Attenuation of cadmium-induced decline in spatial, habituation and recognition memory by long-term administration of almond and walnut supplementation: Role of cholinergic function

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Abstract: Excessive exposure of cadmium which is regarded as a neurotoxin can stimulate aging process by inducing abnormality in neuronal function. It has been reported that supplementation of almond and walnut attenuate age-related memory loss. Present study was designed to investigate the weekly administration of cadmium for one month on learning and memory function with relation to cholinergic activity. Cadmium was administered at the dose of 50 mg/kg/week. Whereas, almond and walnut was supplemented at the dose of 400 mg/kg/day along with cadmium administration to separate set of rats. At the end of experiment, memory function was assessed by Morris water maze, open field test and novel object recognition test. Results of the present study showed that cadmium administration significantly reduced memory retention. Reduced acetylcholine levels and elevated acetyl cholinesterase activity were also observed in frontal cortex and hippocampus of cadmium treated rats. Malondialdehyde levels were also significantly increased following the administration of cadmium. Daily supplementation of almond and walnut for 28 days significantly attenuated cadmium-induced memory impairment in rats. Results of the present study are discussed in term of cholinergic activity in cadmium-induced memory loss and its attenuation by nuts supplementation in rats.

Keywords: Almond, cadmium toxicity, cholinergic activity, memory function, walnut.

INTRODUCTION

Environmental pollution with heavy metals has become one of the major health concerns. Exposure of general population with heavy metals has dramatically increased due to its excessive use (Tchounwou *et al.,* 2012). Cadmium is one of the heavy metal which is recognized as neurotoxin. It is extensively used in household electronic appliances, phosphate fertilizers, as PVC pipe stabilizer, present as color pigment in paints (Järup, 2003). Cigarette smoke, combustion of municipal waste, industrial activities and traffic pollution are also the major sources of cadmium contamination to which general populations are exposed on the daily basis. Cadmium can produce deleterious health effects at much lower levels (Järup and Akesson, 2009). This heavy metal can cross blood brain barrier and hence has shown to produce hazardous effects on central nervous system (Nishimura *et al.,* 2006). In a study acute exposure to cadmium dose-dependently reduced memory retention in Morris water maze (MWM) paradigm in rats (Haider *et al.,* 2015a). Early exposure to cadmium has also shown to produce injurious effects on mental health in children (Wright and Baccarelli, 2007). High concentration of cadmium has been shown to be positively correlated with mental retardation, learning deficits, reduced IQ level and impaired visual-motor tasks (Wright and Baccarelli, 2007). Repeated exposure to cadmium causes reduced levels of neurotransmitters including serotonin, norepinephrine and acetylcholine (ACh) (Rastogi, *et al.,* 1977). Cadmium stimulates the generation of reactive species leading to oxidative stress and cytotoxic effects (Stohs and Bagchi, 1995). This cadmium-induced oxidative stress causes DNA damage, disrupts DNA repair and impairs DNA and protein synthesis (Mitra, 1984; Abshire *et al.,* 1996). The disruption of signal transduction pathway has also been shown previously due to cadmium exposure (Carageorgiou *et al.,* 2005) that may be the cause of reduced neurotransmission. Cadmium exposure has shown to induce cholinergic dysfunction leading to learning and memory disorders (Wang and Du, 2013).

Effects of dietary components on central nervous system are well reported (Gomez-Pinilla and Gomez, 2011). Environmental pollution, nutritional deficiency and senescence are considered as the major causes of neurodegenerative diseases (Chin-Chan *et al.,* 2015; Davinelli *et al.,* 2014). The cost for the treatment of neurological disorders exert financial burden on the global economy, therefore, prevention and management of such diseases by healthy diet has now become a major concern for researchers (Rao and Rao, 2007). Nuts are considered as the rich source of essential and non-

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essential nutrients. Nuts such as pecan, macadamia nut, Brazil nuts, peanuts, almond and walnut contain considerable amount of protein, essential fatty acids, and vitamins including vitamin B-6, tocopherol, β-carotene and choline (Davidson, 1999). Almond and walnut are a rich source of minerals like manganese, copper, potassium, calcium, iron, zinc, and selenium (Morley, 2010). These minerals and vitamins have antioxidative and metal chelating properties (Carageorgiou *et al.,* 2005). Copper, manganese, zinc, and selenium act as co-factors for antioxidant enzymes in cellular system (Higgins *et al.,* 2002). Thus, their adequate availability in cell increases the efficiency of these enzymes. The metal chelating activity of zinc, calcium and L-cysteine has been shown previously by Carageorgiou and co-workers (2005). Furthermore, increased serotonergic and cholinergic functions following long-term administration of almond and walnut have been reported previously from our lab (Haider *et al.,* 2011; Haider *et al.,* 2012; Batool *et al.,* 2016).

Heavy metal-induced neurotoxicity and neurodegenerative diseases assume special attention for the individuals who are being exposed to these neurotoxins on the daily basis. This study was therefore designed to investigate the protective effects of almond and walnut against cadmium-induced cognitive impairment and oxidative stress. The role of ACh in learning and memory functions is well-established (Francis *et al.,* 1999). Since these nuts may also provide essential nutrients for the synthesis of neurotransmitters such as tryptophan and choline for the formation of serotonin and ACh, respectively (Batool *et al.,* 2016). So therefore, this study was further extended to find out the role of cholinergic system in terms of ACh content and acetyl cholinesterase (AChE) activity in memory specific regions i.e. frontal cortex and hippocampus.

MATERIALS AND METHODS

Experimental protocol

Twenty four locally bred Albino Wistar rats having body weight of 150-200 g were purchased from Dow University of Health Sciences, OJHA Campus, Karachi. Experiment was started after a period of three days of acclimatization. Animals were divided into four groups (n=6). Shelled almond and walnut were purchased from local super market, peeled and finely crushed into fine powder which was stored in refrigerator. Fresh almond and walnut suspension was prepared everyday and orally administered to the alm+cad (almond +cadmium) and wal +cad (walnut +cadmium) groups respectively at the dose of 400 mg/kg/day (Batool *et al*., 2016). Cadmium was also given orally to the cadmium, alm+cad and wal+cad groups at the dose of 50 mg/kg/week. Control group was treated with water only. This treatment was continued for four weeks. After this treatment rats were subjected to the memory assessment using MWM, open field test (OFT) and novel object recognition (NOR) task. Rats were decapitated after the completion of behavioral assessment. Frontal cortex and hippocampus were collected from brain samples and stored at -70^{o}C until neurochemical analysis.

Behavioral assessment

Morris water maze (MWM)

The dimensions of the apparatus and procedure were same as described previously (Haider *et al*., 2015b). Briefly the test comprised of training and test sessions during which the escape latency, that is the time taken by each rat to reach the hidden platform, was noted. The test phase was performed 24 h after a training session. The cutoff time was 2 min. A decrease in escape latency during test sessions was taken as an index of memory improvement.

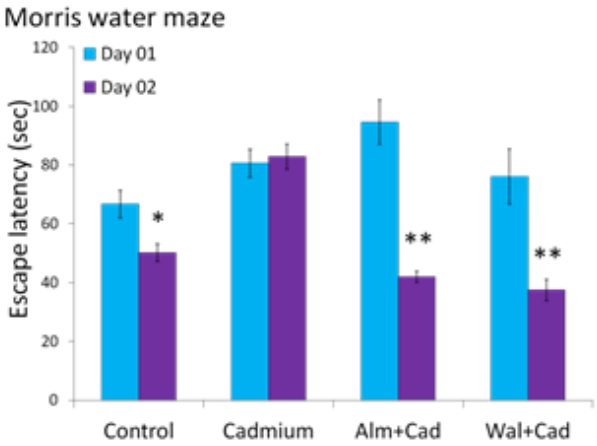

Fig. 1: Effects of co-administration of cadmium with almond and walnut on spatial memory in Morris water maze test in rats. Values are mean±SEM. Data was analyzed by independent *t*-test: *p<0.05, **p<0.01 with respect to day 01.

Open field test (OFT)

The apparatus used in this study was consisted of a square area 76 × 76 cm. Walls of this apparatus were opaque and height of walls was 42 cm. Twenty-five equal squares were drawn on the floor of the apparatus. To determine the activity, animals were removed from their home cages and placed in the center square of the apparatus (one at a time). The number of squares crossed, time (sec) of rearing and number of rearing by each rat was recorded for 5 min. Habituation is a type of non-associative memory and significant decrease in the activity on the repeated exposure is taken as an index of retention of habituation in open field (Kaoud *et al*., 2010).

Novel object recognition (NOR) test

The dimensions of the apparatus and procedure were same as described previously (Batool *et al*., 2016). On the brief account NOR test was conducted to determine recognition ability in rats to distinguish between new and old object. Rats were trained to familiarize with two same

objects during training session. During test session one old object was then replaced by a new object and time to sniff both objects was recorded. Recognition index was calculated by using the formula [time to sniff new object / (time to sniff old object + time to sniff new object)].

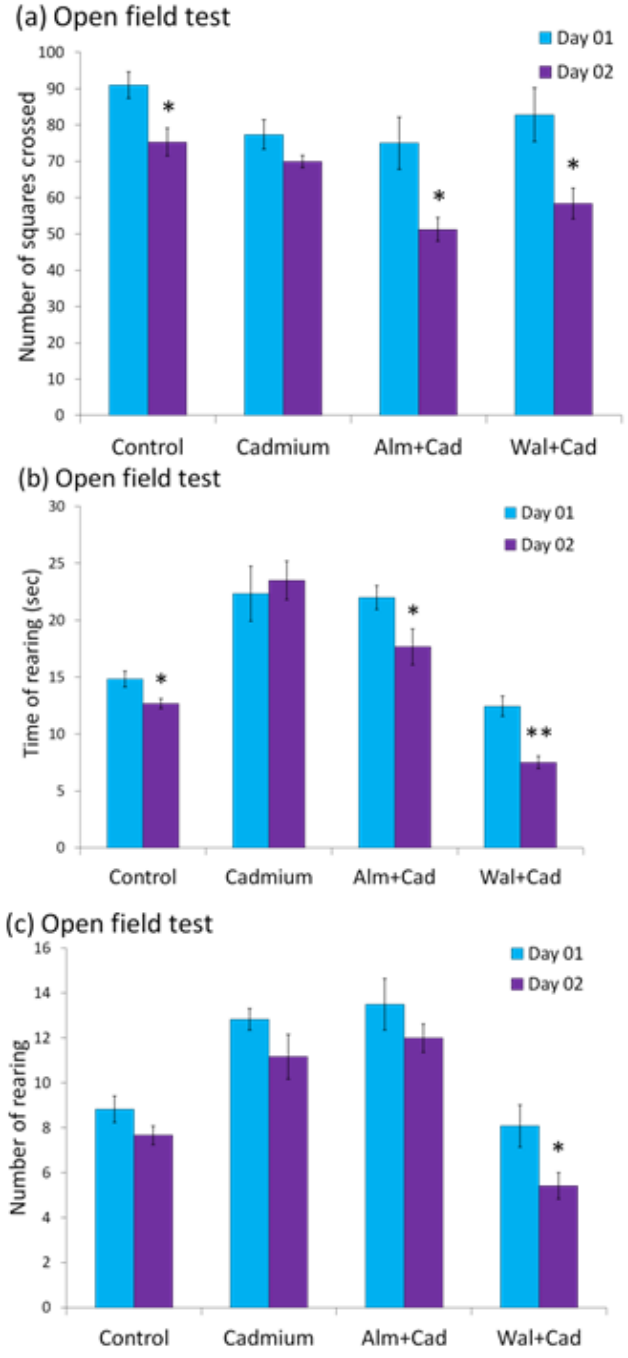

Fig. 2: Effects of almond and walnut supplementation in cadmium treated rats on habituation memory. Memory was assessed in terms of a) number of square crossed, b) time of rearing (sec) and c) number of rearing. Data was analyzed by independent *t*-test: *p<0.05, **p<0.01 with respect to day 01. Values are mean±SEM.

Neurochemical analysis

Malondialdehyde (MDA) levels

Brain MDA levels were estimated as a biomarker of lipid peroxidation as explained by Haider *et al* (2015b). MDA is expressed as µmol/g of brain tissue.

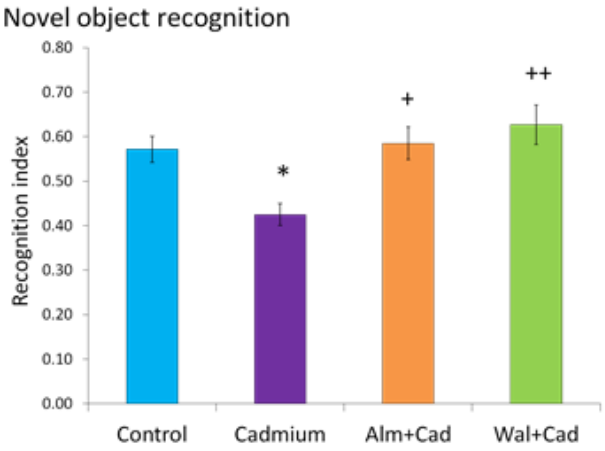

Fig. 3: Effects of almond and walnut supplementation in cadmium treated rats on recognition memory. Values are mean±SEM. Data was analyzed by one-way ANOVA: *p<0.05 from respective controls; +p<0.05, ++p<0.01 from cadmium treated rats.

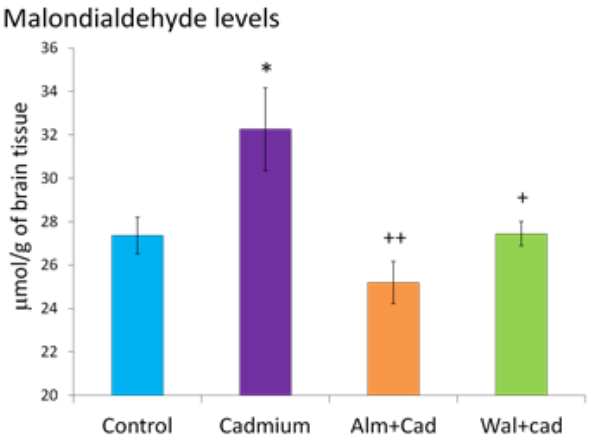

Fig. 4: Effects of almond and walnut supplementation in cadmium treated rats on malondialdehyde levels. Values are mean±SEM. Data was analyzed by one-way ANOVA: *p<0.05 from respective controls; +p<0.05, ++p<0.01 from cadmium treated rats.

Acetylcholine and acetyl cholinesterase

The tissue ACh content was estimated by the method of Hestrin (Batool *et al.*, 2016). The tissue sample was boiled to inactivate the enzyme and release the bound ACh which reacts with ferric chloride and the brown color developed was read at 540 nm against the reagent blank. The concentration of ACh was expressed as µmol/g of brain tissue. Activity of AChE in homogenate was determined according to the method of Ellman *et al.* (1961). The activity of AChE was expressed as µmol/min/g of brain tissue.

STATISTICAL ANALYSIS

Data for MWM and OFT were analyzed by independent *t*-test. One-way ANOVA and Tukey's post-hoc test were used for the analysis of recognition index, MDA levels, ACh and AChE data using SPSS version 20. *p* values <0.05 were considered significant.

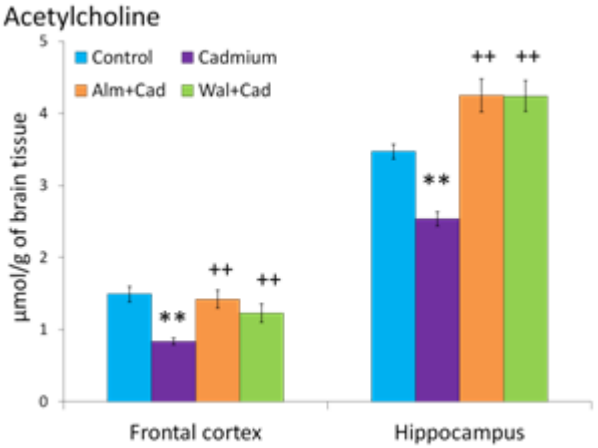

Fig. 5: Effects of co-administration of cadmium with almond and walnut on acetylcholine content (µmol/g of brain tissue) in frontal cortex and hippocampus. Values are mean±SEM. Data was analyzed by one-way ANOVA: **p<0.01 from respective controls; ++p<0.01 from cadmium treated rats.

RESULTS

Morris water maze (MWM)

MWM was used to determine the effects of long term weekly cadmium administration and co-administration of cadmium with almond and walnut on spatial memory. fig. 1 shows significant effects of treatment following independent *t*-test between training and test sessions for each group. It was revealed that cadmium treated rats unaffected the escape latency during test session [$t(10)$ = 0.354, p>0.05] showing the failure of memory retention. Whereas escape latency in control [$t(10)$ = 2.99, p<0.05], alm+cad [$t(10)$ = 6.77, p<0.01] and wal+cad [$t(10)$ = 3.87, p<0.01] rats was significantly decreased after 24 h of training exhibiting memory retention during test session.

Open field test (OFT)

Memory function was also monitored by open field apparatus to assess the non associative memory (fig. 2a-2c). A significant effect of cadmium and co-administration of cadmium with almond and walnut regarding the number of square crossed was observed following independent *t*-test (fig. 2a). Cadmium [$t(10)$ = 1.729, p>0.05] group exhibited comparable number of squares crossed in training and test sessions. Control [$t(10)$ =1.169, p<0.05], alm+cad [$t(10)$ =3.01, p<0.05] and wal+cad [$t(10)$ =2.88, p<0.05] groups exhibited significantly decreased squares crossed during test session as compared to training. Time of rearing (fig. 2b) was significantly lower in control [$t(10)$ =2.64, p<0.05], alm+cad [$t(10)$ =2.31, p<0.05] and wal+cad [$t(10)$ =4.91, p<0.01] groups during test session as compared to that of training session. For number of rearing, independent *t*-test revealed comparable results in all tested groups except wal+cad group [$t(10)$ =2.49, p<0.05] that showed significantly decreased number of rearing after 24 h of training (fig. 2c). These results demonstrated that cadmium administered rats showed impaired habituation memory whereas almond and walnut administration attenuated the memory impairment as the memory retention in alm+cad and wal+cad rats is greater than that of cadmium group.

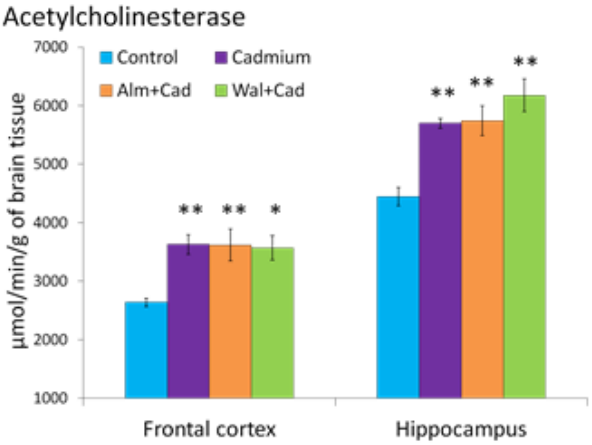

Fig. 6: Effects of co-administration of cadmium with almond and walnut on acetylcholinesterase activity (µmol/min/g of brain tissue) in frontal cortex and hippocampus. Values are mean±SEM. Data was analyzed by one-way ANOVA: **p<0.01, *p<0.05 from respective controls.

Novel object recognition (NOR) test

One-way AVONA for recognition memory revealed significant effects of almond and walnut supplementation in cadmium treated rats [$F(3,20)$=6.539, p<0.01]. Cadmium administration significantly reduced recognition memory (p<0.05) as compared to controls. Whereas, rats treated with Alm+cad (p<0.05) and Wal+cad (p<0.01) showed significantly increased recognition memory as compared to cadmium treated rats.

Malondialdehyde (MDA) levels

Fig. 5 shows one-way ANOVA for MDA levels in rat brain following the administration of cadmium, almond and walnut. Statistical analysis demonstrated significant effects of treatment on MDA levels [$F(3,20)$=6.36, p<0.01]. Tukey's test showed significant increase in MDA following the administration of cadmium as compared to controls (p<0.05). Alm+cad (p<0.01) and wal+cad (p<0.05) groups exhibited significantly reduced MDA levels as compared to cadmium treated rats.

Acetylcholine (ACh) and acetylcholinesterase (AChE)

Fig. 6 shows one-way ANOVA for frontal cortical and hippocampal ACh content to determine the effects of cadmium and co-administration of cadmium with almond and walnut in rats. Statistical analysis demonstrated significant effect of the treatment on ACh content of frontal cortex [$F(3,20)=7.84$, $p<0.01$] and hippocampus [$F(3,20)=22.38$, $p<0.01$]. Post-hoc analysis by Tukeys's test revealed significantly ($p<0.01$) decreased ACh concentration in frontal cortex and hippocampus following weekly administration of cadmium. This decrease in ACh concentration was not observed in alm+cad and wal+cad groups.

Fig. 4 depicts the effects of cadmium and co-administration of cadmium with almond and walnut on AChE activity in frontal cortex and hippocampus. One-way ANOVA demonstrated significant effect of the treatment on AChE activity in frontal cortex [$F(3,20)=6.30$, $p<0.01$] and hippocampus [$F(3,20)=12.95$, $p<0.01$]. Weekly cadmium administration significantly ($p<0.01$) increased AChE activity in frontal cortex and hippocampus following post-hoc analysis by Tukeys's test. This increase in AChE activity was also observed in alm+cad and wal+cad groups in both observed brain regions.

DISCUSSION

Cadmium exposure has shown to induce neurobehavioral aberrations such as depressogenic and anxiogenic behaviors (Haider *et al.,* 2015a). In the present study, weekly administration of cadmium for one month produced impaired spatial, habituation and recognition memory in MWM, OFT and NOR paradigms, respectively. Neurochemical analysis revealed reduced ACh and increased AChE activity following cadmium administration showing cholinergic dysfunction. These behavioral and neurochemical disturbances were significantly attenuated by the co-administration of almond and walnut for 28 days.

Hippocampus and frontal cortex regions of brain are highly implicated in learning and memory processes. These regions play a critical role in everyday memory formation for facts and habituations (Xu and Südhof, 2013). Repeated exposure to a novel environment provides non-associative learning. In present study cadmium treated rats showed decrease in habituation memory as evident by non-significant reduction in exploration behavior in open field on repeated exposure. Similarly, spatial memory and recognition memory were also impaired following cadmium administration in MWM and NOR test respectively. Almond and walnut co-administration, on the other hand, significantly attenuated the cadmium-induced impaired memory function. Hippocampus is specifically essential for the formation of new memories which is then stored in cortex for longer period of time (Kandel *et al.,* 214). Cadmium-induced toxicity and apoptosis in cerebral cortical region has been shown previously. Moreover, morphological changes in cortex following cadmium toxicity were also observed (Wang and Du, 2013). Long-term cadmium intoxication is considered as possible cause of Alzheimer's disease (AD) (Jaishankar *et al.,* 2014). Increased AChE activity plays important role in the progression of AD. AChE activity is also implicated in the determination of environmental contamination of heavy metal (Richetti *et al.,* 2011). Altered AChE activity in hippocampus, cerebral cortex, cerebellum, striatum, and hypothalamus following cadmium exposure for extended duration has been reported previously in adult Wistar rats (Wang and Du, 2013). Consistent with the previous studies, present study also showed changes in AChE activity in hippocampus and frontal cortex following cadmium administration. Cadmium is reported to disrupt signal transduction pathway. Calmodulin protein which is involved in signal transduction is unable to discriminate between calcium and cadmium. This results in inhibition of exocytosis process and ultimately causes decease in the release of ACh from pre-synaptic neuron (Carageorgious *et al.,* 2005). ACh levels in the present study were significantly decreased in cadmium treated rats in both observed memory-specific regions probably by interfering with calcium metabolism. Decreased ACh and increased AChE activity are strongly co-related with neurodegeneration-associated dementia (Mufson *et al.,* 2008).

Oxidative stress due to environmental exposure of heavy metal has been observed previously. Cadmium exposure reduces antioxidant levels (Méndez-Armenta and Ríos, 2007). Altered activity of super oxide dismutase, glutathione peroxidase and catalase after cadmium intoxication has also been shown previously (Méndez-Armenta and Ríos, 2007). Reduced antioxidant levels and altered antioxidant enzyme activity may lead to stimulated generation of reactive species and lipid peroxidation (Wang and Du, 2013). An increased MDA level is regarded as an important biomarker for the determination of lipid peroxidation and oxidative stress (Cui *et al.,* 2006). In the present study cadmium exposure significantly induced oxidative stress as evident by increased brain MDA levels in cadmium treated rats. It has been shown that oxidative stress alters the neurotransmission and neuronal function (Bouayed *et al.,* 2009). Taking together, the altered cholinergic function in cadmium administered rats may be attributed to observed increase in AChE activity and reduced ACh levels possibly due to inhibition of ACh release, change in signal transduction and increased oxidative stress in rat brain.

The everyday dietary pattern plays important role in the modulation of brain function in healthy as well as in pathological condition. Almond and walnut are nutrient rich food. In the present study, administration of almond and walnut along with cadmium significantly attenuated

cadmium-induced impaired spatial, habituation and recognition memories. The findings are consistent with previously reported studies which showed improved memory following nuts consumption in aged rats as well as in scopolamine-induced rat model of amnesia (Pribis and Shukitt-Hale, 2014). The improved memory in nuts treated rats was accompanied by increased ACh levels in hippocampus and frontal cortex. Increased ACh concentration following almond supplementation has been shown previously (Batool *et al.,* 2016). These nuts contain essential nutrients including choline (Morley, 2010; USDA, 2016). Supplementation of phosphatidylcholine has been shown to improve memory function in mice possibly due to increased ACh synthesis (Lim and Suzuki, 2000). Almond and walnut both contain choline as their ingredient. Therefore, it can be postulated from the present study that the daily consumption of these nuts may provide precursor of ACh thus, increasing the synthesis and functioning of this neurotransmitter and improved memory function. Beside this, these nuts also contain other nutrients such as zinc, calcium, copper, cysteine and polyphenols. These components have metal chelating and antioxidant properties (Carageorgious *et al.,* 2005). Therefore, it can be suggested that the long-term administration of almond and walnut in cadmium intoxicated condition may provide nutrients which help in cadmium chelation thereby reducing cadmium-induced inhibition of ACh release. In the present study cadmium-induced increased MDA levels were significantly attenuated by the supplementation of almond and walnut. This suggests the antioxidant role of these nuts. Antioxidant components of these nuts may be helpful in the scavenging of reactive species generated by cadmium. Thus, the availability of precursor and removal of cadmium may be the possible mechanism for enhanced cholinergic function and improved memory in almond and walnut treated rats.

CONCLUSION

Present study shows weekly cadmium administration for one month decreases ACh levels. Increased AChE activity following cadmium administration further decreases the availability of ACh at synapse leading to impaired memory performance. Almond and walnut on the other hand may provide non-nutritive components that may involve in metal chelating activity. Furthermore, antioxidant components of these nuts may augment the antioxidant potential of neuron thereby reducing cadmium-induced oxidative stress and thus improve memory function. Our findings assume special significance for general population exposed to heavy metal through occupation or daily life routine. Daily consumption of nuts may provide beneficial effects against heavy metal-induced toxicity due to environmental pollution.

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