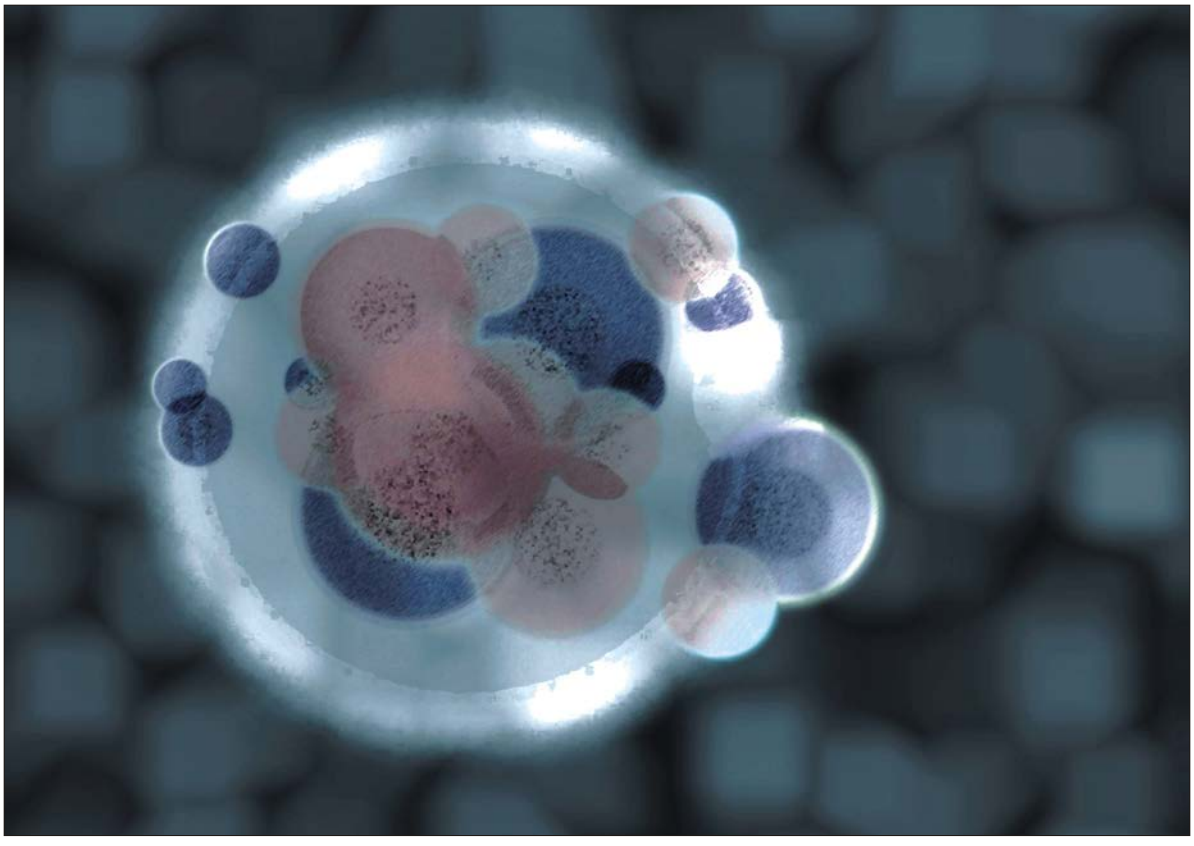

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Research Article
Aqueous Date Fruit Extract can't Ameliorate β-amyloid Induced Memory Impairments in Male Rats

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Abstract

Background: Date (*Phoenix dactylifera* L.) is an important plant in some of Asia and Africa. The importance of date fruit in human nutrition comes from its valuable components such as vitamins, carbohydrates, dietary fibers, salts and proteins. Data have been shown that Aqueous Date Fruit Extract (ADFE) has protective effects in different models of neuronal disorders. Alzheimer's Disease (AD) is a type of dementia that causes problems with thinking, memory and behavior. Symptoms generally develop slowly and get worse over time, becoming severe enough to interfere with daily tasks. In the current study, the effects of ADFE treatment on cognitive impairments and neural degeneration in animal model of AD was investigated. **Methodology:** Adult male rats were divided into three experimental groups including: β-amyloid group that received β-amyloid 25-35 (3 μL) through ICV injection, sham group that undertook the same surgical procedures as β-amyloid group, treated with vehicle (distilled water) and ADFE group was ICV injected with β-amyloid 25-35 and treated with ADFE for 12 consecutive days (4 mL kg^{-1}, gavage). Cognitive behavioral analysis has been performed by Morris Water Maze (MWM) test. Also, neuronal degeneration and cell apoptosis examination was calculated by routine staining as well as TUNEL assay. **Results:** The results of this experimental study revealed that ADFE improves learning process in β-amyloid induced impairments in male rats, however, it could not ameliorate the memory process and neural degeneration. **Conclusion:** This finding may be due to disruption in glucose and insulin regulation in AD and hyperglycemia induced by the ADFE administration.

Key words: *Phoenix dactylifera* L., Alzheimer's disease, β-amyloid, rat, ADFE

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Alzheimer's Disease (AD) is associated with oxidative stress and β-amyloid accumulation[1]. It causes cognitive and mental defects such as impaired memory, intellect and personality disorder in people older than 60 years of age[2]. Dietary influences on AD are in its early years and there are a few numbers of epidemiologic studies investigating association between dietary and AD. Therefore, at this time, we are not sure that any particular dietary component causes or prevents AD. Possibly the antioxidant nutrients, vitamins E and C are the best evidence of disease prevention. Animal models and laboratory studies have shown that AD involves oxidative and inflammatory processes. The final result is usually disruption of neuronal cell function that leading to neuronal cell death. High metabolic activity of brain generates free radical molecules, oxygen molecules that are extremely reactive and thus toxic to neurons[3]. Several animal studies show that the antioxidant nutrients protect neurons from damage due to oxidative and inflammatory mechanisms. Also, histological studies the brains of the antioxidant-fed rodents revealed less damage and neuronal cell death[4,5].

Date palm (*Phoenix dactylifera* L.) is an important plant in Southwest of Asia and North Africa. The importance of dates in human nutrition comes from its valuable components such as carbohydrates, dietary fibers, salts, vitamins and proteins[6]. Date fruits contain 70% of carbohydrates in the form of sugars. On the other hand, Aqueous Date Fruit Extract (ADFE) might be interesting since it has been recently known as promising neuroprotective agent in different models of neuronal degeneration[7-9]. At this time, there is no cure for Alzheimer's disease and new therapeutic methods in AD are urgently needed. Here, using a rodent model of AD, we address if ADFE administration causes a reduction in cognitive impairments and neuronal injury in male rat.

MATERIALS AND METHODS

Chemicals: β-amyloid protein fragment 25-35 (A4559 Sigma) was purchased from Sigma (St., Louis, MO). Whatman No. 2 filter paper was purchased from Whatman International Limited, Kent, England. The terminal deoxynucleotidyl transferase (TdT)-mediated *in situ* dUTP nick end-labeling (TUNEL) the cell death detection kit POD was obtained from Roche (Indianapolis, IN). Folin-Ciocalteu reagent, Na_2CO_3, $NaNO_2$, $AlCl_3.6H_2O$, NaOH, sulphuric acid, sodium phosphate ammoniummolybdate and hematoxylin and eosin were purchased from Merck, Germany. The DPX was bought from Panreac SA, Barcelona, Spain.

Animals: Adult male Sprague-Dawley rats (supplied by Neuroscience Research Animal Center, Kerman, Iran) weighing 210-260 g were kept in a controlled environment at room temperature of 20±1.0°C and automatic day-night schedule (12 h cycle). Animals were divided randomly into three groups: (1) β-amyloid group was ICV injected with β-amyloid 25-35 (3 μm) (n = 8), (2) Sham group, animals which undertook the same surgical procedures as β-amyloid group, treated with vehicle (distilled water) (n = 8) and (3) ADFE group was ICV injected with β-amyloid 25-35 and treated with ADFE for 12 consecutive days (4 mL kg^{-1}, gavage) (n = 8). This study was approved by the ethics committee of Neuroscience Research Center of Kerman University of Medical Sciences (EC/KNRC/92/21). Active β-amyloid peptide fragment 25-35 (A25-35) was dissolved in distilled water at a concentration of 1 mM and stored at -20°C.

Surgery: For ICV injection of β-amyloid 25-35, the animals were anesthetized with ketamine and xylasine (80 and 10 mg kg^{-1}, i.p., respectively) and a stainless steel cannula was inserted stereotaxically (1.5 mm from the middle, 0.9 mm posterior to the bregma and 3.5 mm from the surface of skull) into the right ventricle. Confirmation of the injection site was verified by methylene blue staining.

Preparation of ADFE: Date fruits (Bam, Mozafati, rutab) were purchased from authenticated market. Date fruits were isolated from date kernel and 100 g of dates were soaked in distilled water for 48 h. It was mixed completely in a mixer and then the mixture was centrifuged at 3000 rpm at 4°C for 20 min. After sedimentation, the supernatant part was used for gavages.

Total flavonoids content: Total flavonoid content was determined following a method by Park *et al.*[10]. About 0.3 mL of ADFE was mixed with 3.4 mL of 30% methanol, 0.15 mL of $NaNO_2$ (0.5 M) and 0.15 mL of $AlCl_3.6H_2O$ (0.3 M). After 5 min, 1 mL of NaOH (1 M) was added and homogenized by vortex. The absorbance of samples at 506 nm was compared with a standard curve made from quercetin (0-100 mg L^{-1}). The data were expressed as milligrams of quercetin equivalents per gram of dry matter.

Estimation of Total Phenolic Content (TPC): The phenolic compounds were calculated using the Folin-Ciocalteu method. One milliliter of sample (1 mg mL^{-1}) was mixed with 1 mL of Folin-Ciocalteu reagent followed by addition of 10 mL of a 7% Na_2CO_3 and 13 mL deionized distilled water and mixed thoroughly. The homogenized samples heated for 90 min at

23°C. The absorbance was measured at 765 nm after cooling at room temperature. The data were determined by using a gallic acid standard curve. The data were expressed as milligrams of gallic acid equivalents per gram of dry matter.

Total antioxidant activity (Phosphomolybdate assay): The total antioxidant capacity of the fractions was estimated by phosphomolybdenum method using ascorbic acid as a standard. About 0.1 mL aliquot of 100 μg mL^{-1} concentration of ADFE and standard was mixed with 1 mL of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95°C for 90 min. After the samples were cooled, the absorbance of the mixture was measured at 765 nm. Total antioxidant capacity of extract, expressed as mg g^{-1} of ascorbic acid equivalents. Antioxidant effect of the extract (%) was also estimated using following equation:

$$\text{Antioxidant effect} = \frac{\text{Control absorbance} - \text{Sample absorbance}}{\text{Control absorbance}} \times 100$$

The EC_{50} values calculated to determine the 50% inhibition of Mo reduction by extract.

Learning and memory evaluation using Morris Water Maze (MWM) test: Spatial learning and memory was evaluated using a modified version of the Morris water maze, in which rats learned to get away from the water onto a hidden platform[11]. The experiments were performed in the blurredly light room with spatial cues which were attached to the walls around the maze at different points. Noldus Ethovision system, version 7.1 is a smart video tracing system that recorded the performance of rats. Also, it can be traced on the monitor of the computer. The maze consisted of an iron circular (1.36 m in diameter and 60 cm high) filled with cloudy water with 20±1°C temperature. Animals were challenged to find a hidden platform (10 cm in diameter and 25 cm high) located 1.5 cm below water level (26.5 cm) and in the same place during the entire trial. The platform was always placed 30 cm beyond the rim of the pool in the center of one quadrant with respect to the distal visual cues. The experiment was performed over 4 consecutive days to test their learning, when rats were located into the water facing the rim from 4 different starting points in the pool with the platform kept in the same place and with an interval between each trial. The rats were trained in the maze during (16 trials), 4 sessions on consecutive 4 days except for the last day. The animals had to swim to find the hidden platform in 60 sec (maximum time), where they were allowed to stay for 30 sec. If they failed to find the platform, rats were placed on it for 10 sec to associate spatial cues of the room (pictures, light source and cabinets). Swimming patterns were evaluated and the following factors for spatial learning were computed: Distance traveled to find the hidden platform (cm), time spent to find the hidden platform (Escape latency [sec]) and swimming speed (cm sec^{-1}). One day after the last learning trial, the animals underwent a probe trial to test their long-term spatial memory. For this purpose, percent of distance, time spent and frequency in the target quadrant was recorded and analyzed[12].

Hematoxylin and Eosin (H and E) staining: For quantitative analysis the CA1 subfield of hippocampus, the tissues were processed according to standard histological methods. Paraffinized brains were cut into 7 μm sections on a rotary microtome and the sections were stained with hematoxylin and eosin. Neuronal damage was then estimated for each animal as the rate of the number of degenerated pyramidal neurons to that of both surviving and degenerated in three distinct areas of the CA1 subfield (middle, medial and lateral) of hippocampus in coronal sections.

Immunohistochemistry: The terminal deoxynucleotidyl transferase (TdT)-mediated *in situ* dUTP nick end-labeling (TUNEL) immunohistochemistry was done on the brain sections using the cell death detection kit POD (Roche, Indianapolis, IN) according to the manufacturer's instructions. Briefly, 7 μm sections were deparaffinized and rehydrated. Protein digestion was completed by incubating tissue sections in 20 mg mL^{-1} proteinase K (Roche, Indianapolis, IN) for 20 min at Room Temperature (RT). Endogenous peroxidase was inactivated with 2% H_2O_2 in distilled water for 5 min at RT. The labeling mixture containing biotinylated dUTP in TdT enzyme buffer was added to the sections and incubated at 37°C in a humidified chamber for 1 h. After stopping the enzymatic reaction, the brain sections were rinsed with Phosphate Buffer Solution (PBS) and incubated with anti-digoxigenin peroxidase conjugate (20 min at RT). Then, the sections were incubated in PBS with 0.05% diaminobenzidine (DAB) plus 3% H_2O_2 until color development occurred. Finally, the sections were washed, dehydrated and mounted with DPX[13].

Statistical analysis: Data were offered as Mean±SEM. The means were analyzed using one-way analysis of variance (ANOVA) followed by the Tukey's post hoc test and $p<0.05$ was considered statistically significant.

RESULTS

Measurement of total flavonoids contents and total phenolics: Flavonoids content was 229.3±0.89 mg quercetin equivalents g^{-1} fraction. Total phenolic compounds content as recorded in Table 1, in ADFE was 259±2.14 mg g^{-1} gallic acid equivalent per 1 g of dried extract.

Antioxidant activity of ADFE: Antioxidant activity of ADFE is shown in Table 2. The ADFE showed 76.23% free radical scavenging activity against DPPH radicals (Table 2).

Effect of β-amyloid injection on learning parameters in male rats: There was a significant difference in distance traveled to find the hidden platform ($p<0.05$) and escape latency ($p<0.05$) for β-amyloid compared with sham. Treatment with ADFE significantly ameliorated the aforementioned parameters ($p<0.05$) (Fig. 1, 2). There was no significant difference in swimming velocity among all groups (Fig. 3, $p>0.05$).

Effect of β-amyloid injection on memory parameters in male rats: There was a significant difference in percent of distance ($p<0.01$), time ($p<0.05$) and frequency spent in target quadrant ($p<0.01$) for β-amyloid compared with sham. Administration of ADFE could not ameliorate the deleterious effect of β-amyloid on the memory parameters ($p<0.01$, $p<0.001$ and $p<0.01$, respectively) (Fig. 4-6).

Neuronal degeneration: In the microscopic investigation, the morphology of neurons in sham group was normal including round nuclei with prominent nucleoli and a light pink cytoplasm (black arrow). In contrast, most pyramidal neurons in the β-amyloid group displayed more severe degenerative changes; an eosinophilic cytoplasm and extensively dark piknotic nuclei (white arrow). The severity of the degenerative changes observed in the cytoplasm and nucleus was similar in the ADFE-treated group with β-amyloid group (Fig. 7). Neuronal counting (the percentage of degenerated neurons) in CA1 subfield of hippocampus showed significant differences between the sham (5.46%) and β-amyloid group

Table 1: Total phenolics and flavonoids content of ADFE

Sample	Total phenolics content (mg GAE g^{-1} extract)	Total flavonoids content (mg quercetin g^{-1} extract)
ADFE	259±2.14	229.3±0.89

Table 2: Antioxidant activity of ADFE

ADFE	ASC equivalent (mg g^{-1})	Antioxidant activity (%)
100	56.92	76.23

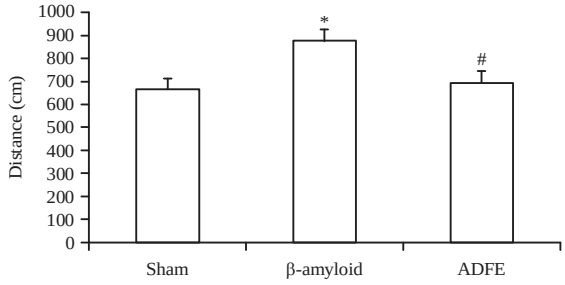

Fig. 1: Effects of sham, β-amyloid and ADFE on distance (cm) traveled by male rats to find the hidden platform. The animals treated with β-amyloid presented more distance compared to sham (*$p<0.05$). ADFEE significantly ameliorated this parameter (#$p<0.05$). Data are expressed as Mean±SEM, ADFE: Aqueous date fruit extract

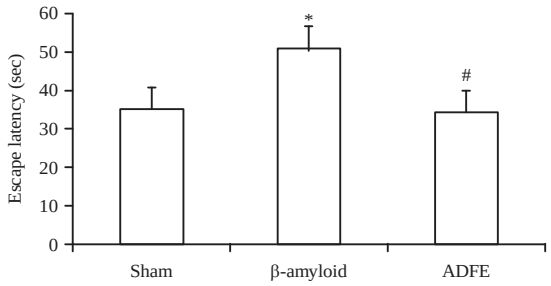

Fig. 2: Effects of sham, β-amyloid and ADFE on time (sec) spent to find the hidden platform. The animals treated with β-amyloid presented more time compared to sham (*$p<0.05$). ADFE significantly inhibited β-amyloid induced learning deficit (#$p<0.05$). Data are expressed as Mean±SEM, ADFE: Aqueous date fruit extract

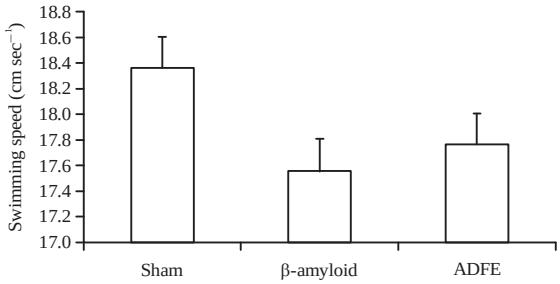

Fig. 3: Effects of sham, β-amyloid and ADFE on swimming speed (cm sec^{-1}) of male rats. There were no differences in the swimming speed between different groups. Data are expressed as Mean±SEM, ADFE: Aqueous date fruit extract

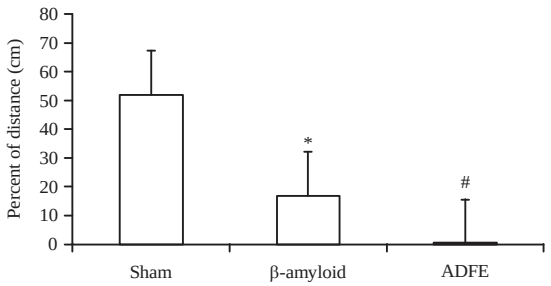

Fig. 4: Effects of sham, β-amyloid and ADFE on percent of distance (cm) spent in target quadrant. The animals treated with β-amyloid presented less distance compared to sham (*p<0.01). ADFE could not improve this parameter (#p<0.01). Data are expressed as Mean±SEM, ADFE: Aqueous date fruit extract

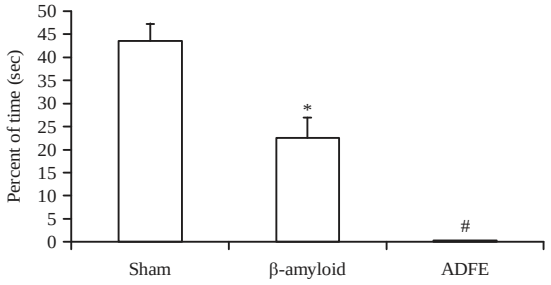

Fig. 5: Effects of sham, β-amyloid and ADFE on percent of time (sec) spent in target quadrant. The animals treated with β-amyloid presented less distance compared to sham (*p<0.05). ADFE significantly ameliorated this parameter (#p<0.001). Data are expressed as Mean±SEM, ADFE: Aqueous date fruit extract

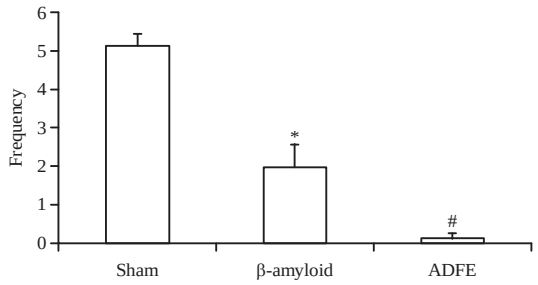

Fig. 6: Effects of sham, β-amyloid and ADFE on frequency spent in target quadrant. The animals treated with β-amyloid presented less frequency compared to sham (*p<0.01). ADFE could not ameliorate this parameter (#p<0.01). Data are expressed as Mean±SEM, ADFE: Aqueous date fruit extract

(54.88%) (p<0.05). Administration of ADFE did not prevent (49.93%) the detrimental effect of β-amyloid induced insults (Fig. 8).

TUNEL study: Neuronal injury to the CA1 area of hippocampus was also examined using the TUNEL method. The ADFE pre-treatment did not decrease the number of TUNEL-positive (dark brown) cells compared with the β-amyloid group.

DISCUSSION

The results of current study demonstrated that short time administration of ADFE improves learning process in β-amyloid induced impairments in male rats. The cognitive-enhancing activity of ADFE on the β-amyloid 25-35 induced memory impairments in rats was investigated using MWM test. Rats treated with β-amyloid showed more prolonged escape latency than rats in the sham group. The ADFE treatment significantly increased escape latency in experimental group which suggested that learning process was impaired by β-amyloid. In addition, during the probe trial session, the β-amyloid induced reduction in swimming times within the target quadrant which wasn't significantly ameliorated by ADFE, indicating a non-positive effect on spatial memory. This finding may be due to hyperglycemic

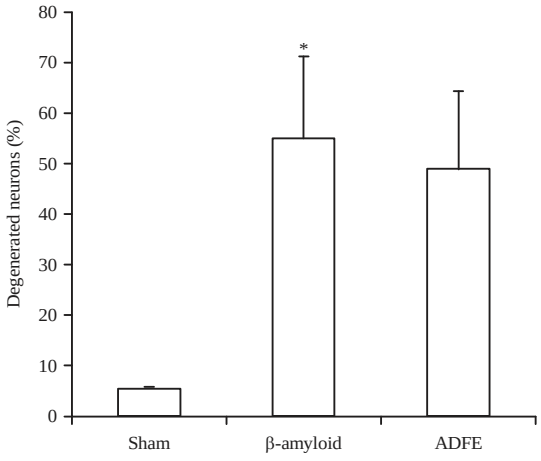

Fig. 7: Effect of sham, β-amyloid and ADFE on neuronal degeneration in male rats. Neuronal damage in CA1 area of hippocampus was assessed using hematoxylin-eosin (H and E). β-amyloid significantly increased (54.88%) degenerated neurons comparing to sham (5.46%) (*p<0.001). Administration of ADFE could not decrease the percent of degenerated neurons in treated group (49.93%). Data are expressed as Mean±SEM, ADFE: Aqueous date fruit extract

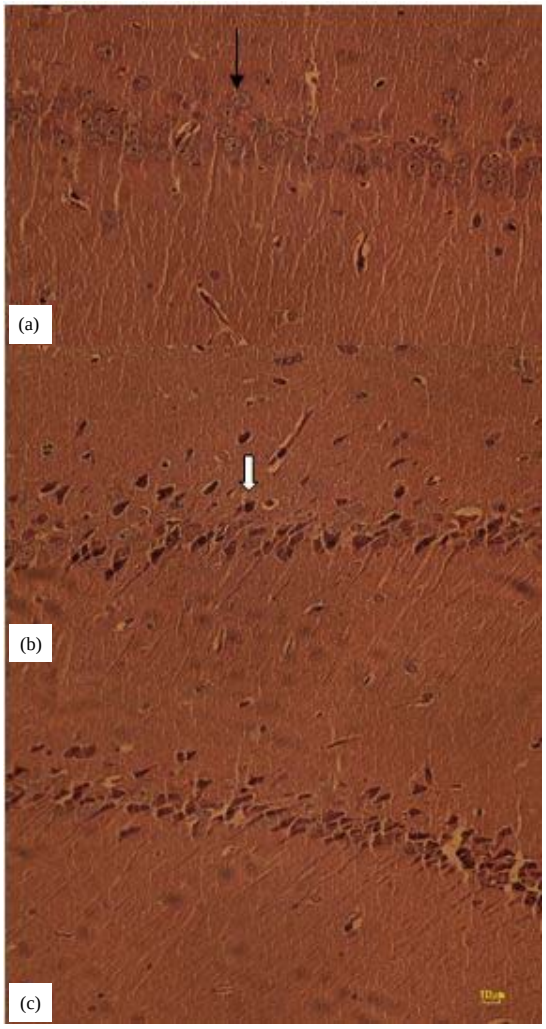

Fig. 8(a-c): Light micrographs of sections of CA1 hippocampal neurons of male rats stained by hematoxylin-eosin (H and E) in different groups, (a) Sham, (b) β-amyloid and (c) ADFE group, (a) Micrograph showing a layer of intact pyramidal neurons with large and prominent nuclei (black arrow), (b) Micrograph showing degenerated neurons (white arrow) and (c) Micrograph showing of ADFE treated group. The severity of the degenerative changes observed in the cytoplasm and nucleus of β-amyloid group was similar with the ADFE-treated (Magnification: ×400)

effect of ADFE because dates have high sugar content (70%) including glucose, sucrose and fructose. In agreement with our data, studies have shown that AD is accompanied by disruption in glucose regulation and utilization that may contribute to its characteristic memory impairment[14,15].

Some brain regions and the cognitive functions supported by them are highly vulnerable to changes in glucose availability[16]. The hippocampus plays a critical role in the conscious acquisition and recall of new information (declarative memory)[17]. Accordingly, declarative memory is impaired when glucose dysregulation affects the hippocampus. Besides, decreased numbers of brain glucose transporters have been documented in AD[18,19].

On the other hand, some evidence suggests that insulin plays a role in brain function[20]. Dense distributions of insulin receptors have been detected in CA1, CA3 and dentate gyrus regions of the hippocampus, the hypothalamus and olfactory bulb distributions which are remarkably similar to the primary areas of neuropathology in AD[21]. *In vitro* studies show that insulin affects hippocampal firing in a dose-dependent manner with moderate levels increasing firing and low or high levels causing inhibition[22]. The ICV administration of insulin has also been shown to increase glucose utilization in entorhinal cortex[18], whereas lesioning of brain insulin receptors with streptozotocin disrupts hippocampal glucose utilization[23]. Finally, peripheral administration of insulin and glucose together enhanced learning in rats[24]. So, it seems that disruption in glucose and insulin regulation may contribute to the characteristic memory impairment in male rats.

Different studies have shown that β-amyloid induce neurodegeneration characteristic of apoptosis[25]. The results of this study also showed that β-amyloid 25-35 induced neuronal degeneration in CA1 hippocampal areas of male rats.

Also, administration of ADFE couldn't protect CA1 neurons against toxic effect of β-amyloid. Altogether, antioxidant analysis of the used extract demonstrated that the extract has potent antioxidant agent but it could not protect the CA1 neurons against deleterious effects of β-amyloid.

CONCLUSION

We may conclude that oral ADFE treatment may attenuate the detrimental effects of β-amyloid injection on learning process in our male rats, although, it could not improve the memory process and neural degeneration in this group of animals. This finding may be due to disruption in glucose and insulin regulation in AD and hyperglycemia induced by the ADFE administration. Further study, however, is necessary to identify the precise mechanism of action.

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