_{1,20}$=42.47, P<0.01). Cadmium administration also exerted a significant effect on latency ($F_{1,20}$=73.34, P<0.01) and time spent in the light box ($F_{1,20}$=22.69, P<0.01), whereas there was a significant interaction effect for walnut×cadmium on latency to move into the dark box ($F_{1,20}$=17.65, P<0.01). Post-hoc tests revealed that rats supplemented with walnut exhibited significantly increased latency (P<0.05) and time spent in the light box

(P<0.01), whereas cadmium intoxication resulted in decreased latency (P<0.01) and time spent in the light box (P<0.01), indicating anxiogenic behavior compared to control animals. The cadmium-induced anxiogenic behavior was significantly reduced by walnut supplementation in Wal+Cad group as evident by elevated latency (P<0.01) and time spent in the light box (P<0.01) when compared to cadmium-treated rats.

Aversive memory

The EPM was used to monitor retention of aversive memory following the administration of nuts and cadmium. Data from training and test sessions were evaluated by three-way ANOVA with repeated measures which showed a significant effect for almond ($F_{1,20}$=20.92, P<0.01), cadmium ($F_{1,20}$=7.23, P<0.05) and sessions ($F_{1,20}$=66.69, P<0.01). Interaction of almond × session ($F_{1,20}$=32.42, P<0.01) and almond × cadmium × sessions ($F_{1,20}$=4.51, P<0.05) were also found to be significant (Fig. 4A). Post-hoc analysis by Bonferroni test demonstrated that almond supplementation (P<0.05) significantly increased retention of memory in EPM, which was significantly impaired by cadmium intoxication (P<0.05) compared to control animals. Cadmium-induced memory impairment was significantly reversed by supplementation with almond in the Alm+Cad group (P<0.05).

Three-way ANOVA with repeated measures also revealed a significant effect of walnut supplementation ($F_{1,20}$=79.08, P<0.01) and sessions ($F_{1,20}$=74.87, P<0.01).
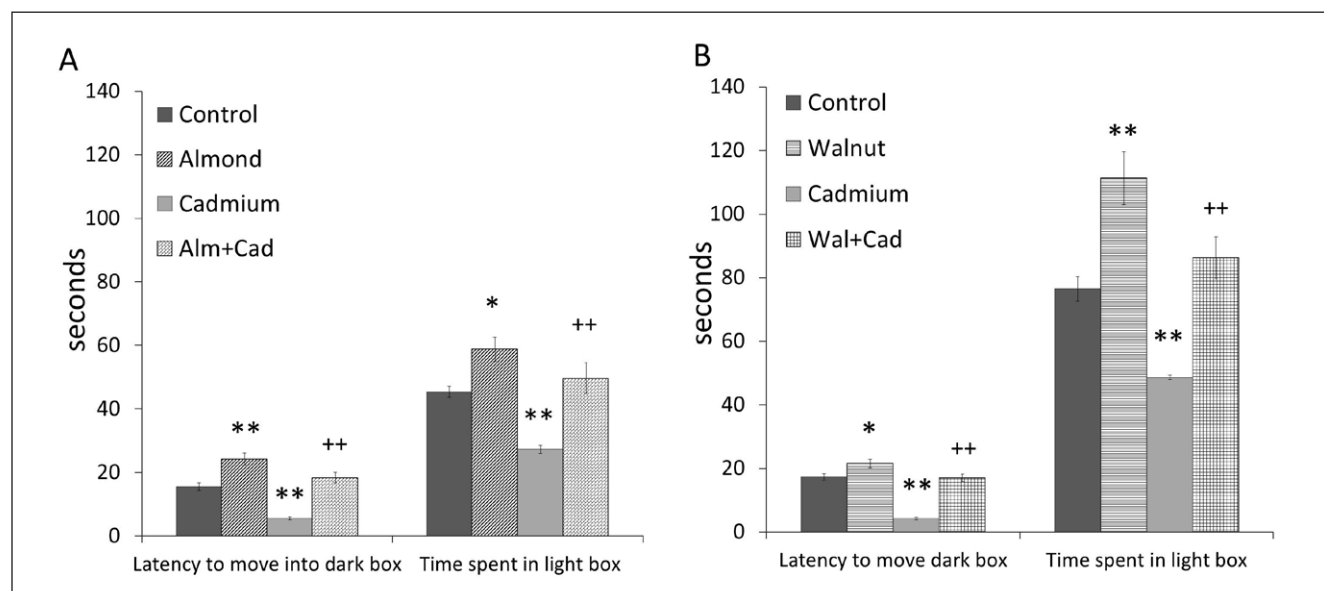

Fig. 3. Anxiety-like behavior was assessed by using a light/dark transition test following the administration of almond (A), walnut (B) in cadmium-intoxicated rats. Data was analyzed by two-way ANOVA using Tukey's post-hoc test. **p<0.01 versus control animals; ++p<0.01 versus cadmium-intoxicated rats. Values are mean±SEM (n=6).
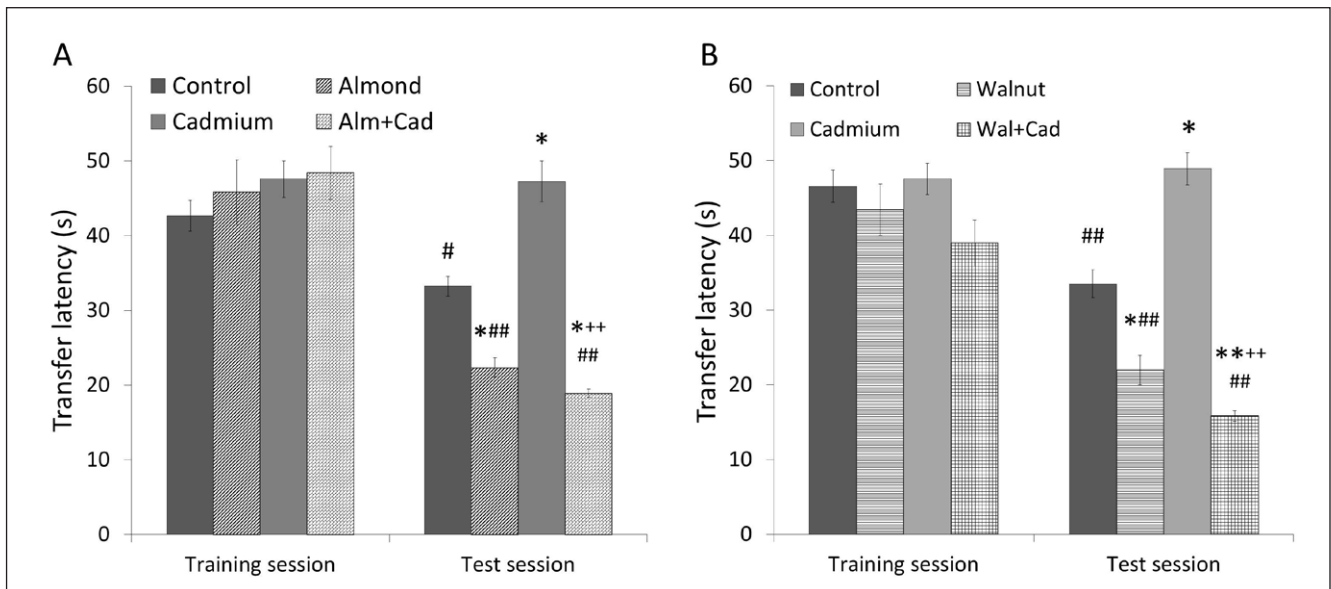
Fig. 4. Elevated plus maze was used to assess the effects of almond (A), walnut (B) administration in cadmium-intoxicated rats on aversive memory. Values are mean±SEM (n=6). Data was analyzed by three-way ANOVA with repeated measure design and Bonferroni post-hoc test. **p<0.01 as compared to controls; ++p<0.01 as compared to cadmium-treated group; #p<0.05, ##p<0.01 as compared to the respective training session.

A significant interaction between walnut×session ($F_{1,20}$=25.43, P<0.01) and walnut×cadmium×sessions ($F_{1,20}$=6.166, P<0.05) was also found in the ANOVA test. Bonferroni post-hoc test showed significantly impaired memory retention following the administration of cadmium (P<0.05), which was significantly attenuated by walnut supplementation in the Wal+Cad group (P<0.01) compared to control groups (Fig. 4B).

Spatial memory

Effects of nuts and cadmium administration on spatial memory were also evaluated by the MWM test. Three-way ANOVA with repeated measures showed significant effects of almond supplementation ($F_{1,20}$=9.40, P<0.01), cadmium administration ($F_{1,20}$=27.06, P<0.01) and sessions ($F_{1,20}$=69.29, P<0.01) and significant inter-
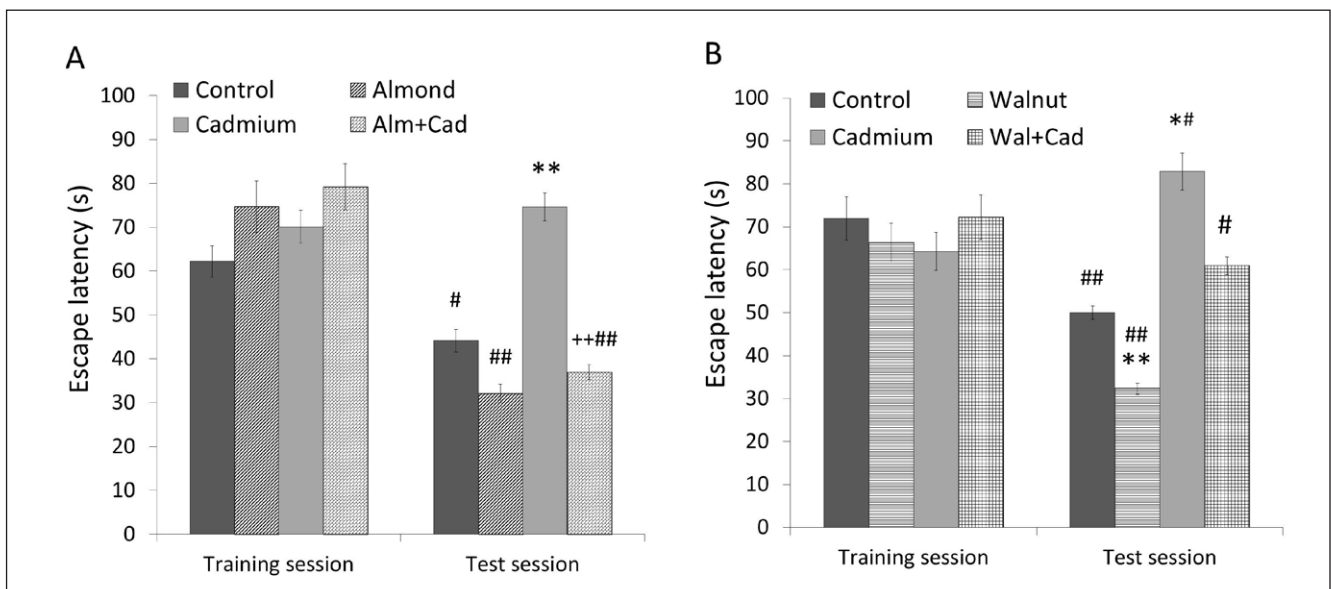

Fig. 5. Spatial memory was monitored by Morris water maze to assess the effects of almond (A) and walnut (B) administration in cadmium-intoxicated rats. Data was analyzed by three-way ANOVA with repeated measure design and Bonferroni post-hoc test. **p<0.01 as compared to controls; ++p<0.01as compared to cadmium-treated group; #p<0.05, ##p<0.01 as compared to the respective training session.

actions for almond × cadmium ($F_{1,20}$=9.98, P<0.01) and almond × sessions ($F_{1,20}$=36.34, P<0.01). Cadmium intoxication resulted in significantly impaired (P<0.01) retention of spatial memory compared to control animals (Fig. 5A), whereas almond supplementation significantly improved retention of the learned task in cadmium-intoxicated rats (P<0.01) compared to rats treated with cadmium alone.

The effects of walnut ($F_{1,20}$=79.08, P<0.01) and sessions ($F_{1,20}$=74.87, P<0.01) and interactions of walnut × cadmium ($F_{1,20}$=18.16, P<0.01), walnut × sessions ($F_{1,20}$=25.43, P<0.01) and walnut × cadmium × sessions ($F_{1,20}$=6.16, P<0.05) were also found to be significant for spatial memory. In these sets of rats, cadmium administration also resulted in impaired spatial memory (P<0.05) compared to control animals and walnut supplementation significantly reduced cadmium-induced impaired memory retention in the Wal+Cad group as shown in Fig. 5B.

Recognition memory

Effects of nuts and cadmium administration on recognition ability are shown in Fig. 6. There was a significant effect of treatment for almond ($F_{1,20}$=27.30, P<0.01) and cadmium ($F_{1,20}$=70.71, P<0.01) on cognition function. Post-hoc analysis by Tukey's test showed significantly increased recognition memory (P<0.01) following the supplementation of almond compared to control animals. Impaired cognitive ability (P<0.01) was found in the cadmium group which was not observed in cadmi-

um-intoxicated rats (P<0.01) that were co-administered almond for 28 days (Fig. 6A).

Significant effects of walnut supplementation ($F_{1,20}$=40.79, P<0.01) and cadmium treatment ($F_{1,20}$=5.85, P<0.05) were also found on recognition memory (Fig. 6B). Walnut supplemented rats showed significantly increased (P<0.05) recognition ability and cadmium-intoxicated rats exhibited impaired (P<0.05) discrimination between a new and old object compared to control animals. Cadmium-induced impaired recognition memory was significantly attenuated by walnut supplementation in the Wal+Cad group when compared to cadmium-treated rats (P<0.01).

Brain biogenic amines

Cadmium-induced neurotoxicity was observed in terms of levels of brain biogenic amines and their metabolites. Fig. 7A shows the effects of cadmium and almond, as well as their co-administration, on brain catecholamines. Two-way ANOVA revealed a significant effect of almond on NA ($F_{1,20}$=15.5, P<0.01), DA ($F_{1,20}$=30.8, P<0.01) and DOPAC ($F_{1,20}$=15.7, P<0.01) levels but a non-significant effect on HVA ($F_{1,20}$=1.38, P>0.05) levels. Administration of cadmium also had a significant effect on NA ($F_{1,20}$= 146.5, P<0.01) and DA ($F_{1,20}$=10.06, P<0.01) levels but a non-significant effect on DOPAC ($F_{1,20}$=3.17, P>0.05) and HVA ($F_{1,20}$= 2.16, P>0.05). Moreover, there was a significant interaction effect for almond and cadmium on NA ($F_{1,20}$=5.17, P<0.05), DOPAC
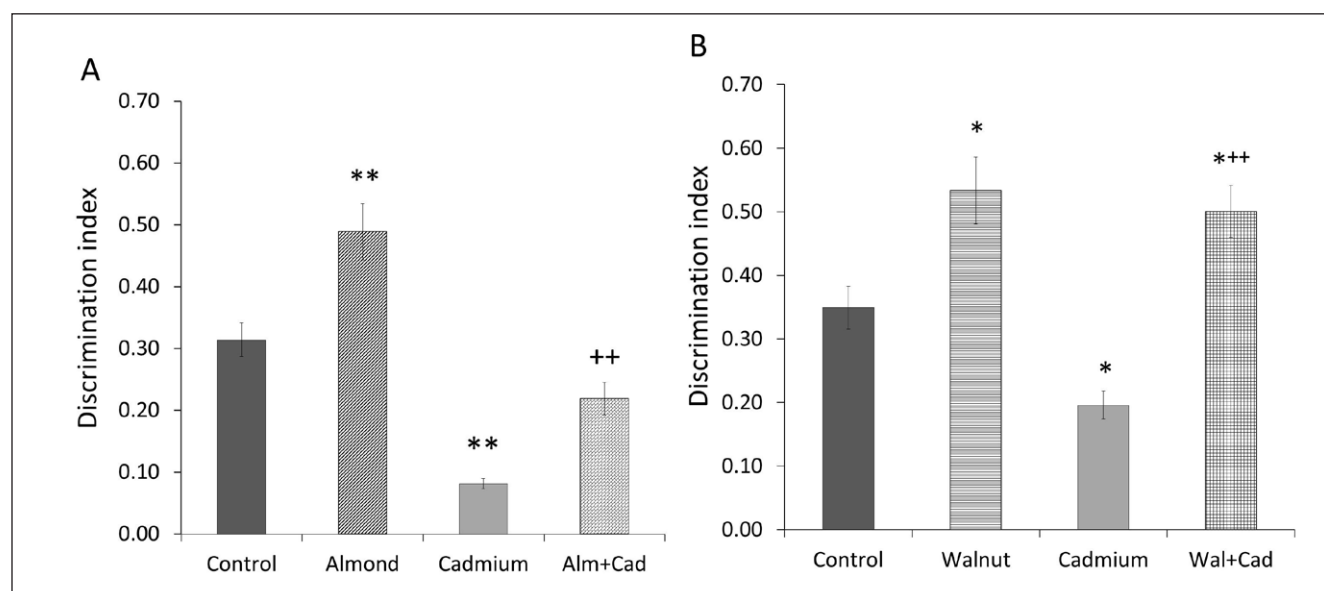

Fig. 6. Novel object recognition test was used to evaluate the cognitive ability of rats supplemented with almond (A) and walnut (B) following co-administration with cadmium. Two-way ANOVA was used to analyze the data followed by Tukey's post-hoc test. Values are mean±SEM (n=6). **p<0.01 versus control animals; ++p<0.01 versus cadmium-intoxicated rats.
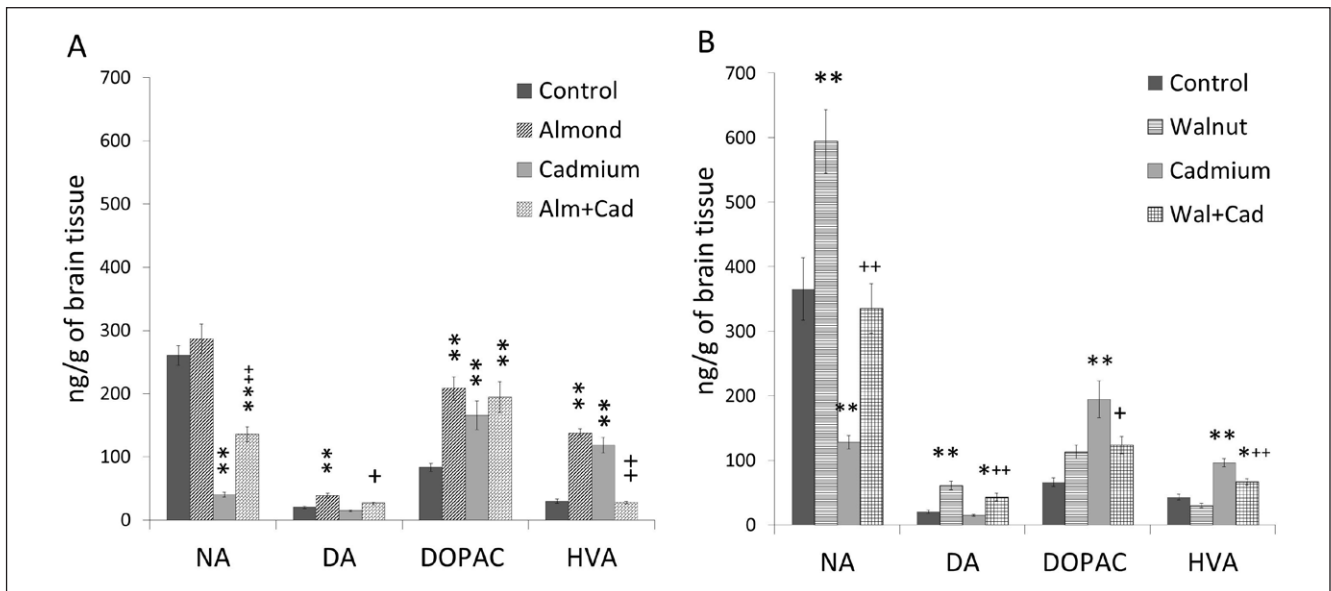
Fig. 7. Effects of almond (A) and walnut (B) supplementation on catecholamine levels was examined in cadmium-intoxicated. Values are mean±SEM (n=6). Data was analyzed by two-way ANOVA followed by Tukey's post-hoc test. **p<0.01 as compared to control animals; ++p<0.01 as compared to cadmium-intoxicated rats.

($F_{1,20}$=15.5, P<0.01) and HVA ($F_{1,20}$=185.97, P<0.01) levels but a non-significant effect on DA ($F_{1,20}$=1.24, P>0.01) levels. It was observed following the Tukey's test that almond administration for 28 days significantly increased DA, DOPAC and HVA levels (P<0.01) compared to controls. Cadmium intoxication resulted in decreased levels of NA (P<0.01) but significantly increased levels of DOPAC and HVA (P<0.01) relative to control rats.

Alm+Cad rats exhibited significantly increased levels of NA and DA (P<0.01) compared to cadmium-treated rats. DOPAC levels of Alm+Cad rats were significantly (P<0.01) increased versus the control group, whereas HVA levels were significantly (P<0.01) decreased when compared with cadmium exposed rats.

Fig. 7B shows the levels of brain catecholamines following the administration of walnut or cadmium. Wal-
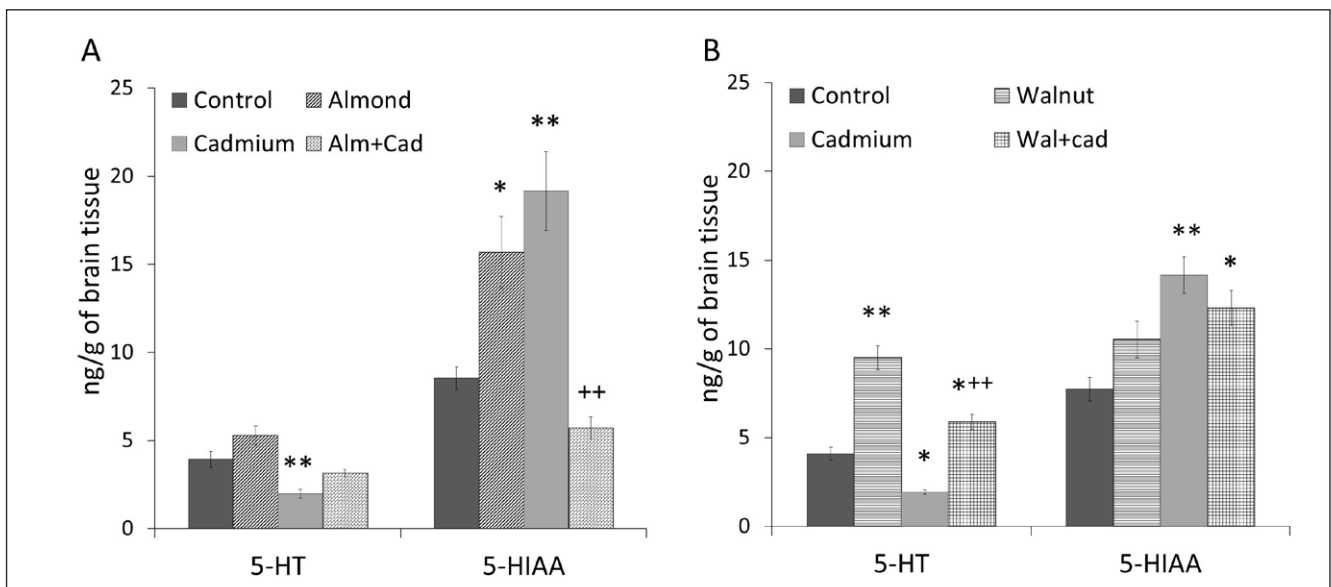

Fig. 8. Effects of almond (A) and walnut (B) supplementation on indolamine levels in cadmium-intoxicated rats were also assessed. Values are mean±SEM (n=6). Data was analyzed by two-way ANOVA followed by Tukey's post-hoc test. **p<0.01 versus control animals; ++p<0.01 versus cadmium-intoxicated rats.

nut administration significantly affected NA ($F_{1,20}$=30.17, P<0.01), DA ($F_{1,20}$=5.74, P<0.01) and HVA ($F_{1,20}$=20.52, P<0.01) levels. The effect of walnut on DOPAC ($F_{1,20}$=0.499, P>0.05) levels was non-significant. Cadmium treatment significantly altered NA ($F_{1,20}$=39.11, P<0.01), DA ($F_{1,20}$=6.48, P<0.05), DOPAC ($F_{1,20}$=16.88, P<0.01) and HVA ($F_{1,20}$=90.52, P<0.01) levels. There was a significant interaction effect for walnut×cadmium on DOPAC ($F_{1,20}$=12.33, P<0.01) levels only. Tukey's test revealed that walnut administration significantly increased NA (P<0.01) and DA (P<0.01) levels compared to control rats. In the second experiment, cadmium intoxication also resulted in significantly decreased (P<0.01) NA levels, but it significantly increased (P<0.01) metabolites of DA (DOPAC and HVA) when compared to control group. Walnut administration in cadmium-intoxicated rats significantly increased the levels of NA (P<0.01) and DA (P<0.01) while it significantly decreased the levels of DOPAC (P<0.05) and HVA (P<0.01) compared to rats treated with cadmium alone.

Results for serotonin and its metabolite are shown in Fig. 8. Data was analyzed by two-way ANOVA and it was observed that administration of almond ($F_{1,20}$=30.88, P<0.01) and cadmium ($F_{1,20}$=10.06, P<0.01) significantly affected the concentration of 5-HIAA (Fig. 8A), while the interaction of almond and cadmium exerted significant effects on 5-HT levels ($F_{1,20}$=43.12, P<0.01). Post-hoc analysis by Tukey's test revealed a significant increase in 5-HIAA levels following the oral treatment of almond (P<0.05) and cadmium (P<0.01) compared to control rats. Cadmium intoxication also resulted in significantly decreased (P<0.01) levels of 5-HT in rat brain.

Supplementation with almond in cadmium-intoxicated rats resulted in a drastic decrease (P<0.01) in 5-HIAA levels compared to cadmium-treated rats, whereas 5-HT levels became comparable to control rats.

Fig. 8B depicts the effects of walnut and cadmium administration on 5-HT and 5-HIAA levels. Walnut administration significantly altered the levels of 5-HT ($F_{1,20}$=112.61, P<0.01), whereas cadmium treatment exerted significant effects on 5-HT ($F_{1,20}$=3.10, P<0.01) and 5-HIAA ($F_{1,20}$=19.03, P<0.01) levels. Walnut × cadmium also resulted in significant interaction effect on the concentration of 5-HIAA ($F_{1,20}$=6.14, P<0.05). Tukey's test revealed a significant increase (P<0.01) in 5-HT levels following the administration of walnut compared to controls. Cadmium intoxication showed significantly decreased (P<0.05) 5-HT levels and increased degradation of 5-HT as evident by significantly increased (P<0.01) 5-HIAA levels compared to levels in control rats. Co-administration of walnut in the cadmium-treated group resulted in significantly increased (P<0.01) 5-HT and decreased (P<0.05) 5-HIAA levels when compared to the group treated with cadmium alone.

In this study turnover of biogenic amine was also calculated in terms of the ratio between metabolite and neurotransmitter. Fig. 9 shows the turnover ratio of DA and 5-HT following the administration of nuts and cadmium. Two-way ANOVA showed significant effects for almond administration on 5-HT turnover ($F_{1,20}$=38.37, P<0.01). Cadmium intoxication significantly affected the turnover of both DA ($F_{1,20}$=33.27, P<0.01) and 5-HT ($F_{1,20}$=27.99, P<0.01), whereas the interaction
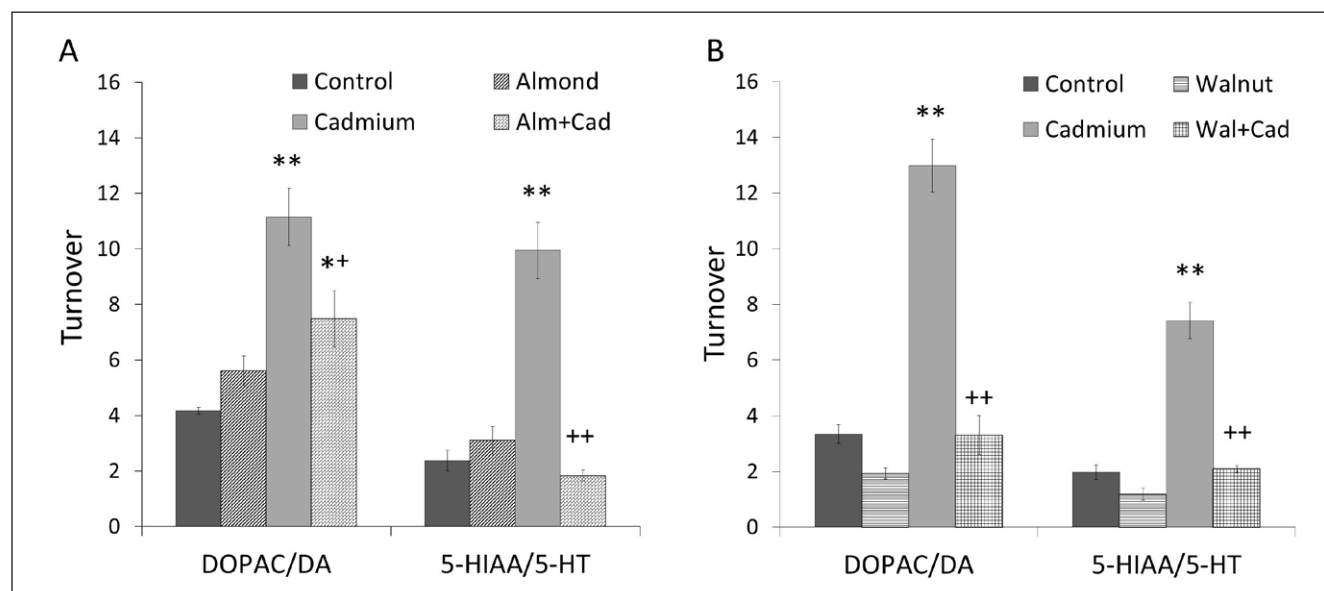

Fig. 9. Biogenic amine turnover was calculated by taking the ratio of a metabolite's concentration versus its neurotransmitter. Effects of almond (A) and walnut (B) supplementation were analyzed by two-way ANOVA with Tukey's post-hoc test. *p<0.05, **p<0.01 versus control animals; +p<0.05, ++p<0.01 versus cadmium-intoxicated rats. Values are mean±SEM (n=6).

of almond×cadmium also resulted in altered turnover ratios of DA ($F_{1,20}$= 1.09, $P<0.01$) and 5-HT ($F_{1,20}$= 5.01, $P<0.01$) (Fig. 9A). Tukey's test showed a significant increase in DA ($P<0.01$) and 5-HT ($P<0.01$) degradation in cadmium-intoxicated rats compared to controls, while the administration of almond in cadmium-treated rats resulted in significantly decreased turnover of DA ($P<0.05$) and 5-HT ($P<0.01$) compared to the cadmium group. These results depict the attenuation of cadmium-induced alterations in neurotransmitters by almond supplementation.

Fig. 9B shows the effects of walnut and cadmium administration on DOPAC/DA and 5-HIAA/5-HT ratios. There was a significant effect of walnut administration on DA ($F_{1,20}$=80.53, $P<0.01$) and 5-HT ($F_{1,20}$=67.29, $P<0.01$) turnover ratios. Significant effects of cadmium treatment on DA ($F_{1,20}$=79.27, $P<0.01$) and 5-HT ($F_{1,20}$=72.83, $P<0.01$) turnover were also revealed. In addition, a walnut×cadmium interaction resulted in altered DOPAC/DA ($F_{1,20}$=44.65, $P<0.01$) and 5-HIAA/5-HT ($F_{1,20}$=36.88, $P<0.01$) ratios. Post-hoc analysis showed a significant increase in turnover of DA ($P<0.01$) and 5-HT ($P<0.01$) following the administration of cadmium compared to controls. This increase in turnover ratios was not observed in the cadmium group that was co-administered walnut, representing the protective effects of walnut supplementation against cadmium-induced neurotoxicity.

DISCUSSION

Cadmium is toxic even at very low doses. Cadmium toxicity results in damaging effects on human health (Wang and Du 2013). It has been suggested that the nutrients and nutritional status of an individual may modify susceptibility to metal toxicity (Fernstrom and Fernstrom, 2007). In the present study, weekly exposure to cadmium at a dose of 50 mg/kg produced deleterious effects on the levels of brain neurotransmitters. There was a significant decrease in NA, DA and 5-HT levels, whereas the concentrations of their metabolites were significantly increased compared to untreated animals. The variation in levels of neurotransmitters resulted in altered neurobehavioral effects. Cadmium administration caused depressogenic, anxiogenic and memory impairing effects. It has been shown that the toxic effect of cadmium on the CNS is due to an increase in permeability of the BBB, which enhances the penetration and accumulation of cadmium in brain (Andrade et al., 2017). Cadmium exerts its neurotoxic effects by blocking Ca^{++}-dependent intracellular signaling pathways and inhibiting the fusion of storage vesicles, resulting in decreased neurotransmitter at the synapse (Wang and Du, 2013).

It has also been observed that exposure to cadmium increases the release of excitatory neurotransmitters and inhibits the release of inhibitory neurotransmitters leading to excitotoxicity and synaptic disruption thus causing behavioral impairments (Marchetti et al., 2014). Therefore, the observed reduced levels of brain biogenic amines may be attributed to impaired signal transduction induced by cadmium administration. In this study cadmium-induced changes in neurotransmitter levels and behavior were significantly attenuated by the long-term co-administration of almonds and walnuts in rats.

Entrance of heavy metals into the nervous system is tightly controlled but they can still interfere with cellular response by mimicking physiological ions. Cadmium exposure affects the concentration and function of ions that have important physiological function, including zinc, iron, magnesium and calcium (Guo et al., 2017). The normal function of vitamin B2, B12 and B6 is also shown to be affected by cadmium exposure. Cadmium interferes with the absorption of calcium from the intestine. It also interrupts the binding of zinc with metallothionein, a small protein which is involved in the transport of essential metals, thus creating a deficiency of zinc and other physiologically important metals (Flora, 2002). Indispensable metal ions are necessary for the synthesis of coenzymes such as pyridoxal-5-phosphate and flavin adenine dinucleotide (Churchich et al., 1989) which are essential for the synthesis of biogenic amines (Dakshinamurti et al., 1990). It has been suggested that neuronal communication can be affected by diet, either by modulating the amount of neurotransmitter precursors or by affecting the concentration of nutrients which are required as cofactor for neurotransmitter synthesis (Fenech et al., 2017). In various studies supplementation of micronutrients have been reported to modify metal toxicity and may also act as a chelating agent. Calcium, zinc and iron have been shown to protect against the toxicity of heavy metals including lead, mercury and cadmium in all age groups (Flora, 2002). Copper is another important physiological ion required for the maintenance of neurotransmitters in the brain. It has been shown that cadmium exposure disturbs copper metabolism and intake of copper has been suggested to reverse cadmium-induced alteration by replacing the cadmium from metallothionein (Matovic et al., 2004). Deficiency of these nutrients has been shown to exacerbate metal toxicity. It has been postulated that cognitive dysfunction, depression and other neuropsychiatric behaviors are directly associated with mineral and/or vitamin deficiencies (Brenner, 2017). Previously, deficiencies of folic acid and magnesium were found to induce depression in human

subjects (Sylvia et al., 2013). Therefore, it is possible that cadmium exposure may induce deficiencies of essential nutrients, leading to impaired neurotransmission, neurocognitive and neuropsychiatric behaviors

as observed in this study. Nuts, including almonds and walnuts, contain a variety of indispensable micronutrients such as zinc, calcium, iron, magnesium, copper, selenium, thiamine, folic acid and tocopherol.
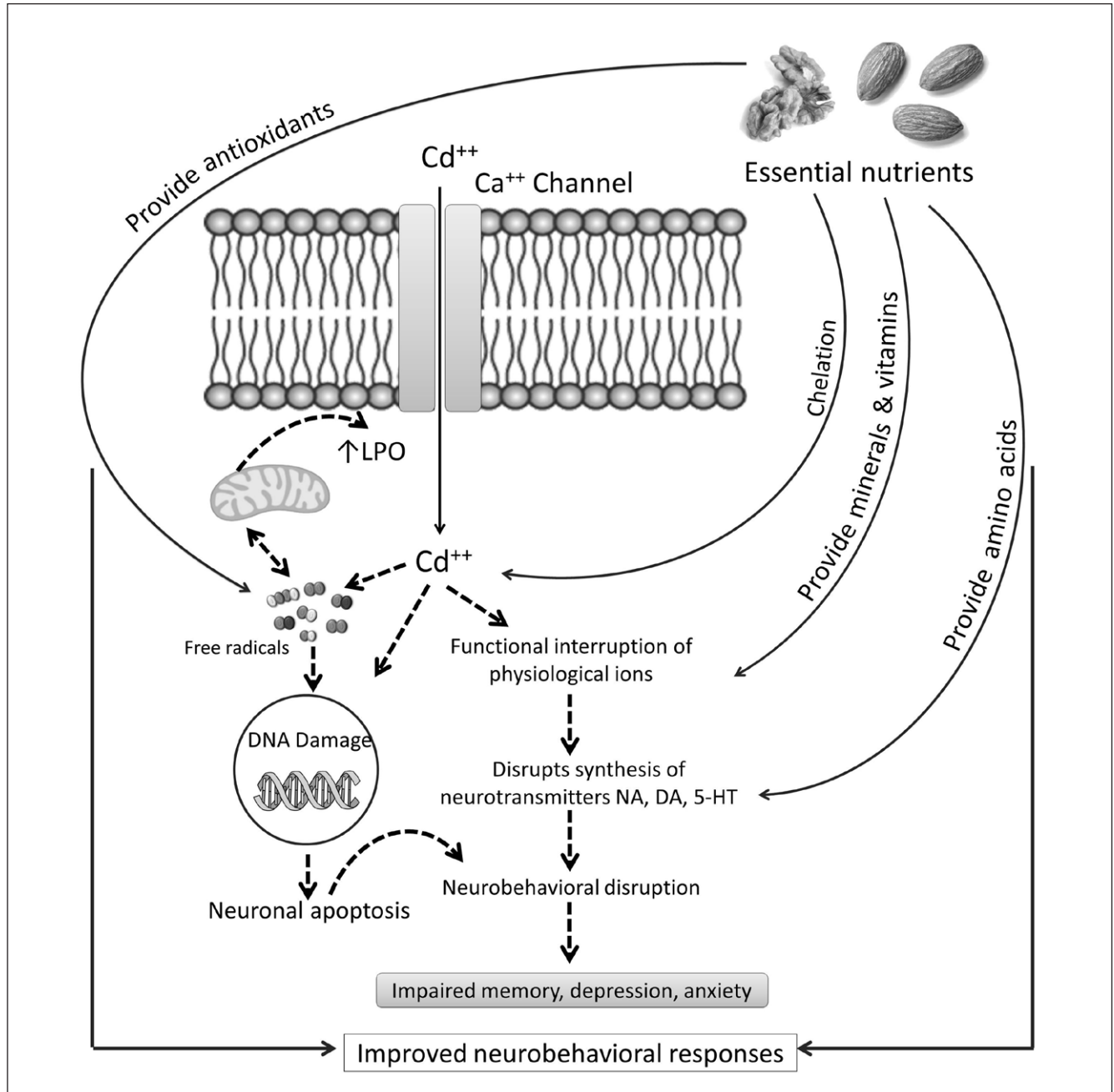

Fig. 10. Schematic diagram showing ameliorative effects of nut consumption on cadmium neurotoxicity. Exposure to cadmium increases the permeability of blood brain barrier which results in increased penetration and accumulation of cadmium in the brain. Cadmium exerts its neurotoxic effects by blocking Ca++-dependent intracellular signaling and inhibiting the release of neurotransmitters into the synapse leading to their increased degradation by monoamine oxidases. Increased aberrations of neurotransmitters may lead to behavioral deficits such as impaired memory function, depression and anxiety. Nuts, including almond and walnuts, are a natural source of multi-nutrients including essential amino acids and minerals which are endogenously required for the synthesis of indolamines and catecholamines. They are also an important source of antioxidants which may provide protection against oxidative stress induced by cadmium intoxication. Moreover, nutrients present in nuts are reported to have heavy metal chelating properties and thus provide protection against metal toxicity. In the present study daily administration of almond and walnut resulted in improved metabolism of neurotransmitters and an attenuation of behavioral deficits caused by the exposure of rats to cadmium.

Supplementation with nuts could be considered as an important source of vital micronutrients (Gorji et al., 2017). In this study, supplementation of vitamins and essential minerals through nuts may be the reason for the observed attenuation of cadmium-induced neurotoxicity. Gubrelay et al. (1998) showed the preventive effects of thiamin against cadmium-induced biochemical alteration in male rats. This group suggested the formation of an excretable thiamin-cadmium complex which reduced cadmium intoxication in male rats. Multi-nutrients have also been suggested to reduce metal toxicity. A combination of vitamin C, tocopherol and flavonoid was tested against hepatotoxicity induced by cadmium. This nutritional supplementation was shown to reduce hepatotoxic markers and improve hepatic antioxidant enzymes in cadmium-exposed rats (Prabu et al., 2011). Thus in the present study, the availability of micronutrients from almonds and walnuts may be involved in the reversal of cadmium-induced impaired neurotransmission and neurobehavioral deficits.

Data published by the US department of Agriculture Nutrient Database described the presence of ample amounts of protein, essential amino acids, including tyrosine, phenylalanine and tryptophan, and choline in almonds and walnuts (Batool et al., 2016; Haider et al., 2018). Brain catecholamines and serotonin neuronal mechanisms are involved in a number of physiological actions such as behavior, mood and memory functions. Supplementation of essential amino acids in nutritional deficiency may lead to improved synthesis and release of neurotransmitter and enhanced neurobehavioral responses of biogenic amines (Ney et al., 2017). In active neurons, tyrosine concentration controls the synthesis of catecholamines by modulating the activity of tyrosine hydroxylase, which is the rate-limiting enzyme for catecholamine synthesis. It has been shown that diet influences DA and NA levels by directly affecting tyrosine uptake and levels in the brain (Fernstrom and Fernstrom, 2007). The administration of tyrosine in rats pre-treated with reserpine was shown to stimulate synthesis and release of DA and NA (Sved et al., 1979). Likewise, the supplementation of tryptophan, another essential amino acid for the synthesis of 5-HT, increased serotonergic neurotransmission (Haider et al., 2006). Administration of tyrosine and tryptophan has been shown to improve memory function and reduce psychiatric illnesses under neurologically stressful conditions by raising catecholamine and serotonin synthesis and release (Haider et al., 2006; Ogawa et al., 2018). It could be suggested that, in the present study, weekly cadmium exposure in rats resulted in reduced levels of biogenic amines via interference with metabolic pathways and thus induced memory dysfunction and behavioral despair in rats. Food-induced increases in brain tyrosine, tryptophan and choline may result in behavioral changes (Batool et al., 2016; Ng and Anderson, 1992). Previously, improved memory function and increased plasma and brain tryptophan levels have been reported following the long-term administration of tryptophan, almonds and walnuts in healthy rats (Haider et al., 2006; Batool et al., 2016; Haider et al., 2018). Therefore, a possible mechanistic explanation for the improved behavioral responses observed in cadmium-treated rats after the administration of nuts may involve an induced adequate supply of precursors reducing cadmium-induced nutritional deficiency. Acetylcholine may also be another plausible brain chemical playing a role in the nuts-associated improved memory function in cadmium-treated rats. Previously, acetylcholine and acetylcholinesterase activity were monitored in frontal cortex and hippocampal regions of cadmium-intoxicated rats. Enhanced cholinergic function was observed in these memory-specific areas, along with improved cognitive ability, with almond and walnut supplementation in cadmium-treated rats (Batool et al., 2017). Brain acetylcholine concentration and function also depends upon sufficient provision of its precursor. Lecithin and choline supplementation in animals and human subjects resulted in enhanced memory function (Moré et al., 2014). Intake of choline from early life is also recommended for better cognitive ability and healthy brain function (Tabassum et al., 2017).

CONCLUSIONS

From the present study, we conclude that the essential nutrients in nuts provide precursors for the synthesis of neurotransmitters. The chelating properties of some of the nutrients present in nuts may also help in the elimination of cadmium. This study suggests that a combined action of multi-nutrients in nuts reduces cadmium-induced neurotoxicity and behavioral deficits in rats (findings of the current study are summarized in Fig. 10). The present findings emphasize the utility of nuts in a daily diet to attenuate cadmium intoxication, especially in individuals who are unavoidably exposed to cadmium through cigarette smoke and environmental or occupational activities.

ACKNOWLEDGEMENT

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