

Detrimental effects of chia (*Salvia hispanica* L.) seeds on learning and memory in aluminum chloride-induced experimental Alzheimer's disease

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Polyphenols and omega-3 fatty acids are thought to have beneficial effects in Alzheimer's disease, the most common cause of dementia. Seeds of chia (*Salvia hispanica* L.) are highly rich in these nutrients, and thus, the present study investigated the effects of chia seeds on behavior and cognition in an aluminum-induced Alzheimer's disease model in rats. Experimental animals received chia supplementation either during the generation of the model (i.e., pretreatment) or after the model was established (i.e., treatment). A battery of behavioral and cognitive tests were performed, including open-field, elevated plus maze, Porsolt's forced swim, and Morris' water maze, to evaluate anxiety- and depression-like behaviors, and learning and memory. Results showed that chia supplementation was ineffective against Alzheimer's-related anxiety, whereas depression-like behaviors were attenuated with both pretreatment and treatment. There was no improvement in learning and memory with chia treatment. Rather, cognitive performance in chia-pretreated animals was remarkably worse as compared to their non-treated disease-induced counterparts. Hippocampal concentrations of amyloid- β 42, amyloid precursor protein, and total tau protein were similarly increased in all disease-induced animals (despite chia supplementation), as compared to the controls. Based on these findings, chia supplementation during the progression of Alzheimer's disease may exacerbate the disease. Although the results presented here emerge from an experimental/preclinical study, we suggest cautious and careful use of chia, especially in early-stage Alzheimer's patients, until future research in different experimental settings is conducted.

Key words: Alzheimer's disease, chia, anxiety, depression, learning, memory

INTRODUCTION

To date, all of the neurodegenerative diseases, including Alzheimer's disease, remain without a curative treatment. Nevertheless, ceaseless efforts of neuroscientists are paving the way with remarkable palliative treatment options towards discovering a curative treatment. Alzheimer's disease is the most common form of dementia (Alzheimer's Association 2018). Since the first identification of Alzheimer's disease, our knowledge about its pathophysiology has substantially grown and carried us to the point where we can identify and test potential medical and nutritional

interventions to break the neurodegeneration cycle. Among the possible interventions, polyphenols such as rosmarinic acid, quercetin, rutin, and caffeic acid are extensively studied and shown to possess promising antidegenerative actions (Bhullar and Rupasinghe 2013, Kelsey et al. 2010, Nabavi et al. 2015). In addition to polyphenols, several reports underline disease relieving features of an omega-3-rich diet in Alzheimer's (Barberger-Gateau et al. 2007, Huang et al. 2005, Kalmijn et al. 1997). Although clinical data regarding benefits of omega-3 fatty acids in Alzheimer's disease are controversial to some extent (see Canhada et al. 2017), the systematical review by Hooijmans et al.

(2012) suggests that omega-3 supplementation can alleviate Alzheimer's pathology in experimental models. Hooijmans et al. (2012) also noted that contradictory clinical reports likely result from inadequate duration of the supplementation.

The seeds of chia (*Salvia hispanica* L.), a native Latin American herbaceous plant, are highly rich in the abovementioned polyphenols (Pellegrini et al. 2018) as well as omega-3 fatty acids (especially alpha-linolenic acid) (Sargi et al. 2013). These ingredients have caused chia seeds to be called a “superfood” and a “functional food” (Muñoz et al. 2013, van den Driessche et al. 2018). Supplementation with chia seeds is generally considered to be safe, with relatively rare and nonspecific adverse effects (Ulbricht et al. 2009). In recent years, there has been an increase in research regarding health benefits of chia, including antioxidant, antiobesogenic, antidiabetic, cardioprotective, and antitumoral effects. Surprisingly, however, there are no studies examining the effects of chia seeds on neurodegenerative diseases and in particular, Alzheimer's disease.

Based on the ingredients of chia seeds, the present study was designed to test the hypothesis that chia seed supplementation results in antidegenerative effects in an experimental model of Alzheimer's disease. To test this hypothesis we investigated cognitive, behavioral and Alzheimer's-associated parameters in an aluminum chloride-induced disease model.

METHODS

Laboratory conditions and preparations

The study was conducted in the Application and Research Center for Experimental Researches at Hatay Mustafa Kemal University under standardized laboratory conditions (22±2°C temperature, 55±10% relative

humidity, 12:12-h light/dark cycle). The animals were provided with *ad libitum* water and standard or chia-rich pellets. Chia seeds were purchased from local suppliers, and finely ground and nicely blended in crushed standard rat chow [36.2% (w/w)]. Aluminum chloride (AlCl₃) (Merck, Germany) and D-galactose (D-gal) (Sigma-Aldrich, Germany) were dissolved in physiological saline in separate beakers (0.07 M and 0.8 M, respectively). Chia-rich pellets were freshly prepared every other day, whereas solutions were prepared weekly and stored in the refrigerator (4°C). The study was approved by the local ethics committee at Hatay Mustafa Kemal University (#2017/4-1 rev.#2018/6-3).

Experimental design

Adult male Wistar albino rats were randomly assigned into one of four groups: (1) Control (Con, n=8), (2) Alzheimer (Alz, n=10), (3) Pretreatment (Pre, n=10), and (4) Treatment (Tre, n=10). The animals in Alz, Pre, and Tre groups intraperitoneally received 10 mg/kg/day AlCl₃ and 150 mg/kg/day D-gal for 21 days whereas the Con group received saline injections. The combination of AlCl₃ and D-gal was preferred based on previous reports (Xiao et al. 2011, Chiroma et al. 2018), which note that this combination generates pathologies resembling to Alzheimer's disease such as memory deficits, neuronal loss, increased acetylcholinesterase activity, and tauopathy in Wistar albino rats. Con and Alz groups were fed standard rat chow throughout the study. The animals in the Pre group were fed chia-rich pellets during the induction of the experimental model, whereas the Tre group were fed chia-rich pellets for 21 days following the induction. All animals were subjected to a battery of behavioral and cognitive tests to evaluate anxiety- and depression-like behaviors, and learning and memory. The study plan is shown at Fig. 1.

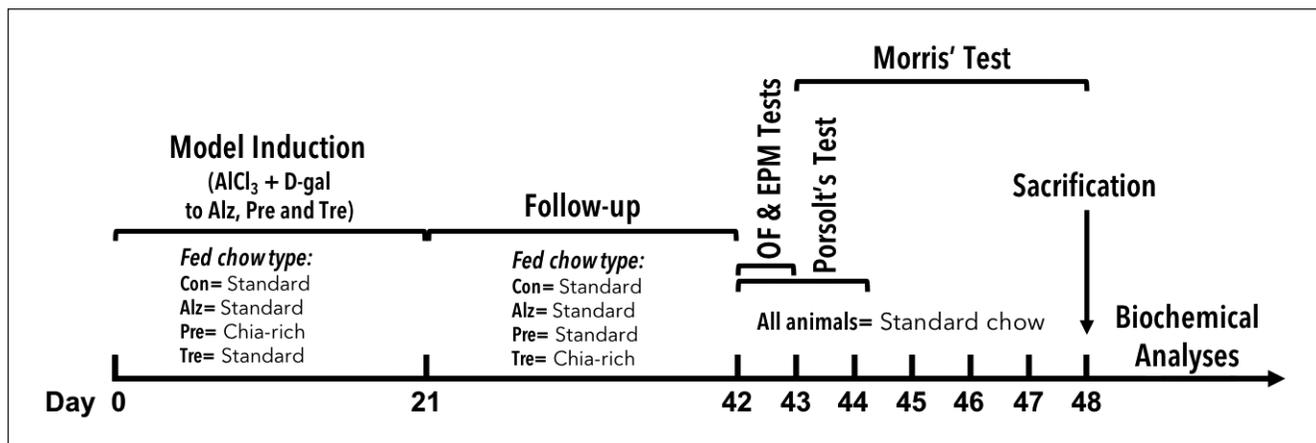


Fig. 1. Study plan. Con: Control, Alz: Alzheimer, Pre: Pretreatment, Tre: Treatment.

Behavioral and cognitive tests

Open-field and elevated plus maze tests were performed to assess anxiety-like behaviors. Depression-like behaviors were evaluated by using Porsolt's forced swim test. Learning and memory performance of the animals was examined by means of Morris' water maze test. The methods for each test are briefly explained below.

Open-Field (OF) test

The test apparatus consists of an open-top cube-shaped box (70x70 cm) which is virtually divided to central (40x40 cm) and peripheral zones (30 cm to the wall). The animals were gently released into the apparatus and left to move freely for 5 min. Total distance moved (cm), velocity (cm/s) and time in center zone (s) were estimated with an animal tracking software (EthoVision XT, Noldus, The Netherlands) whereas numbers of defecations and rearing behaviors were manually recorded. The apparatus was cleaned with 70% ethanol between trials to eliminate olfactory cues.

Elevated plus maze (EPM) test

The elevated plus maze test was conducted in a plus-shaped apparatus with two open and two closed arms (50x10 cm arms and 10x10 cm center). The closed arms were surrounded by opaque walls with the height of 50 cm. The animals were gently placed into the apparatus and left to explore freely for 5 min. Time in open/closed arms (s) was measured with an animal tracking software (EthoVision XT, Noldus, The Netherlands), and numbers of rearing and head-dipping behaviors were manually noted. The apparatus was cleaned with 70% ethanol between trials to eliminate olfactory cues.

Porsolt's forced swim test

The forced swim test was performed after anxiety tests in a cylindrical pool (25 cm dia.) that was filled with warm water (25±1°C) to the height of 35 cm. In the acclimation (pre-test) session, the animals were released into the pool and left to habituate for 15 min. The test session, in which the ethological analysis was carried out from video recordings by using a software (Behavioral Observation Research Interactive Software, Italy) (Friard and Gamba 2016), was conducted 24-h later. In the test session, the animals were allowed to swim for 5 min and behaviors except for those required

to keep the nose above the water surface (i.e., swimming, climbing, diving) were considered mobility. The water was changed and pool was cleaned thoroughly between trials.

Morris' water maze test

A round pool with the diameter of 150 cm was filled with warm water (maintained at 25±1°C with water heaters) to the height of 50 cm and the water was darkened with food coloring. A platform was placed 2 cm below the water level on one of the virtually designated quadrants. The animals were taught the location of the platform for 4 consecutive days in learning sessions. Each learning session was consisted of 4 trials with 90 s cut-off and 30 s inter-trial interval. On the day after the last learning session, the platform was removed and the animals were left to swim freely for 90 s (probe trial). All trials were video-recorded and latency to the platform (s), total distance moved (cm), velocity (cm/s), time in the target quadrant (s), distance moved in the target quadrant (cm), and average distance to the platform zone (cm) were estimated by using a software (EthoVision XT, Noldus, The Netherlands).

Biochemical analyses

The animals were exsanguinated under ketamine/xylazine (80/12 mg/kg) anesthesia following the behavioral/cognitive tests. The brains were excised and hippocampi were dissected on ice. The hippocampal tissues were homogenized in a proprietary lysis reagent (T-PER, Thermo Fisher Scientific, USA) and tissue levels of amyloid-β42, amyloid precursor protein (APP), and total tau protein (t-tau) were measured with commercial ELISA kits (Elabscience, China) according to the instructions of manufacturer. Amyloid-β42, which accumulates in Alzheimer's disease, is the cleavage product of APP and tau protein is a microtubule-associated protein which is strongly connected with the progression of the disease (Kamentani et al. 2018). Bradford's (1976) method was used to quantify total protein contents. The results were reported as ng/mg protein.

Statistical analyses

Parametric data were analyzed with ordinary or repeated measures one-way ANOVAs, post-hoc Tukey's test, or paired Student's t-tests, as appropriate. Kruskal-Wallis test, post-hoc Dunn's test, or Mann-Whitney U tests were used for non-parametric data, as appropri-

ate. Results are shown as mean \pm standard error of the mean (SEM) for parametric data, and 25–75% percentiles for non-parametric data. Results were considered statistically significant at a $p < 0.05$ threshold.

RESULTS

Body weights and chow consumption

Initial body weights of the animals were similar among groups ($p > 0.05$). There was no difference between final body weights (data not shown), although Tre animals had significantly higher body weight

change than Alz and Pre groups ($F_{(3,34)} = 7.21$, $p = 0.001$; post-hoc $p < 0.001$ and $p = 0.035$, respectively) (Fig. 2A). No significant difference in body weight was found between groups consuming standard vs. chia-rich chows ($p > 0.05$), as depicted in Fig. 2B.

Locomotor activity

The total distance moved and velocity were examined for locomotor activity. Both parameters did not differ between groups in either open-field test (Fig. 3A, B) or in the probe trial of Morris' water maze test (data not shown).

Anxiety-like behaviors

We employed the elevated plus maze test to determine anxiety-like behaviors. More time spent in the open-arms is thought to reflect lower anxiety-like

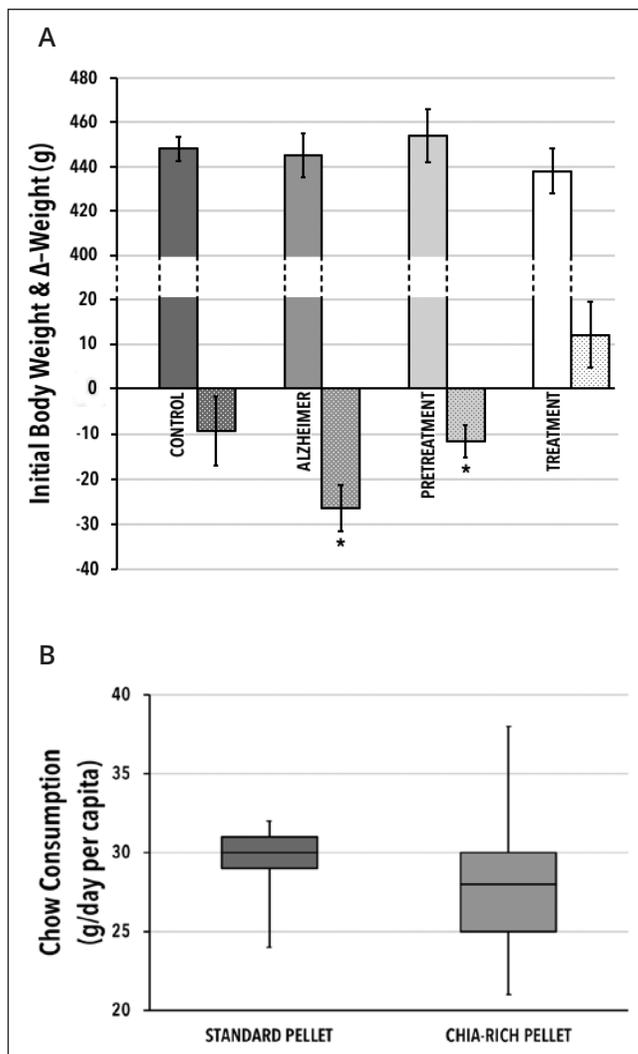


Fig. 2. (A) Initial body weights & Δ -Weight (B) Chow consumption. (A) Initial body weights are shown with solid bars whereas cumulative body weight changes (Δ -Weight) are shown with patterned bars. Asterisks (*) indicate significance versus Treatment ($p < 0.05$). (B) There was no statistical significance for the consumption of standard and chia-rich pellets ($p > 0.05$) (Control: Con, Alzheimer: Alz, Pretreatment: Pre, Treatment: Tre).

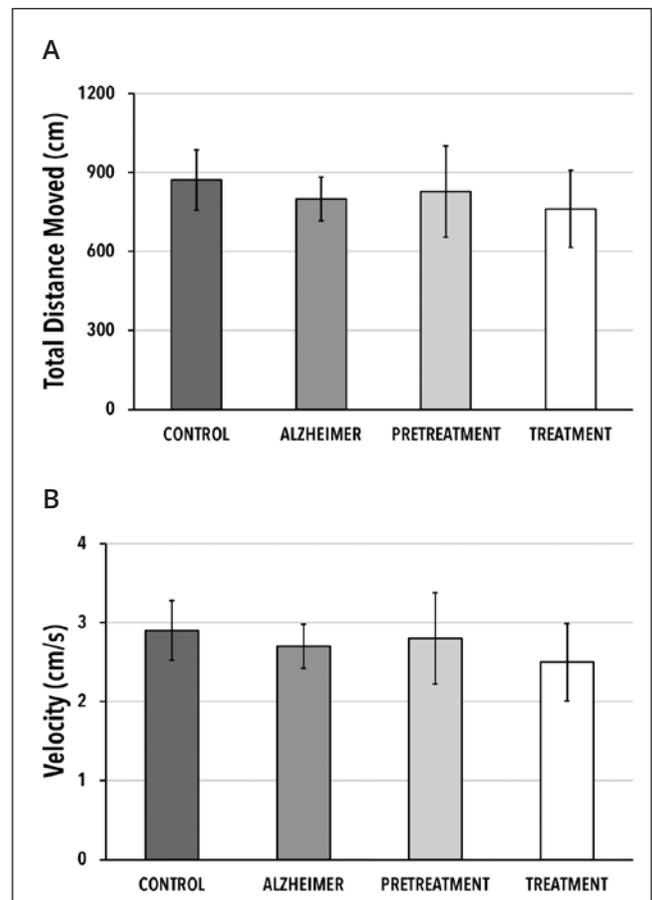


Fig. 3. Locomotor activity in the open-field test. (A) Total distance moved and (B) Velocity. There was no statistical significance for either (A) total distance moved or (B) velocity ($p > 0.05$) (Control: Con, Alzheimer: Alz, Pretreatment: Pre, Treatment: Tre).

behavior. Compared to the controls, Alz, Pre and Tre animals spent significantly less time in the open-arms ($F_{(3,34)}=6.9$, $p=0.001$; post-hoc $p=0.037$, $p<0.001$ and $p=0.006$, respectively) and there was no difference between these groups (Fig. 4A). Except for rearing, which represents exploratory behavior, we did not find statistically significant differences between groups for other ethological measures, including defecation, stretching and head-dipping (data not shown). The number of rearing behaviors was significantly lower in the Pre group relative to controls (Fig. 4B).

Depression-like behaviors

As shown at Fig. 5A, in the Porsolt's forced swim test, which was performed to assess depression-like behaviors, Alz animals had significantly lower mobility

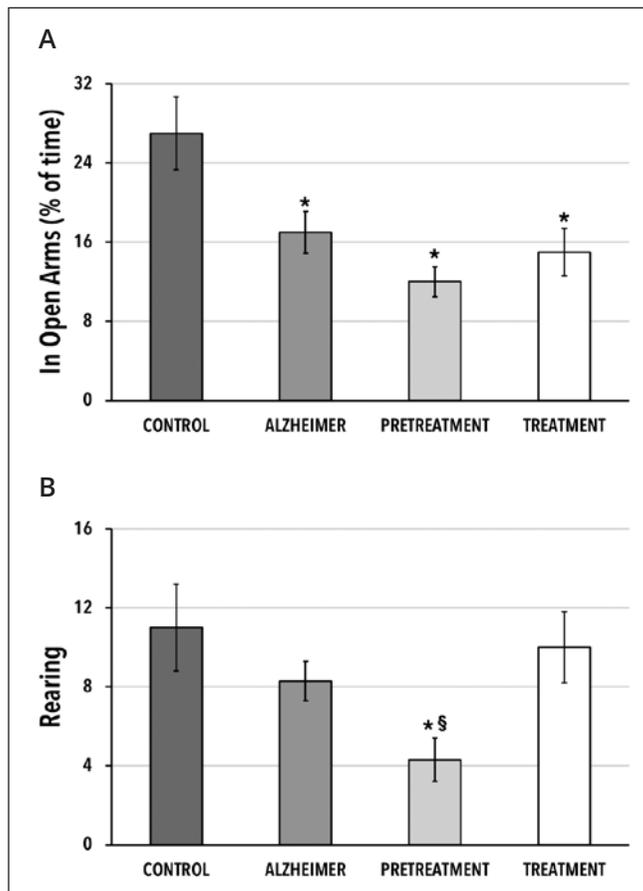


Fig. 4. (A) In the elevated plus maze test, the animals in which the experimental Alzheimer's disease was induced displayed anxiety-like behaviors, but neither pretreatment nor treatment with chia seeds had any effect on these behaviors. (B) Exploratory behavior, inferred from the number of rearing, was decreased in chia-pretreated animals. Asterisks (*) indicate significance versus Control, and section sign (§) versus Treatment ($p<0.05$). (Control: Con, Alzheimer: Alz, Pretreatment: Pre, Treatment: Tre).

compared to other groups ($F_{(3,34)}=5.6$, $p=0.003$; post-hoc $p=0.004$ vs Con, $p=0.032$ vs Pre, and $p=0.020$ vs. Tre). No difference in mobility was found between Con, Pre and Tre groups ($p>0.05$). The proportion of climbing time (to total mobility) did not between groups (data not shown). Since diving had a rare occurrence, it could not be analyzed. There were no group differences in the number of head twitches ($p>0.05$) (Fig. 5B).

Learning and memory

The spatial learning and memory performance of the animals was evaluated using Morris' water maze test. As depicted at Fig. 6, all groups took gradually less time to locate the escape platform in learning trials (RM-ANOVA, $p<0.001$), but in the last learning session, controls showed significantly faster latency to the platform than other animals (KW-test's $H=17.5$, $p<0.001$; post-hoc

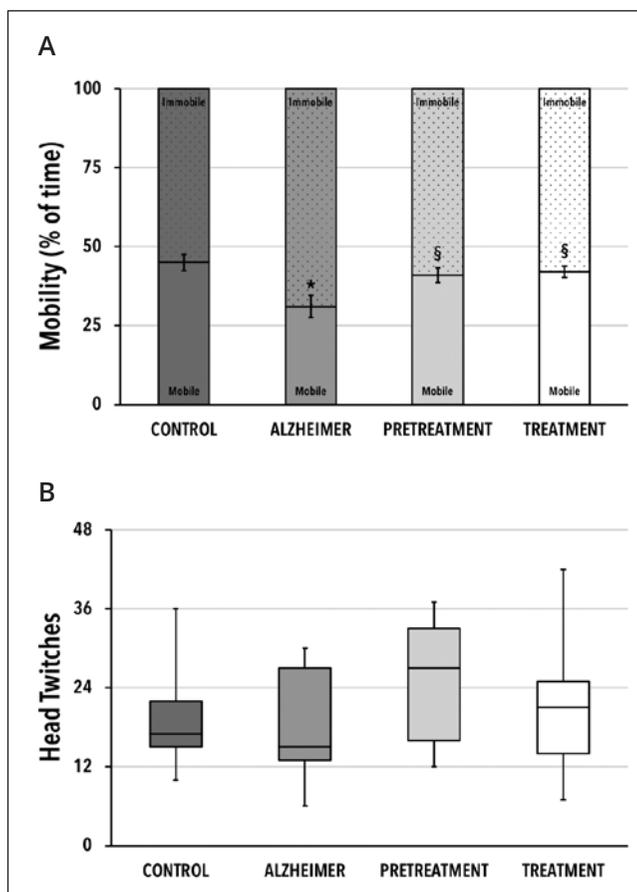


Fig. 5. (A) Mobility and (B) Number of head twitches in the Porsolt's forced swim test. (A) Non-treated disease-induced animals exhibited significantly less mobility than both controls and chia-supplemented groups. Asterisk (*) indicates significance versus Control, and section sign (§) versus Alzheimer ($p<0.05$). (B) There was no statistical significance for the number of head twitches between groups ($p>0.05$) (Control: Con, Alzheimer: Alz, Pretreatment: Pre, Treatment: Tre).

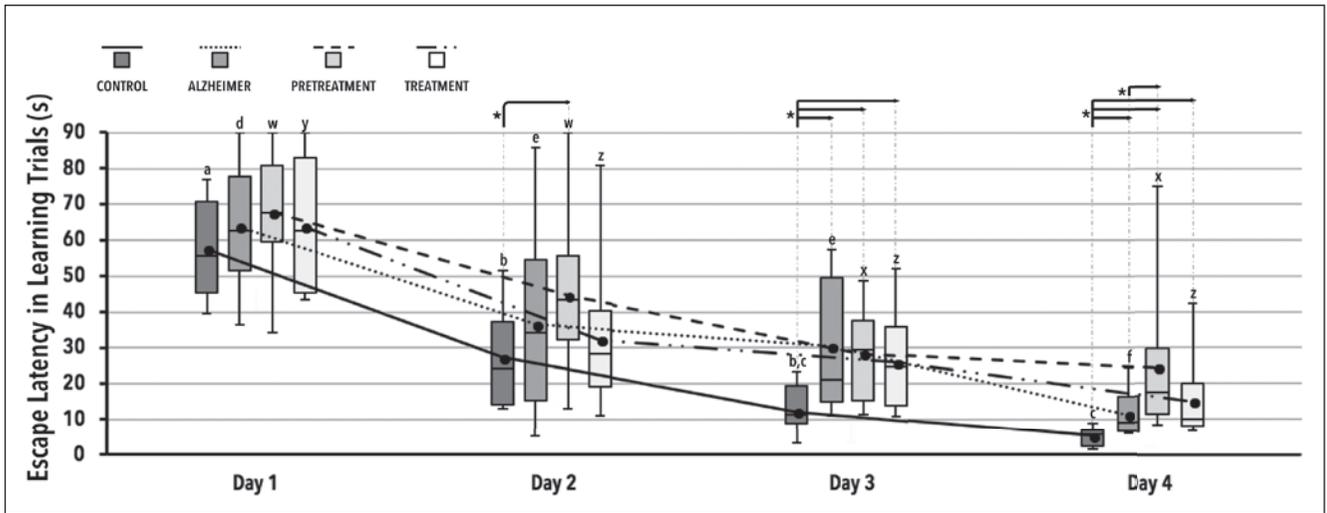


Fig. 6. Learning trials of the Morris' water maze test. All animals gradually showed better performance at finding the platform; however, controls spent significantly lesser time than others in the last learning trial. Different letters in each group indicate statistical significance between learning sessions (in-group comparison) (p < 0.05). Asterisks (*) indicate significance between groups shown with arrow begin and end in each day (inter-group comparison) (p < 0.05) (Control: Con, Alzheimer: Alz, Pretreatment: Pre, Treatment: Tre).

p=0.037 vs. Alz, p < 0.001 vs. Pre, and p=0.003 vs. Tre), In addition, the Pre group took longer to find the platform compared to Alz group (p=0.036). In the probe trial, the

time spent in the target quadrant, which is interpreted as the main measure of memory retention, was significantly greater in controls than other animals. Further-

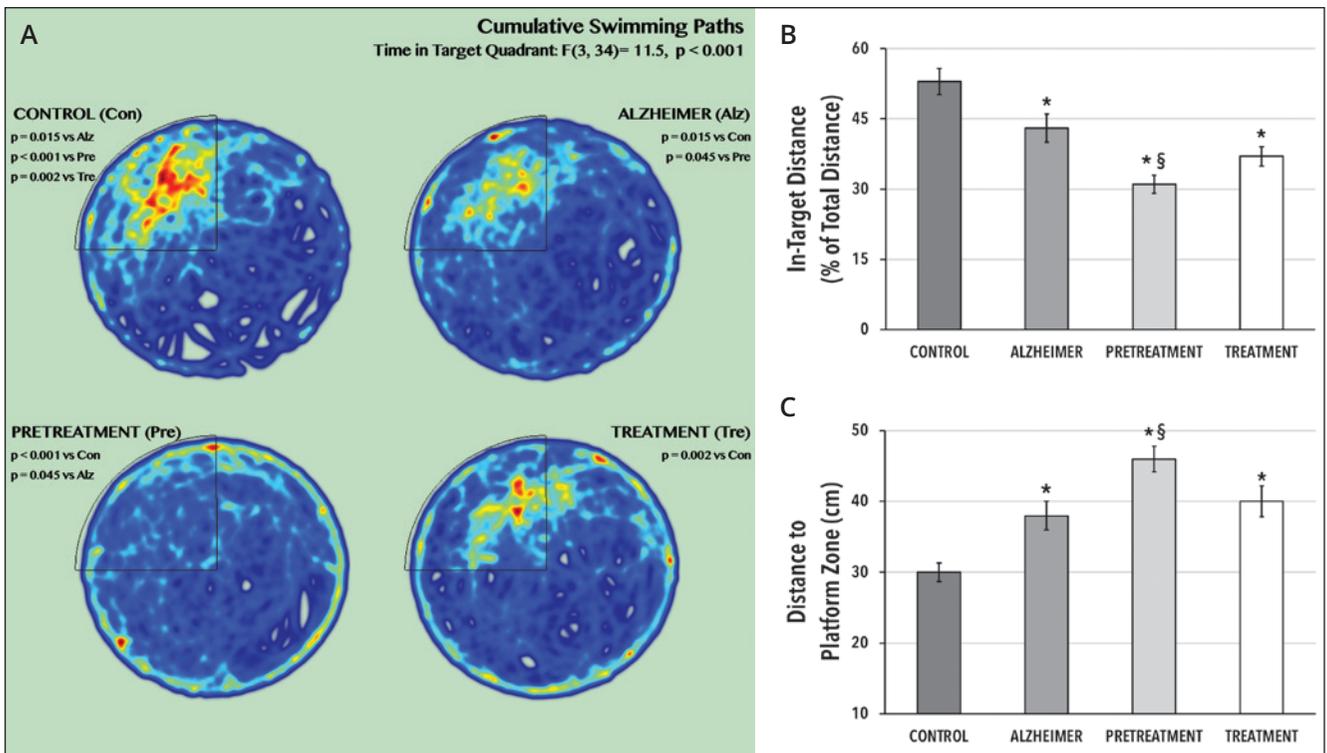


Fig. 7. (A) The time spent in the target quadrant, (B) Ratio of in-target distance to total distance moved and (C) Average distance to the platform zone. (A) Disease-induced animals, either chia supplemented or not, spent lesser time than controls in the target quadrant. Also, the animals pretreated with chia elapsed less time in the target quadrant compared to non-treated disease-induced animals. Both for (B) the proportional in-target distance and (C) distance to the platform zone, the results were alike. Asterisks (*) indicate significance versus Control, and section sign (§) versus Alzheimer (p < 0.05) (Control: Con, Alzheimer: Alz, Pretreatment: Pre, Treatment: Tre).

more, the Pre group spent less time than Alz and Tre groups in the target quadrant, as illustrated at Fig. 7A. Results for additional parameters were consistent with these results, specifically the ratio of distance moved in the target quadrant to total distance moved (Fig. 7B; $F_{(3,34)}=13.6$, $p<0.001$; post-hoc Con vs. Alz: $p=0.044$, Con vs. Pre: $p<0.001$, Con vs. Tre: $p<0.001$, Alz vs. Pre: $p=0.006$) and the average distance to the platform zone (Fig. 7C; $F_{(3,34)}=11$, $p<0.001$; post-hoc Con vs. Alz: $p=0.049$, Con vs. Pre: $p<0.001$, Con vs. Tre: $p=0.009$, Alz vs. Pre: $p=0.016$).

Biochemical measurements

As shown at Fig. 8A, the hippocampal concentrations of amyloid- β 42 were higher in Alz, Pre and Tre animals as compared to controls (KW-test's $H=14.6$, $p=0.002$; post-hoc $p<0.001$, $p=0.017$ and $p=0.002$, respectively). As expected, the concentration of APP was similarly increased in the experimental Alzheimer's disease-induced groups (KW-test's $H=8.4$, $p=0.038$; post-hoc Con vs. Alz: $p=0.012$, Con vs. Pre: $p=0.031$ and Con vs. Tre: $p=0.011$) (Fig. 8B). Also, higher amounts of t-tau were found in Alz, Pre and Tre groups as compared to Con group (KW-test's $H=15.2$, $p=0.002$; post-hoc $p=0.001$, $p<0.001$ and $p=0.007$, respectively) (Fig. 8C). There was no difference between Alz, Pre and Tre animals in regard to aforementioned hippocampal measurements ($p>0.05$).

DISCUSSION

The present study investigated the behavioral and cognitive effects of chia-rich feeding in rats, using an aluminum chloride-induced experimental Alzheimer's disease model. Our main results can be summarized as follows: the disease (*i*) generated anxiety-like behaviors, but neither pretreatment (i.e., supplementation while the model is being established) nor treatment (i.e., supplementation after the model is established) with chia had any anxiolytic effects, (*ii*) provoked depression-like behaviors, and both pretreatment and treatment alleviated depressive behaviors, (*iii*) impaired the learning and memory performance of animals, and pretreatment (but not treatment) exacerbated the impairment in learning and memory, and (*iv*) increased the hippocampal concentrations of Alzheimer's-associated parameters [amyloid- β 42, APP and t-tau] which remained elevated with pretreatment or treatment.

Modern humans are inevitably exposed to aluminum through polluted air, contaminated diet, medications, and even the skin (Exley 2013). Although this environmental exposure is believed to be in minute amounts (Campbell 2002), aluminum has been shown to

accumulate in aging neurons due to lifetime exposure (Walton 2006). Previous studies also show elevated levels of aluminum in the brains and cerebrospinal fluid of Alzheimer's disease patients (Virk and Eslick 2015). Several authors have emphasized the role of chronic aluminum exposure in the pathophysiology of Alz-

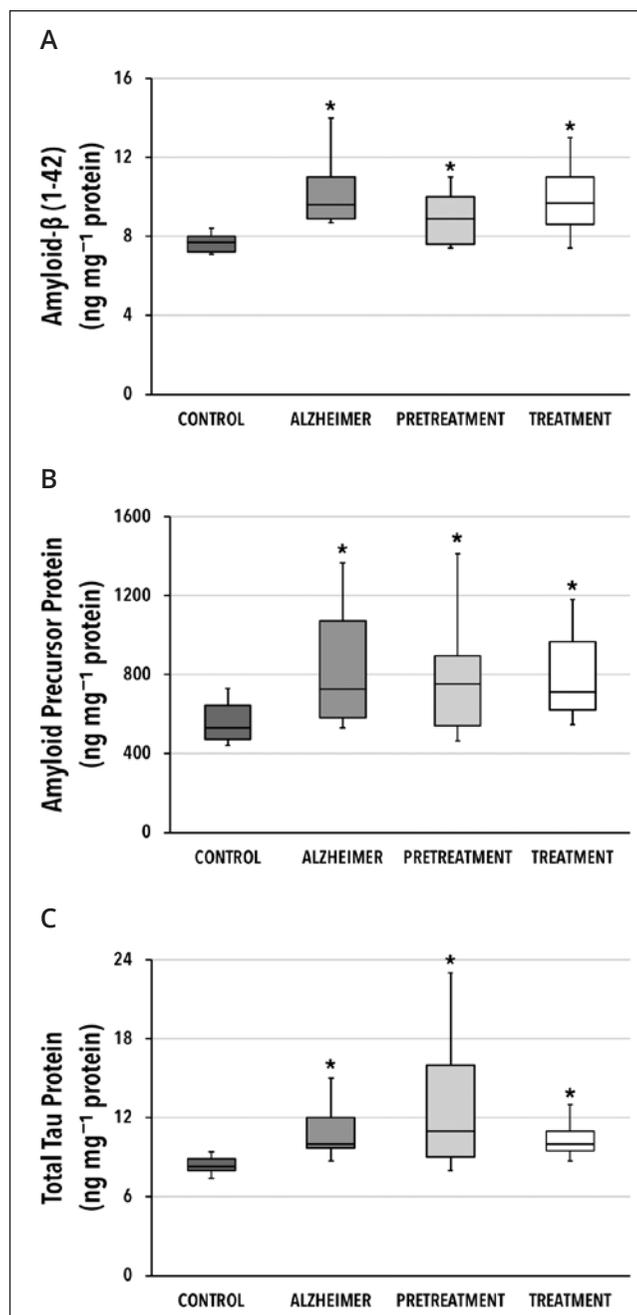


Fig. 8. Hippocampal concentrations of (A) Amyloid- β 42, (B) Amyloid precursor protein and (C) Total tau protein. Controls had significantly lower concentrations of amyloid- β 42, APP, and t-tau. There was no difference between disease-induced animals. Asterisks (*) indicate significance versus Treatment ($p<0.05$) (Control: Con, Alzheimer: Alz, Pretreatment: Pre, Treatment: Tre).

heimer's disease (Gupta et al. 2005, Wang et al. 2016); however, no direct evidence exists that supports or rejects a causal relationship between these variables. Nevertheless, the existing experimental data indicate that aluminum-based experimental models show neuronal alterations that resemble those observed in Alzheimer's disease (Castorina et al. 2010, Shaw and Tomljenovic 2013, Walton 2007, 2014).

A significant proportion of Alzheimer's patients suffer from comorbid affective disorders, even though the disease is fundamentally a memory deteriorating disease (Even and Weintraub 2010, Novais and Starkstein 2015). Affective disorders and Alzheimer's disease seem to be reciprocally linked based on abnormal myelination (Nihonmatsu-Kikuchi et al. 2013). Furthermore, they share some other similar neuroimmunologic, neuroendocrine and oxidative disturbances (Rodrigues et al. 2014). Despite these similarities, depressive symptoms are not correlated with the severity of Alzheimer's disease, which suggests distinct pathophysiological mechanisms (Lee and Lyketsos 2003). Hence, an intervention that relieves depression is not expected to have antidegenerative efficacy in Alzheimer's disease.

Despite an extensive literature search, we are aware of only a few reports examining the behavioral and cognitive effects of chia seeds. In the behavioral study by Nemeth et al. (2014), which was conducted in guinea pigs, no influence of chia seeds on locomotion was reported. These results are in accordance with our own data in rats. Although depression-like behaviors were not evaluated in the study by Nemeth et al. (2014), saliva cortisol concentrations were found to be lower in chia-supplemented animals. Cortisol is physiologically a stress confronting hormone, but in depression, dysregulated hypothalamic-pituitary-adrenal (HPA) axis response leads to increased cortisol levels, which can be reduced by antidepressants (Maric and Adzic 2013). Although cortisol is not a depression marker by itself, but rather an associated hormone, the observed antidepressant-like effects of chia in the present study may be derived from its action on the HPA axis. Also, the finding of ineffectiveness of chia supplementation on social stress in the study of Nemeth's team may be interpreted as absence of any influence on anxiety-like behaviors, which is also similar to our results.

Regarding the cognitive effects of chia seeds, a recent nutritional intervention trial was conducted in undergraduate students by Onneken (2018). The author founds an improvement in both memory and intelligence tests following chia seed supplementation (5 g/day) for 21 days. Based on these results, the author of the study concluded that "chia consumption is highly recommendable for dealing with Alzheimer's disease", despite the fact that the included partici-

pants were all young, healthy individuals (average age: 21.3 years). Our results instead suggest that chia seeds while an experimental Alzheimer's disease is progressing tremendously impairs learning and memory. We also found no benefit of chia seed supplementation after the disease has already emerged. Recently, Rui et al. (2018) reported similar findings in a senescence-accelerated mouse-prone 8 mouse line, which displays the phenotype of accelerated aging. In their study, they emphasized the absence of cognitive improvement with chia supplementation and increased activity in both amyloidogenic and non-amyloidogenic pathways, which subsequently bolstered the amyloid pathology. The ineffectiveness of chia in cognitive improvement was also confirmed in male guinea pigs by Nemeth et al. (2015). Undoubtedly, it is clear that experimental/preclinical researches cannot be directly extrapolated to clinical practice; however, these studies are invaluable in terms of translational medicine, especially where scant knowledge exists.

To further explain our cognitive findings, we should revisit the chemical properties of aluminum. Aluminum is a prooxidant, but redox-inactive metal. Exley (2004) and Ruipérez et al. (2012) have attributed the prooxidant potency of aluminum, in part, to its iron reducing action. Aluminum stimulates iron overload in tissues by altering the cellular uptake of iron (Cannata Andia 1996). Polyphenols, which are generally known antioxidants, exert prooxidant activity in this reduced iron-enriched medium (Decker 1997, Margină et al. 2015, Osborn and Akoh 2003). Given that chia seeds are highly rich in omega-3 fatty acids and polyphenols, this study was designed to observe chia's effects on both progressing and already emerged disease. We found that the learning and memory impairing effect of chia is apparent in animals in which the disease was progressing, but not in those in which the disease has already emerged. We hypothesize that this result may be due to the prooxidation favoring environment. Presumably, the animals in which the disease already emerged had time to excrete aluminum and were able to escape from its devastating consequences. Furthermore, these animals may have had a positive energy balance via the help of increased beta-oxidation of alpha-linoleic acid (Fu and Sinclair 2000). In this context, how can our results of Alzheimer's-associated biochemical parameters be interpreted? Indeed, amyloid/tau hypothesis is insufficient to completely explain the pathophysiology, although amyloidogenic accumulation remains to be a prominent feature of the disease (Kametani and Hasegawa 2018). Hence, one should also consider other hypothesis, including oxidative stress. Therefore, no change in these parameters in our study supports the assumption of prooxidant action of the

chia supplementation within the perspective of the oxidative hypothesis.

Although this is the first study to examine the behavioral and cognitive effects of chia seeds in an experimental Alzheimer's disease model, it has some limitations that should be taken into account when interpreting results. Although we demonstrated an impairment in learning and memory with chia supplementation, we speculate without any relevant analysis that this impairment may be the result of oxidative stress. We also restricted our analyses to basic Alzheimer's-associated biochemical parameters. Further examination, probably including of the cholinergic pathway, protein modifications, neuroimmune reactions and neuromodulations, are needed to clarify the exact mechanism(s). Next, we employed an aluminum-induced model, which is reported to resemble the pathophysiological features of Alzheimer's disease; however, our results need to be confirmed in other non-transgenic and transgenic models before deprecating chia supplementation in patients with Alzheimer's disease. Nonetheless, based on the knowledge of the neuronal accumulation of aluminum in Alzheimer's disease, we suggest cautious and careful use of chia supplementation – particularly in early-stage patients whose disease is progressing – until further research in different experimental settings is conducted.

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