NUTRITIONAL NEUROSCIENCE
https://doi.org/10.1080/1028415X.2022.2108255

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Almond intake during pregnancy in rats improved the cognitive performance of adult male offspring

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ABSTRACT

Background: Based on evidence there are accepted links among early nutrition, epigenetic processes, and cognitive performance. Almond as a nutritious food could exert neuroprotective effects and improve anxiety, learning, and memory.

Methods: In the current study, female rats were fed with a diet containing 5% (w/w) almonds during the mating period (two days) and gestation period (21 consecutive days). Then, the effect of the almond diet on short-term memory (Y maze), anxiety (elevated plus maze), and stress adaptation (forced swimming test) were investigated in the adult male offspring. The hippocampus (HIP), prefrontal cortex (PFC), and amygdala (AMY) of offspring were collected, and the level of cyclic AMP response element-binding proteins (CREB), brain-derived neurotrophic factor (BDNF) was assessed by western blotting. Also, Monoamine oxidases (MAO)-A and B activity were evaluated via enzymatic assays.

Results: Our results indicated that prenatal almond consumption improved memory, made a modest reduction in anxiety-like behavior, and increased stress adaptation in adult male offspring. Also, molecular assessments showed an increased level of CREB phosphorylation and BDNF in the HIP and PFC of the almond group, while the activity of MAO-A and MAO-B was inhibited by almond consumption in mentioned areas.

Discussion: These findings introduce almonds as a beneficial diet during pregnancy, for improving short-term memory, stress adaptation, and cognitive performance in adult offspring.

KEYWORDS
Almond; maternal feeding; adult offspring; memory; stress adaptation; CREB; BDNF; MAO

1. Introduction

Persian medicine (PM) has special approaches toward pregnancy. Healthy lifestyle of parents during childbearing age and pregnancy to give birth to a healthier offspring had been proposed by Avicenna (980–1037 A.D.) [1,2]. Based on PM literature, the most important topics in a healthy lifestyle during pregnancy are divided into four main groups: Nutrition, physical activity, sex, and psychological issues [3].

The perinatal period is a window to enhance plasticity as the basis for future consequents in anatomy, physiology, and behavior. Maternal diet during pregnancy is crucial for the maturation of vital organs and neural connection development. Based on the evidence, early life nutrition could alter the epigenetic regulation of genes, leading to persistent metabolic and physiological alterations in offspring and disease susceptibility later in life [4].

Nuts are nutritious foods with a complex matrix rich in unsaturated fats, minerals, fiber, high-quality vegetable protein, phenolic compounds, and phytosterols [5]. In 2003, U.S. Food and Drug Administration (FDA) announced nuts as a 'heart-healthy' component [6], with an accepted role in improving endothelial function, lipid, and apolipoprotein levels, besides oxidative stress and inflammation reduction [7].

Almond [*Prunus dulcis* (Mill.) D.A.Webb] as a member of the Rosaceae family, belongs to the subgenus Amygdalus inside the genus Prunus. Nowadays, as food with medicinal properties, sweet almond is the most valuable nut in terms of commercial production [8].

From ancient times, almond has been noted for its positive effects on mentality [9] and is considered to supply the brain and contribute to mental alertness, memory, concentration, and sleep quality in the PM

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literature [10]. The levels of dietary fiber, lipid and protein contents, vitamin E, phytosterols, and several key micronutrients in almonds have recently been found as the components of a healthy nutrient profile [8,9]. Studies suggest that almonds prevent brain atrophy and improve memory in rats [11]. By means of Morris water maze, elevated plus maze, forced swimming, and open field test, Komati and colleagues demonstrated that treatment with almonds significantly improved spatial learning and memory being impaired by alcohol in male rats [12]. It has also been observed that almonds can inhibit lipid peroxidation and reduce oxidative stress in rat's brains [13].

The cyclic AMP response element-binding proteins (CREB) is defined as cellular transcription factor responsible for memory-related synaptic plasticity. It was demonstrated that the increased phosphorylation of the CREB improves the cognitive performance of rodents during the behavioral tasks [14]. Besides, the brain-derived neurotrophic factor (BDNF) as a neurotrophin is one of the important CREB downstream target genes. Based on the literature, there is a relationship between BDNF and activity-dependent regulated CREB, in which its expression contributes to synaptic plasticity, neural development, and neuroprotection [15].

On the other hand, several enzymes and their activity level could influence the CNS performance in different aspects of learning, memory, anxiety, etc. Monoamine oxidases (MAO-A and MAO-B) are enzymes that oxidatively destroy several neurotransmitters, such as norepinephrine, dopamine, tyramine, serotonin, and some other amines [16]. Inhibition of mentioned enzymes could increase the effectiveness of neurotransmitters by elevation of their exposure time. Changing the balance in MAO-A is associated with conditions including anxiety, depression, schizophrenia, and other psychiatric disorders [17]. Besides, increased MAO-B activity has been seen in neurodegenerative disorders [18]. It has been reported that the prevention of monoamine oxidation associated with MAO could be a target to improve cognitive state [19].

In this study, we aim to investigate the effects of almond consumption during pregnancy on offspring's memory, and its anxiety and stress adaptation. Moreover, to find out the molecular basis we determined the alterations in the level of CREB phosphorylation, BDNF, and monoamine oxidase activity in different brain regions including the hippocampus (HIP), prefrontal cortex (PFC), and amygdala (AMY).

2. Materials and methods

2.1. Plant material and diet

The fruits of almond trees growing in Vaneshan, Isfahan Province, Iran, were used in the current study. The plant identification (voucher specimen NO. PMP-1753) was carried out by the herbarium of Tehran University of Medical Sciences, Tehran, Iran. The almonds were stored in dark, at room temperature. The diet for the experiment group was prepared in pellet form by adding the almond powder to the normal food (5% w/w).

2.2. Animals

Adult male and female Wistar rats (200 ± 20 g) were obtained from Pasteur Institute (Tehran, Iran). Rat's housing had controlled temperature and humidity with a standard 12 h light/dark cycle; they could freely get food and water. All the procedures in this study were under the supervision of the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 80-23, revised 1996). The Animal Care Committee of the Shahed University (IR.SHAHED.REC.1399.149) has also approved this study.

2.3. Experiment design

We randomly assigned female rats (weight 200–220 g; age two to three months) to the control and almond diet groups. Rats in the almond group were fed a diet containing 5% (w/w) of almonds from mating (two days) and during pregnancy. The control group was fed a control diet.

Then, based on the mothers' diet, pups were allocated to two experimental groups. Two to three male offspring were selected from four mothers randomly. The control group (Mother fed control diet throughout pregnancy, $n = 10$) and the almond group (Mother fed almond diet throughout pregnancy, $n = 10$). Immediately after behavioral tests, at the 11th postnatal week, animals were decapitated. Brain areas, including the hippocampus (HIP), prefrontal cortex (PFC), and amygdala (AMY), were separated. We then rapidly froze the tissues in the liquid nitrogen and kept them at −80°C for subsequent enzymatic and molecular analyses (Figure 1).

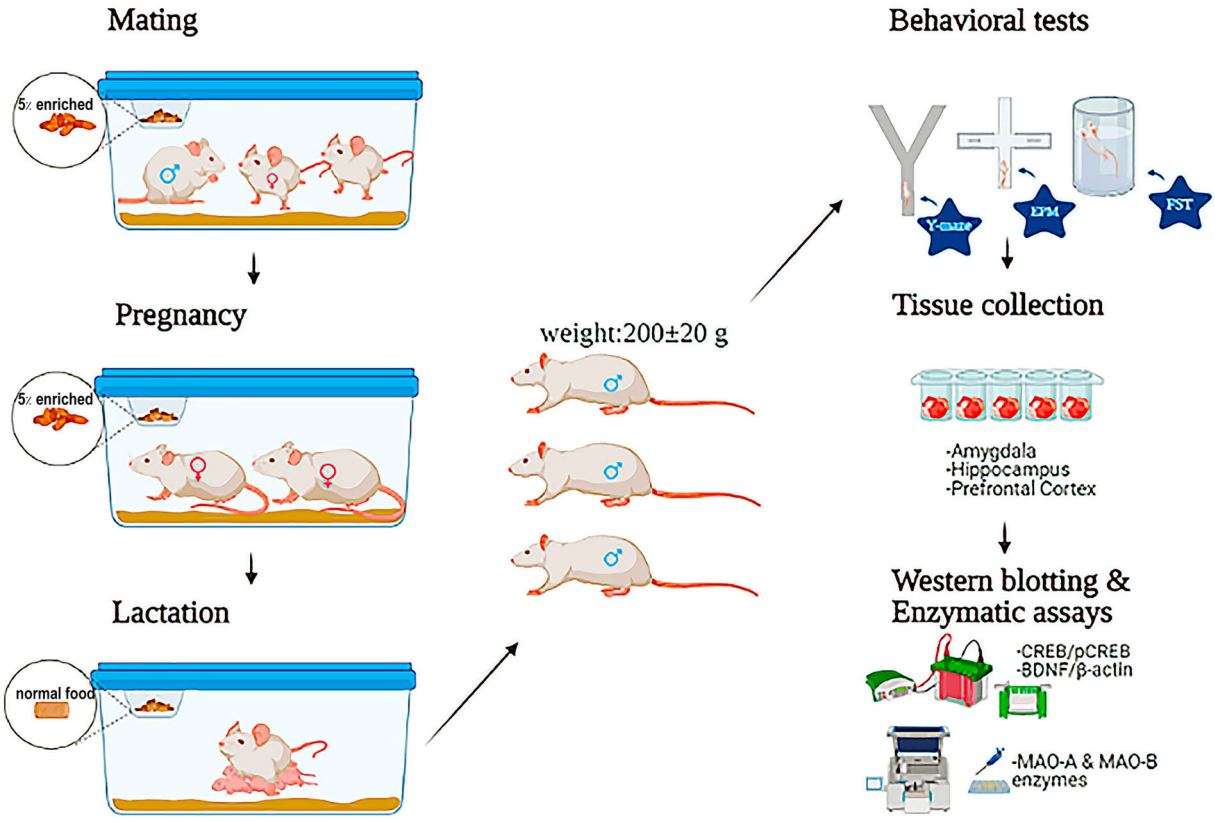

Figure 1. Schematic order of the experimental procedure. The figure was created on www.biorender.com.

2.4. Behavioral tests

At the age of 11 weeks, the behavioral tests were conducted. One hour before testing, animals were allowed to acclimatize to the test room. The effects of an almond-based diet throughout pregnancy were analyzed on the pup's spatial working memory (Y maze), anxiety-like behaviors (Elevated plus maze), and behavioral adaptation, and survival (Forced swim). All the behavioral experiments were conducted between 8 am and 1 pm

2.4.1. Y-maze test

Working memory was evaluated with a Y-maze test. The Y-maze apparatus is composed of three equal-length gray plastic arms (50 cm × 10 cm) with 20 cm high walls and a bottom plate at a 120° angle. The test duration was 8 min, and rats were allowed to move freely through the maze. When all four limbs of the rat entered an arm, this would be counted as arm entry. Alternation is considered as the rats sequentially entered into all three arms without repetition of a single arm. The spontaneous alternation percentage is calculated as below:

$$\text{Spontaneous alternation} = \left(\frac{\text{Number of alternatives}}{\text{Total number of arm entries-2}}\right) \times 100 \text{ arm entries}) \times 100]$$

2.4.2. Elevated plus maze (EPM) test

In a silent environment under dim light, rats were allowed to discover the surroundings freely for 5 min in the EPM test apparatus. The EPM device has two open and two closed arms making a cross sign, with a central sheath 50 cm above the floor. Anxiety index is defined as the time percentage that rats spent in open arms [%OAT: (time in open arm/time in open + closed arm) × 100] and open arm entry percentage [%OAE: (number of open arm entries/number of open + closed. The total number of arm entries was measured to show the locomotor activity. When all four limbs enter into an arm, this would be counted as arm entry. The

apparatus was disinfected using 70% ethanol between consecutive tests [20].

2.4.3. Forced swim test

A transparent, cylindrical plastic tank 20 cm in diameter was used to conduct a forced swimming test. The height of the tank was 45 cm and tap water was filled to a depth of 30 cm (24 ± 1°C) in the tank. For more reliable results rats were gently placed into the water the day before the test, after 15 min they were taken out of the water and allowed to dry and returned to their cages. The test duration was 6 min and rats were placed in the cylindrical tank again. Total immobility time was measured as the time that the rat remained floating and made only minor movements to keep its head out of the water [21].

2.5. Western blot assay

The brain tissues (HIP, PFC, and AMY) were homogenized in a lysis buffer containing phosphatase inhibitors (NaF, Na_2MoO_4, and $NaVO_3$), a complete protease inhibitor and a Bradford protein assay were used to measure protein concentrations [22]. Proteins were then separated based on their molecular weight (60 µg per gel lane) by means of a 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system which was electroblotted onto polyvinylidene difluoride (PVDF) membranes. A blocking solution was used to incubate the membrane (at room temperature) and then primary antibodies (CREB, p-CREB, BDNF, and β-actin) were added. The next step was using horseradish peroxidase (HRP)-conjugated antibody as the secondary antibody. Immunoreactive polypeptides were identified using an Enhanced Chemiluminescent (ECL) detection kit, and eventually, results on X-ray film scans were analyzed by Image J software. Our internal control was β-actin.

2.6. Measurement of MAO-A and MAO-B

The mitochondrial fraction was isolated based on the previously described method [23]. MAO-A activity was measured by adding samples to the wells with sodium phosphate buffers (100 mM, pH 7.4) and 5-hydroxytryatpamine, 4 mM (Sigma-Aldrich, St. Louis, U.S.A.). Changes in the absorbance were recorded by a microplate reader (Biotek, Synergy HTX) at a wavelength of 280 nm against the blank containing sodium phosphate buffer (100 mM) and 5 hydroxytryptamine (4 mM). MAO-B activity was measured using a buffer of sodium phosphate buffer (100 mM, pH 7.4) and benzylamine, 0.1 M (Sigma-Aldrich, St. Louis, U.S.A.) which was added to the samples. The absorbance change was recorded at 250 nm wavelength against the blank (sodium phosphate buffer and benzylamine) [24].

2.7. Statistical analysis

All data are represented as the mean ± SEM. Graphpad Prism 6 Software was used for data analysis. The statistical analysis of behavioral and molecular studies was conducted using an unpaired *t*-test to find the difference between the control and almond groups. Statistical significance was considered as $P < .05$.

3. Results

3.1. Almond diet during pregnancy period improved the spatial working memory in adult male offspring

The percentage of spontaneous alternation in the Y-maze test was used as an indicator for spatial working memory. As shown in Figure 2(A), the spatial working memory was improved by 1.24-fold in the offspring of the almond group ($P < .001$) compared with the control group. General locomotion or the calculated total number of rat entries to the arms remained unaffected in both experimental groups (Figure 2(B)).

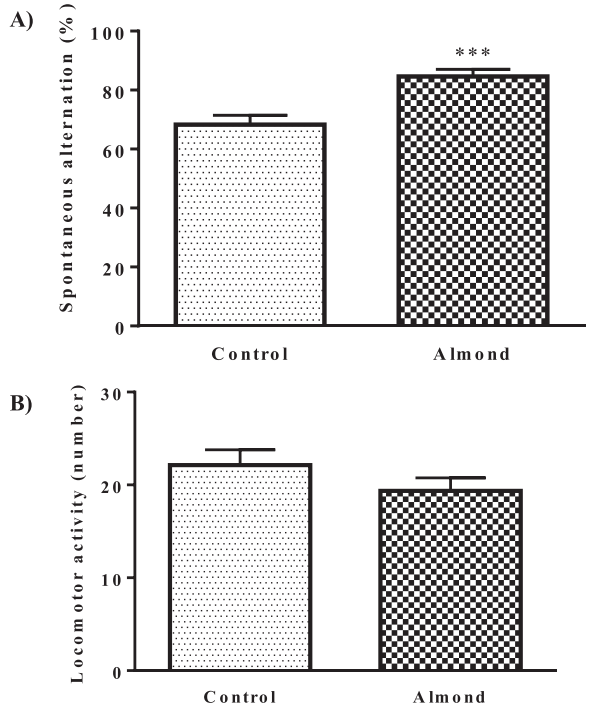

Figure 2. Y-maze test in adult male offspring with prenatal (A) Spontaneous alternation percentage and (B) locomotor activity ($n = 10$). ***$P < .001$ as compared to the control.

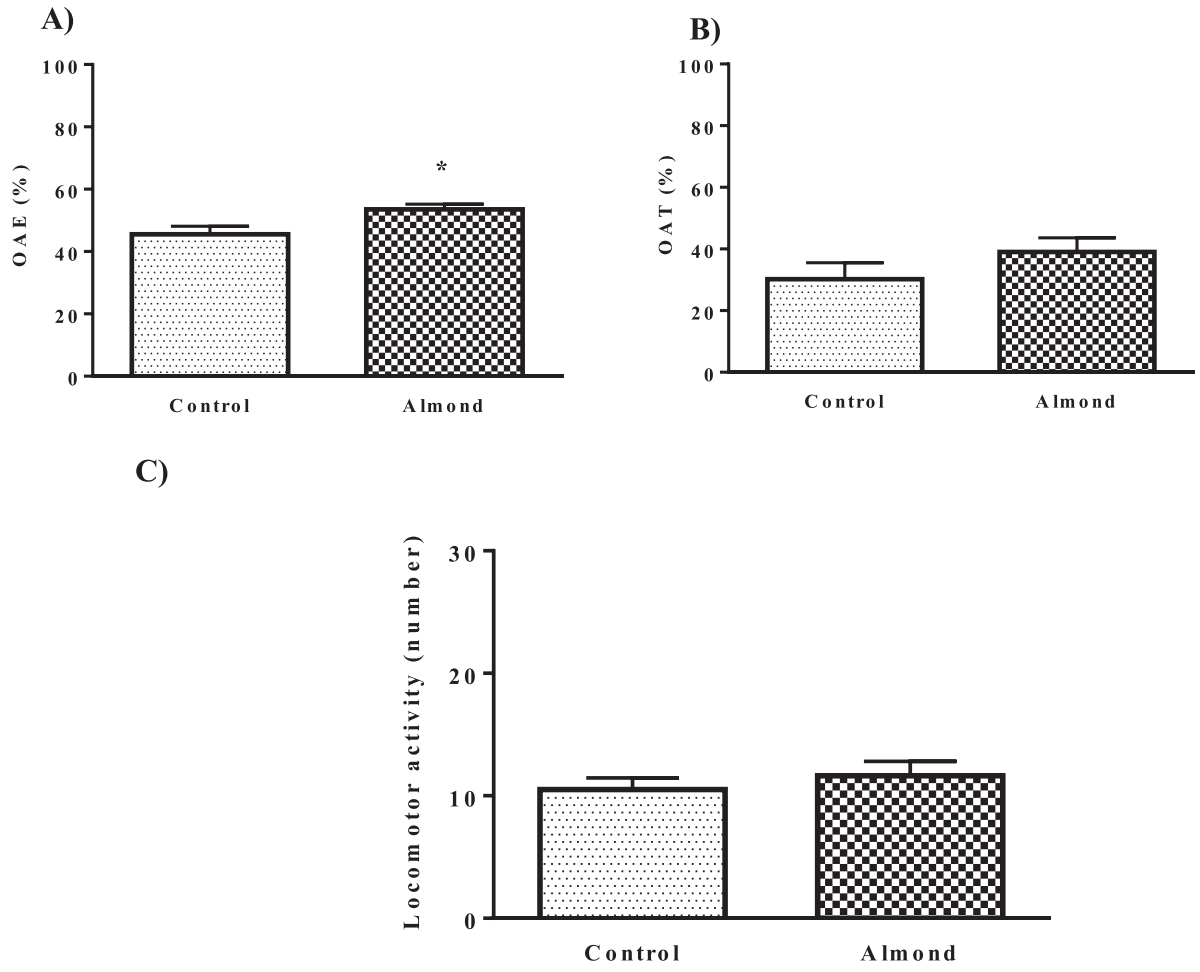

Figure 3. EPM test in adult male offspring with prenatal almond intake (A) Open arm entries percentage (OAE%), (B) open arm time percentage (OAT%), and (C) locomotor activity ($n = 10$) *$P < .05$ as compared to the control.

3.2. Almond consumption during pregnancy slightly reduced anxiety-like behaviors in adult male offspring

The percentage of open arm entry (%OAE), open arm time (%OAT), and locomotor activity in the EPM test is represented in Figure 3. Based on the evidence, rats with lower levels of anxiety remain for a longer period in the open arms with increased %OAT. EPM data analysis indicated that almond consumption during pregnancy increased %OAE by about 1.2-fold in the male offspring in comparison to the control group ($P < .05$, Figure 3 (A)). However, the %OAT and locomotor activity didn't show any significant alteration (Figure 3(B,C)).

3.3. Almond consumption during pregnancy increased the stress adaptation and survival in the adult male offspring

The Forced Swim Test (FST) is used to show the ability to overcome acute inescapable stress in rodents. Based on the test explanation, it provides unique insight into the neural limb of the stress response and adaptation to stress [25]. According to Figure 4, the immobility time, the most important outcome of FST, was reduced by about 79% in the almond group as compared to the control ($P < .001$), showing a significant reduction.

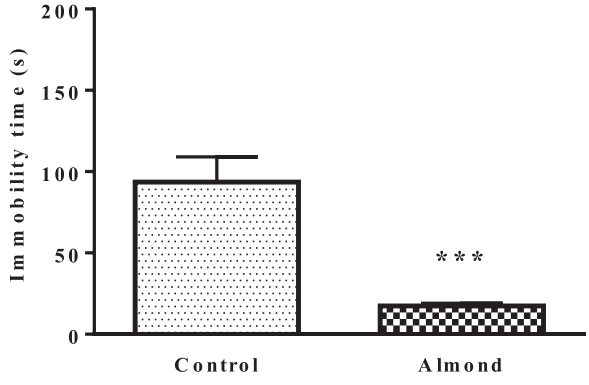

Figure 4. Forced swimming test in adult male offspring with prenatal almond intake, indicating stress adaption and survival ($n = 10$), ***$P < .001$ as compared to the control.

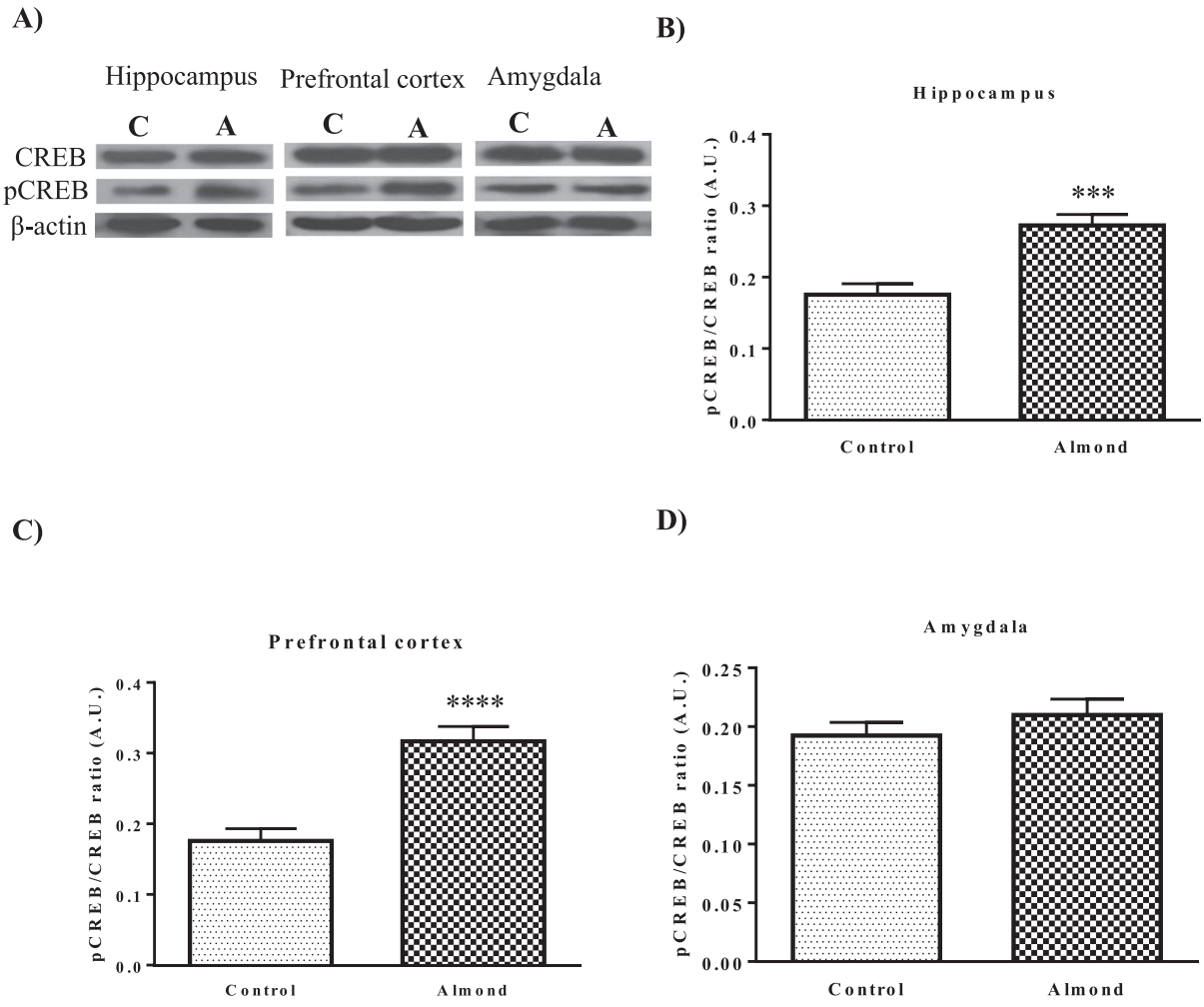

Figure 5. Effect of almond diet during pregnancy on (A) p-CREB, CREB, and β-actin expression in the hippocampus, prefrontal cortex, and amygdala of rat's brains. (B) The ratio of p-CREB/CREB expression in the hippocampus, (C) prefrontal cortex, and (D) amygdala of the adult male offspring ($n = 6$) ***$P < .001$ and ****$P < .0001$ as compared to the control.

3.4. Almond consumption during pregnancy-induced CREB phosphorylation in the HIP and PFC of the adult male offspring

Phosphorylated CREB (pCREB) has an accepted role in neuronal plasticity, long-term memory, and spatial memory formation in the brain [26].

As shown in Figure 5, western blot assessments revealed that p-CREB/CREB ratio increased in the Almond group compared to the control, and this was significant in the HIP and PFC, but the AMY region showed no substantial change (Figure 5(D)). Figure 5 (B,C) show that almond consumption during pregnancy significantly increased the p-CREB/CREB ratio by about 1.6- and 1.8-fold in the HIP and PFC of the adult male offspring, respectively ($P < .001$).

3.5. Almond consumption during pregnancy increased BDNF level in the HIP and PFC of the adult male offspring

As a neurotrophin, the brain-derived neurotrophic factor (BDNF) is involved in the survival of nerve cells of the brain that regulate emotion, memory, learning, and sleep [27]. Our data showed that almond consumption increased BDNF in some brain regions (Figure 6). As shown in Figure 6(A,B), the almond diet enhanced BDNF levels in the HIP and PFC of rat's brains by about 1.3- and 1.9-fold when compared to the control group, respectively ($P < .01$ and $P < .001$). However, this diet didn't affect the level of BDNF in the AMY area, as indicated in Figure 6(D).

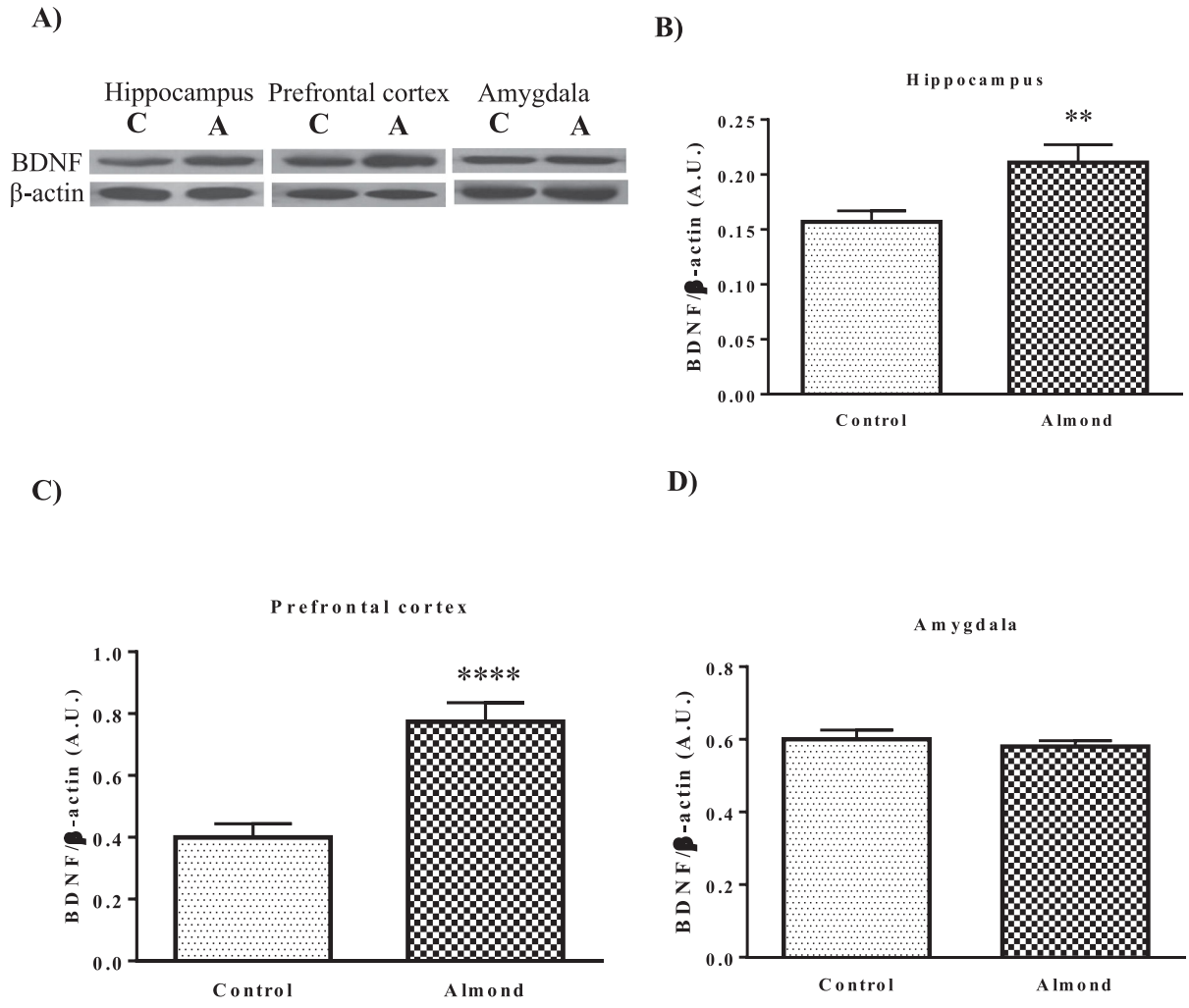

Figure 6. Effect of almond diet during pregnancy on (A) BDNF and β-actin protein expression in the hippocampus, prefrontal cortex, and amygdala of rat's brains. (B) Densities of BDNF to β-actin expression in the hippocampus, (C) prefrontal cortex, and (D) amygdala of the adult male offspring ($n = 6$) $^{**}P < .01$ and $^{****}P < .0001$ as compared to the control.

3.6. Almond consumption during pregnancy inhibited MAO-A activity in the HIP and PFC of the adult male offspring

Monoamine oxidase A (MAOA) is a mitochondrial enzyme involved in the catabolism of catecholamines [28]. Unpaired *t*-test analysis showed that almond consumption during pregnancy significantly inhibited MAO-A activity in the HIP and PFC of the offspring's brain up to 60 and 56%, respectively ($P < .05$ and $P < .01$, Figure 7(A,B)). Besides, our investigations in the AMY revealed no differences between groups (Figure 7(C)).

3.7. Almond diet during pregnancy decreased MAO-B activity in the HIP and PFC of the adult male offspring

In the brain tissue, MAO-B has a key role in oxidative signaling by producing hydrogen peroxide and metabolizing monoamines [28]. As shown in Figure 8, MAO-B had the same trend of change as MAO-A. The activity of this enzyme decreased in the HIP and PFC significantly in the almond group to about 65 and 40% as compared to the control group ($P < .05$ and $P < .01$, Figure 8(A,B)), while the MAO-B activity didn't reveal any alteration in the AMY region between both experimental groups (Figure 8(C)).

4. Discussion

Based on our results it can be assumed that almond consumption during pregnancy could influence the programming of the neurological system in male adult offspring; a hypothesis that seems can be valuable to be investigated in more detail. This maternal diet affected memory, stress adaptation, and anxiety-like

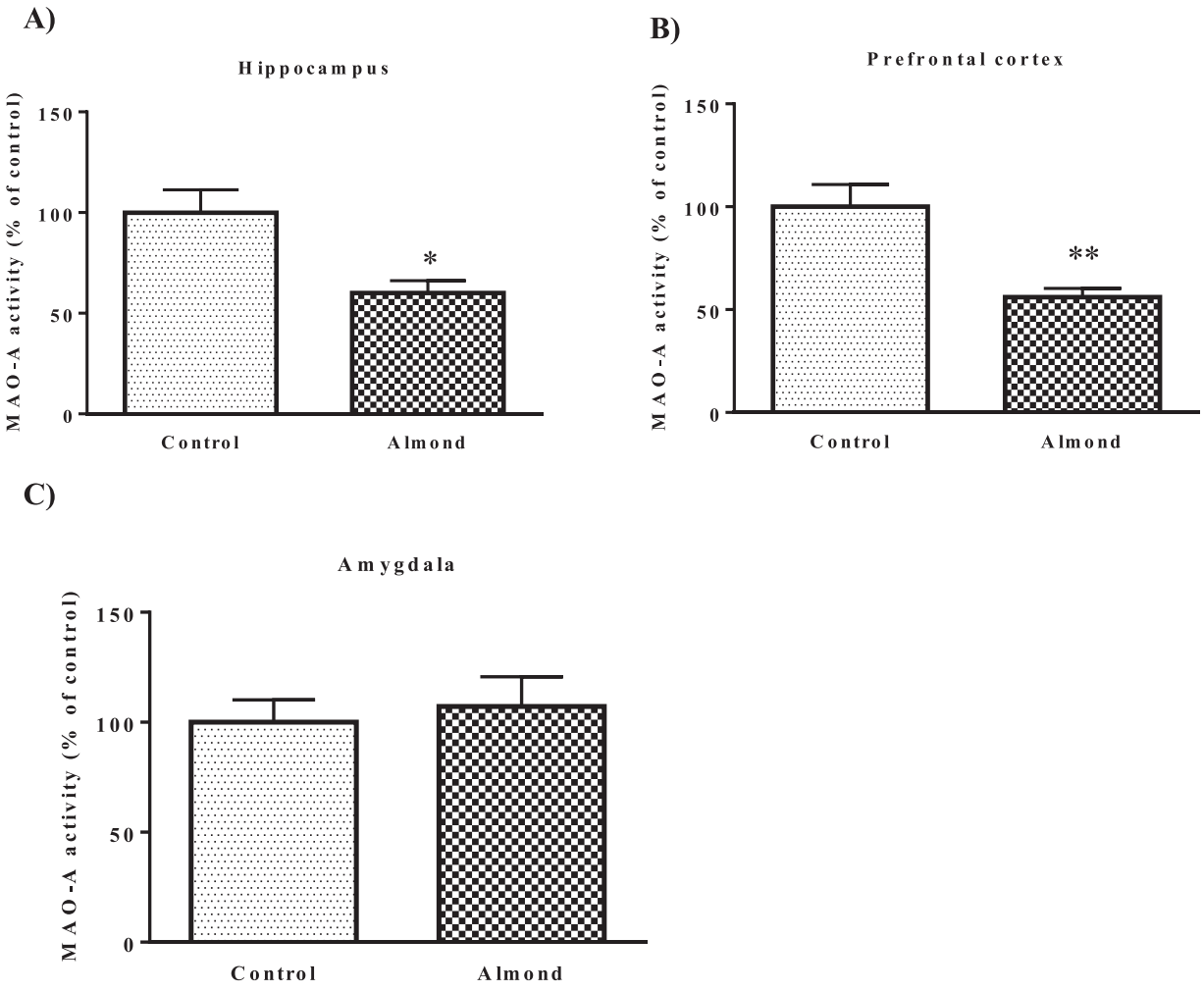

Figure 7. Effect of almond diet during pregnancy on (A) MAO-A activity in the hippocampus, (B) prefrontal cortex, and (C) amygdala of the adult male offspring ($n = 10$) *$P < .05$ and **$P < .01$ as compared to the control.

behavior via alteration in the level of p-CREB, BDNF, and MAO enzymes.

In recent years, nutraceuticals have received much attention due to their immune, nutritional, and therapeutic effects to promote health and prevent disease [29]. On the other hand, diet is one of the most important factors related to lifestyle in determining the health status and predisposing children to several diseases. Various studies have pointed out the positive and negative long-term effects of prenatal diet on adolescents' and adulthood health status [30–32]. Also, the impact of maternal diet on the permanent changes of offspring's CNS has been evidenced [33]. However, the effect of maternal dietary supplementation with nuts as a rich source of vitamins, minerals, fibers, nutrients, and non-nutrients in offspring has not been well studied. We have observed that almond consumption during pregnancy has long-term effects on adult male offspring and causes molecular changes. Almonds are composed of several ingredients including macronutrients (carbohydrate, protein, fat), micronutrients (vitamins and minerals), phytochemicals, and essential oils [34]. Almonds are a rich source of amino acids like tryptophan [35], vitamins and minerals including a variety of B and E vitamins [36], and polyphenols and phytosterols [37]. Interestingly, studies have shown that all active compounds of almonds can cross the placenta and affect the development of fetus organs [38].

The regular consumption of almonds as a dietary supplementation increased memory performance [12]. Assessment of different behavioral parameters showed that almond consumption significantly improved spatial learning and memory that has been impaired by alcohol [12]. Based on the mentioned effects behavioral tests were done as the first step. We evaluated the memory performance by Y maze [39] and stress adaptation by forced swimming test [25]. Our results showed significant improvement in the Y maze and forced swimming evaluations in the group that takes almond prenatally.

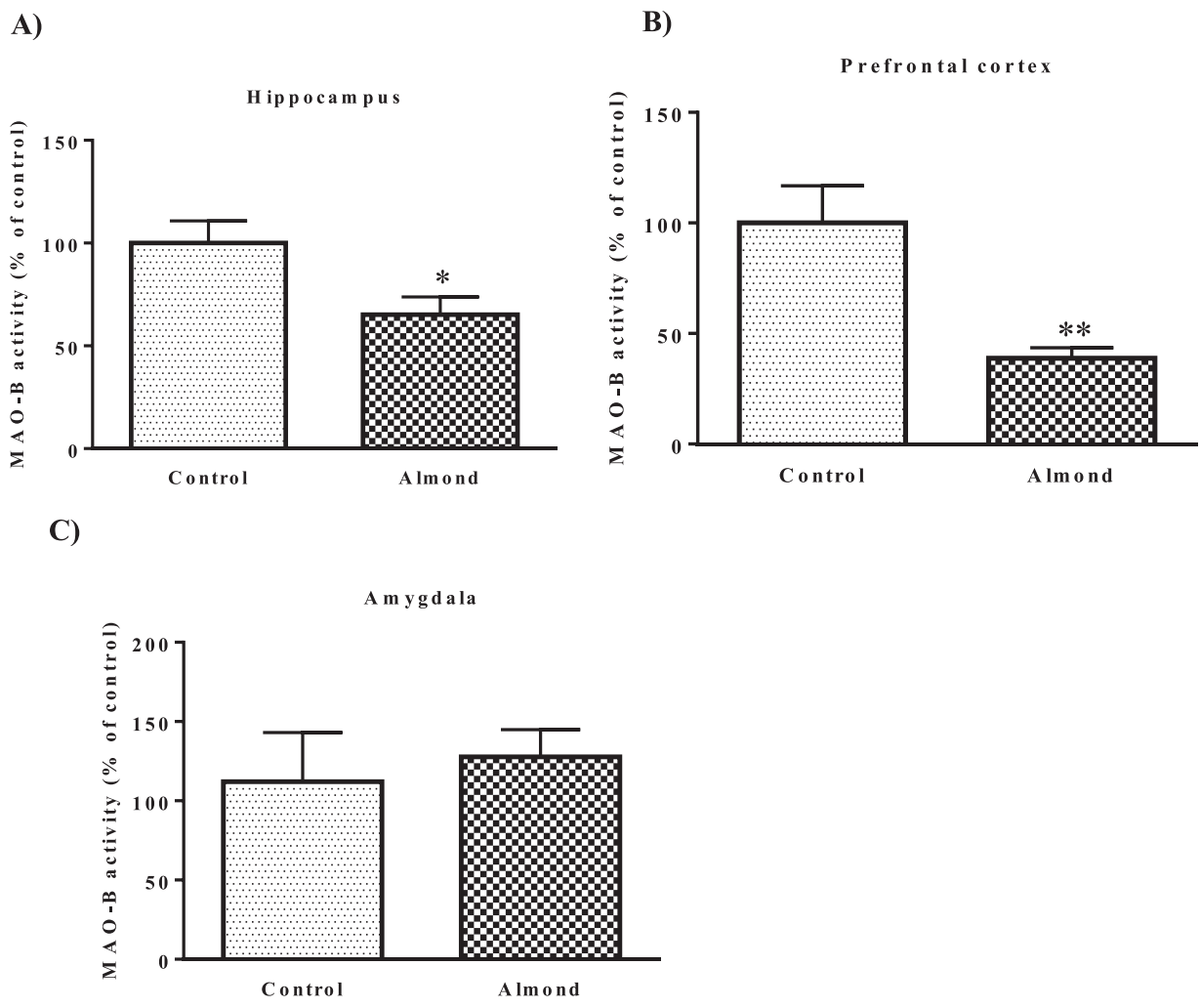

Figure 8. Effect of almond diet during pregnancy on (A) MAO-B activity in the hippocampus, (B) prefrontal cortex, and (C) amygdala of the adult male offspring ($n = 10$) *$P < .05$ and **$P < .01$ as compared to the control.

During early life, offspring received the polyphenols, tryptophan, and vitamins of almonds through the placenta.

Previous investigations have shown positive effects of polyphenols on mental health and memory function [40], targeting different molecular pathways[41]. Interestingly, the recent study by Sakakibara et al shed light on the anti-stress and stress adaptation effects of the polyphenols [42]. In addition, some studies pointed out the anti-depressive effects of almonds which they attributed to the antioxidant content of this nut [43]. Furthermore, tryptophan and vitamins of almonds could play a crucial role in anti-depressive effect [44] Almond diet has also been shown to inhibit the MAO enzyme which might be another mechanism for its effect on depression. Hypothetically, almond consumption by mothers could influence maternal care leading to more stress adaptation capacity and less depression in the offspring.

On the other hand, Haider and colleagues revealed an increase in the brain tryptophan and serotonergic turnover of rat's brains following oral intake of almonds leading to memory improvement in rats [45]. Enhanced serotonin activity of the brain has been shown to improve cognitive performance in both animals and human studies, whereas decreased serotonergic levels by acute tryptophan depletion would impair cognition [46,47]. The role of the serotonergic system in neuroplasticity has been explored, which is critical during brain development [48]. Serotonin acts as a growth factor during embryogenesis and is involved in brain structural changes. The serotonergic system interacts with chemical messengers of the GABAergic, glutamatergic, and dopaminergic neurotransmitters [49]. Given the above-mentioned data, tryptophan can be considered an effective part of almond in improving the memory of adult offspring rats.

Furthermore, almond is a good source of vitamins and minerals that may affect offspring if taken prenatally. Alpha-tocopherol is the major vitamin E isomer in almonds [50], which is associated with fetal growth by increasing the blood flow and nutrient supply to the fetus [35]. Vitamin E has been proposed to prevent certain neurological disorders [51]. Natural products, containing vitamin E, improve cognitive function and behavioral performance. Some of the key nutrients in almonds, such as vitamin E and L-carnitine, have a major role in the development of neural pathways, increasing the activity of the brain, and enhancing memory [12].

Despite the positive effect of almonds on stress adaptation and memory, our results showed only a slight anti-anxiety effect of this nut evaluated by the EPM test. We didn't find any alteration in the time spent in the open arm, which is a better indicator of anxiety compared to the open arm entries percentage. It is noteworthy to mention that in this study we compared an almond diet with healthy control rats, but previous studies on the anxiolytic effect of almonds and other nuts, such as hazelnut, have been reported in different animal models of neurological disorders [52].

We also explored the molecular pathway responsible for memory improvement in offspring who has prenatally received almond. For this purpose we assessed the levels of CREB phosphorylation and BDNF in the HIP, PFC, and AMY. Our results showed that almond consumption in pregnant mothers elevated the ratio of p-CREB/CREB and level of BDNF in the HIP and PFC of adult male offspring. Several studies demonstrated that CREB is integral for synaptic plasticity, neurogenesis, and cognitive performance [53]. Previously proven data indicated that improved cognitive performance may be attributed to the p-CREB/BDNF signaling in the HIP and PFC of different animal models [54–56].

Polyphenols and vitamins of almonds could be responsible for targeting the p-CREB/BDNF signaling. It has been reported that polyphenols phosphorylate CREB protein and subsequently increase BDNF [57]. Approximately 130 different polyphenols have been identified in almonds, including proanthocyanidins, tannins, flavonoids, phenolic acids, and aldehydes as the main contents. It seems that their synergistic actions should be considered as the mechanism of action [58]. Besides, the activating effect of vitamin E on p-CREB/BDNF signaling has been investigated recently [59].

Finally, we evaluated the MAO-A and B activity in the mentioned brain regions. Our data revealed a significant reduction of both enzymes in the HIP and PFC of adult male offspring. Monoamine oxidases (MAO), as mitochondrial enzymes, catalyze the oxidation of monoamines leading to increased epinephrine, norepinephrine, serotonin (for MAO-A), phenylethanolamine, and benzylamine (for MAO-B) in the brain. Animal and human studies have shown the alteration of MAO-A and B in the HIP and PFC to be in relation to memory and cognitive performance [60,61]. A recent study by Oyeniran et al. showed an inhibitory effect of almond leaves on MAO activity [62]. It has been demonstrated that protective polyphenols exert their effect via a modulatory action on neurotransmitter pathways like MAO inhibition [63]. In addition, anthocyanins as one of the almond flavonoids [58], inhibited MAO-A and MAO-B in vitro [64]. So, MAO inhibition is a possible mechanism that improves cognitive function in offspring which prenatally received almonds in its diet.

At the molecular level, we didn't find any significant changes in the AMY region of rats' brains. It may be because of this fact that the major AMY responses are fear and anxiety [65]. As, our results didn't show any considerable alterations in the EPM test, which could be related to our experimental design as well.

Despite the importance of maternal diet and the availability of rich resources such as nuts, there is little evidence about prenatal consumption of nuts effect on cognitive performance in the offspring. Although some investigations showed the effect of maternal diet supplementation with nuts such as, cashew and walnut during pregnancy improves memory in offspring [66–68], the molecular mechanism, modulating these effects, is not clear.

5. Conclusion

Taken together, the present study suggests that almond consumption during pregnancy is associated with improved memory, slightly reduced anxiety-like behavior, and increased stress adaptation in adult male offspring. On the molecular level our results revealed a significant elevation in p-CREB/CREB ratio and BDNF level, besides a reduction of MAO-A and B enzymes in the HIP and PFC of adult male offspring that received almond prenatally in their diet. Further studies are needed to explore other underlying molecular pathways of these effects.

Acknowledgements

The authors would like to thank the Neuroscience Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran for the laboratory support given to this study.

Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

All data of this study are available on request from the corresponding author [Z. B.].

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