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EFFECT OF DEHYDROEPIANDROSTERONE (DHEA) ON MEMORY AND BRAIN DERIVED NEUROTROPHIC FACTOR (BDNF) IN A RAT MODEL OF VASCULAR DEMENTIA

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The effect of dehydroepiandrosterone (DHEA) on memory and cognition in experimental animals is well known, but its efficacy in clinical dementia is unproven. So, the aim of the present study was to investigate the effect of DHEA on learning and memory activities in a rat model of vascular dementia (VD). Forty-eight male rats that positively passed the holeboard memory test were chosen for the study before bilateral permanent occlusion of the common carotid artery. They were divided into four groups (n=12, each) as follows (i) untreated control, (ii) rats exposed to surgical permanent bilateral occlusion of the common carotid arteries (BCCAO) leading to chronic cerebral hypoperfusion, (iii) rats exposed to BCCAO then received DHEA (BCCAO + DHEA) and (i.v.) rats exposed to BCCAO then received donepezil (BCCAO + DON). Holeboard memory test was used to assess the time, latency, working memory and reference memory. Central level of acetylcholine, norepinephrine and dopamine in the hippocampus were measured. Furthermore, the expression of brain derived neurotrophic factor (BDNF) in the hippocampus was determined. Histopathological studies of the cerebral cortex and transmission electron microscope of the hippocampus were performed. BCCAO decreased the learning and memory activities in the holeboard memory. Also, it decreased the expression of BDNF as well as the central level of acetylcholine, noradrenaline and dopamine as compared to control rats. Treatment with DHEA and donepezil increased the working and reference memories, BDNF expression as well as the central acetylcholine in the hippocampus as compared to BCCAO rats. DHEA produced neuroprotective effects through increasing the expression of BDNF as well as increasing the central level of acetylcholine and catecholamines which are non-comparable to donepezil effects.

Key words: *dehydroepiandrosterone, bilateral occlusion of the common carotid arteries, vascular dementia, acetylcholine, holeboard memory test, brain derived neurotrophic factor*

INTRODUCTION

Disorders of the cerebral circulation are the causes of numerous neurological and psychiatric illnesses. A sudden disruption of the blood supply to distinct brain regions leads to stroke while a moderate but persistent reduction in regional cerebral blood flow (CBF) compromises memory processes and contributes to the development and progression of vascular dementia (VD). VD is characterized primarily by the gradual loss of cholinergic neurons. In addition to a deficiency of acetylcholine (ACh), decreases in other neurotransmitters systems, especially, serotonergic and dopaminergic have also been noted. Due to this, current therapies for neurodegenerative diseases mainly affect alterations in appropriate neurotransmitter systems, as well as the symptoms of the disease. Thus, searching for drugs that enhance acetylcholine and other neurotransmitters levels is an area of interest nowadays (1-3).

Dehydroepiandrosterone (DHEA) has several crucial actions in the brain. DHEA exerts antioxidant, antilipidperoxidative,

antiinflammatory and thereby antiaging actions (4). The possibility of using DHEA in the management of different diseases has attracted extensive attention over recent years. DHEA therapy seems to be successful in treating patients with cognitive decline, depression, cardiovascular disease, osteoporosis and sexual dysfunctions. Further research is needed to assess the efficacy and safety of DHEA supplementation in patients with neurodegenerative disorders associated with advanced age. It is now well accepted that serum dehydroepiandrosterone level declines progressively with aging in men (5). This decline is associated with alterations in body composition; diminished energy, muscle strength, and physical function; reduced sexual function; depressed mood; and decreased cognitive function (6).

DHEA and DHEA-S are produced in the brain in significant amounts, although adrenal cortex and gonads are the major sources of these compounds in the body. There were coherent reports of higher brain and CSF levels of DHEA in AD, which have been correlative to the neuropathological stages of the

disease, but there are variable reports of altered DHEA in blood in AD (7-10). On the other hand, the level of DHEA-S derived from DHEA has been reported to be significantly less in CSF and blood in AD compared to control, although the studies are limited in the number (8). The serum level of DHEA-S is not altered compared to control in vascular dementia subjects in this study, which contrasts with some earlier reports (9). To the best of our knowledge in the literature, no data investigated the effect of DHEA in the cognition and learning in a rat model of vascular dementia. Therefore, we investigated the possible role of DHEA in changing the concentration of the central neurotransmitters (acetylcholine, norepinephrine and dopamine) in a rat model of vascular dementia.

Brain-derived neurotrophic factor (BDNF), a member of the neurotrophin family, is a key protein in the regulation of the maintenance, growth and survival of neurons (11). BDNF is necessary for cell proliferation, cell differentiation, neuronal protection, and the regulation of synaptic function in the central nervous system (CNS) *via* stimulating key intracellular signaling cascades (12). In addition, BDNF plays a vital role in various aspects of neural plasticity, such as neurogenesis, long-term potentiation (LTP), learning and memory, and mood changes (13). The mutation or deletion of the BDNF gene in mice results in learning deficits and LTP impairment, whereas re-expression of BDNF restores LTP (14). In patients with Alzheimer's disease or major depression, the brain or serum levels of BDNF were decreased (15).

In the present study, the holeboard food search task has been used in rats to analyze their learning ability and different types of memory; working and reference memory. Vawter and Van Ree confirmed that the performance in the holeboard memory test is sensitive to the degree of food deficiency. A higher level of food deprivation resulted in a superior performance of the animals, but the processes implicated in learning and memory were less affected. The data obtained by them indicated that both external and internal characteristics can influence the results of the holeboard food search task, and thus the calculated scores for learning and memory (16).

The first experimental study that evaluated the role of the cholinergic transmission in the storage and retrieval of new data were performed by Deutsch (17). Moreover, the effects of cholinergic antagonists and lesions of cholinergic nuclei are often related to cognitive deficits similar to those observed in aging and dementia (18). Recently Croxson *et al.*, (19) concluded that in the absence of acetylcholine innervations to inferotemporal cortex, the retrieval of episodic memory is impaired and the amnesia caused by the structural injury is more dangerous.

Seymour Kety suggested that emotionally arousing experiences may be associated with stimulation of the locus coeruleus, sending adrenergic projections to different regions of the brain (such as hippocampus, cortex and cerebellum) (20). Moreover, he proposed that activation of β -adrenoreceptors by released norepinephrine (NE) could result in facilitation of synaptic transmission through the mechanism involving increases in the intracellular cAMP concentration and new protein synthesis, thus contributing to the memory acquisition and maintenance. It is currently hypothesized that synaptic plasticity, specifically long-term potentiation (LTP), in the neural circuits of learned behaviors could provide a cellular substrate of memory storage (21). Consistent with Kety's proposal, it has been demonstrated recently that direct activation of the locus coeruleus initiated protein synthesis-dependent LTP at the perforant path input to the dentate gyrus in awake rats (22). Furthermore, electrophysiological studies *in vivo* and *in vitro* point to a specific role for dopamine in the temporal persistence of long-term potentiation (LTP) (23).

So, the aim of the present study was to investigate the possible protective effects of DHEA on the memory, acetylcholine, dopamine and norepinephrine, as well as BDNF expression in a rat model of vascular dementia.

MATERIAL AND METHODS

All experimental procedures were approved by the medical research ethical committee at King Khalid University and according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996).

Animals

The animals employed in the present study comprised 96 male Sprague-Dawley rats weighing 170–200 g. All rats were bred and housed in the research center of King Khalid college of medicine (Abha, Saudi Arabia) at a temperature of $23\pm 1^\circ\text{C}$, and a 12:12 hours light:dark cycle. All rats had a free access to tap water and fed standard laboratory chow.

Experimental design

After 1 week of acclimatization to the laboratory environment, the animals were randomly allocated into four groups (each, 24 rats) as follows:

- 1) Normal rats (control group): were healthy rats non exposed to bilateral common carotid artery occlusion.
- 2) Rats exposed to bilateral common carotid artery occlusion (BCCAO).
- 3) Rats exposed to bilateral common carotid artery occlusion and treated with DHEA (dissolved in 5% DMSO in saline) orally in a dose of 250 mg/kg body weight/day (24), (BCCAO + DHEA)
- 4) Rats exposed to bilateral common carotid artery occlusion and treated with donepezil 3 mg/kg/ day; *i.p.* (BCCAO + DON).

The Schematic representation of the experimental procedure is shown in *Fig. 1a*. After the 7th day of training, the number of rats per group was reduced to 12 rats.

Psychometric assessment (holeboard memory test)

To measure the speed of learning and memory capacity in rats, the holeboard memory test was used (25). The testing area of the holeboard apparatus contains 16 holes in a 4'4 array. The holes are 20 cm apart and filled with plastic cups (2.5 cm deep, 3 cm in diameter) for sweetened cereal food pellets. Some were spilled on the floor to exclude the influence of smell (olfactory stimuli) on the search for food. The rats had to collect pellets in specified periods of time.

The test consists of 3 phases: habituation, training and retests. During the habituation and training phases and for one day before the retests, food was restricted for the rats to enhance their motivation to search for it (water was available *ad libitum*).

The habituation phase was for 4 consecutive days. Each hole in the board was baited with 50 mg of cereals. The trial started when the rat entered the testing area and it was stopped after 10 min or earlier, *i.e.* the time necessary to collect all food pellets. The monitoring included the time and the number of visits and revisits to the baited holes. Rats which found at least 14 of 16 pellets on the 4th day, qualified for the training phase. The ones with poorer results were excluded from further experiments (1st exclusion criterion).

Training phase started 3 days after habituation and continued for 7 consecutive days with a pause on the 5th and 6th day. The

rats were trained to collect pellets from 4 holes: A1, B3, C2 and D4 (Fig. 1). During one training session, four trials were carried out for each animal. The maximum time given to collect 4 food pellets was 5 min. There were intervals of about 1 min between the trials for cleaning and baiting A1, B3, C2 and D4 holes with pellets. Apart from the time of performance, the registration included latency time (time between the beginning of the trial and the first hole visit) and the number of visits and revisits to the baited and empty holes. Based on these parameters, two distinct memory functions - working and reference memory - were evaluated. The parameters were evaluated based on results of the 7th day of training. Only rats with WM ratio >50% and RM ratio >40% qualified for the surgical procedure (BCCAO). Animals with poor results were excluded as non-intelligent. So, all the groups were halved to 12 rat/group.

Working memory ratio (WM) was presented as a percentage of all visits to the baited set of holes that had been supplied with food (calculated as the number of food rewarded visits divided by the number of visits and revisits to the baited set of holes). Reference memory ratio (RM) was expressed by the number of visits to the baited set of holes as a percentage of the total number of visits to all holes (calculated as the number of visits and revisits to the baited set of holes divided by the number of visits and revisits to all holes). The parameters were evaluated based on results of the 7th day of training.

The first retest (R1) was performed one week after the surgery, while each subsequent test was 5–7 days apart, with a new order of baited holes: A4, B2, C3, D1 (rotated by 90° as compared with the training phase) (Fig. 1). The course for the retests, as well as the registered parameters, were the same as during the training phase.

Bilateral common carotid occlusion (BCCAO)

Bilateral common carotid arteries were occluded as previously described under ketamine hydrochloride (50 mg/kg, i.m.) and xylazine (5 mg/kg, i.m.) anaesthetics. To prevent respiratory distress, the rats were also administered atropine sulfate (0.1 mg/kg, i.m.). The common carotid arteries were carefully separated from surrounding tissues, including the vagus nerve and ligated with coated Vicryl (R)

Plusantibacterial/Polyglactin 910 3/0 absorbable surgical suture (Ethicon, Johnson & Johnson, UK), approximately 1 cm inferior to the origin of the external carotid artery. The control rats were subjected to the same surgical procedure without occlusion of the arteries.

Pharmacological treatment

DHEA and donepezil were obtained from Sigma Aldrich. Starting the day after BCCAO the rats were treated daily with DHEA (dissolved in 5% DMSO in saline) orally through gastric gavage (250 mg/kg/day) and donepezil (3 mg/kg/ day; i.p.) The treatments were continued for 6 consecutive days.

Sample preparation and biochemical evaluations

The rats were euthanized after the 3rd–4th retest, i.e. 2 or 3 weeks following the operation. The brain was quickly removed from the skull; the hippocampus and the rest of the brain were dissected. Tissue samples from the hippocampus were immediately frozen in liquid nitrogen and kept at –70°C until assayed. The dopamine, norepinephrine and acetylcholine concentrations were measured by ELISA obtained from Abnova. BDNF was detected by ELISA technique according to the method of Barakat-Walter (26) The assay is based on monoclonal antibody specific for BDNF precoated onto a microplate. When the standard and samples are pipetted into the wells, any BDNF present is bound by the immobilized antibody. Then, the enzyme-linked monoclonal antibody specific for BDNF is added to the wells and, following a wash to remove any unbound antibody enzyme, a substrate solution are added to the wells. The color develops in proportion to the amount of BDNF bound in the initial step. The color development is stopped and the intensity of the color can be measured at 450 nm.

RNA isolation and cDNA synthesis

Total RNA was extracted from hippocampus homogenate using GStruct™ RNA isolation kit II (SA-40005, Maxim BioTech, Inc. San Francisco, USA) guanidium thiocyanate method. The purity and concentration of RNA were quantified

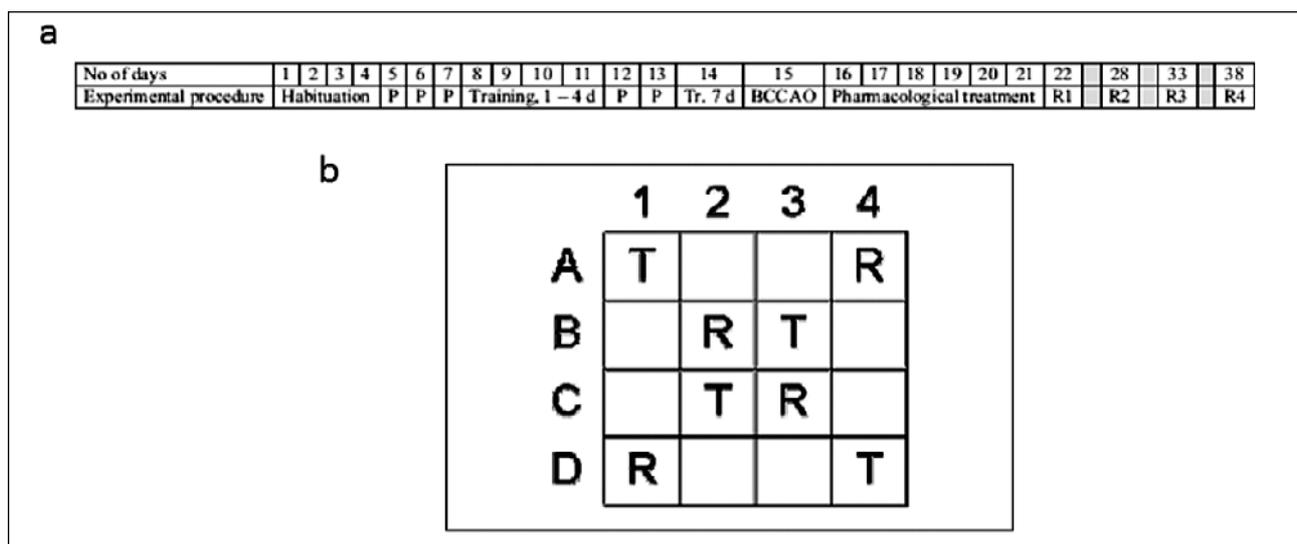


Fig. 1. (a): The experimental protocol for psychometric test. P - pause, Tr - training, d - day, R - retest; see text for details. (b): Testing area of holeboard apparatus - position of baited holes during training phase (T) and retests (R). Modified after Lannert and Hoyer, 1998.

by spectrophotometry. Reverse transcription reaction was performed using oligo (dT) primers (USA). The 25 μ L cDNA synthesis reaction consisted of 2.5 μ L (5 \times) buffer with $MgCl_2$, 2.5 μ L (2.5 mM) dNTPs (Pharmacia Biotech), 1 μ L 10 pmol Oligo d(T) primer (Pharmacia Biotech), 2.5 μ L RNA (2 mg/ml) and 0.5 unit reverse transcriptase enzyme (Qiagen, US). The mixture was incubated at 37°C for 1 hour. PCR amplification was performed in a thermal cycler (Applied Biosystems (ABI), USA) programmed at 42°C for 1 hour, 72°C for 10 min (enzyme inactivation) and the product was stored at 4°C until used.

Real time PCR and quantitative estimation of BDNF R1 mRNA

For qRT-PCR, a set of primers: Forward 5'-AGTGATGACCATCCTTTTCCTTAC3'. Reverse 5'-CCTCAAATGTGCATCCAAGGA -3_ (Invitrogen Life Technologies) were designed from the published cDNA sequences of the rat adiponectin gene which amplified a 89 bp product. The reaction was carried out using Rotor-Gene6000system (Qiagen, USA) and consisted of 12.5 μ L of 2X QuantitechSYBR®.

Green RT Mix (Fermentas, Germany), 1.0 μ L of 25 pm/ μ L adiponectin primers, 2 μ L cDNA (100 ng) and 9.25 μ L of RNase free water. Samples were spun well before loading in the Rotor's wells. The real time PCR program was performed as follows: initial denaturation at 95°C for 10 min.; 40 cycles of 95°C for 15 s.; annealing at 60°C for 30 s and extension at 72°C for 30 s. for 35 cycles and final extension 10 min. As a negative control, 2L of the RT-PCR product synthesized in the absence of avian myeloblastosis virus (AMV)-transcriptase was used as a template. PCR products were electrophoresed on a 1.3% agarose gel stained with ethidium bromide and visualized *via* light UV Transilluminator (Model TUV-20, OWI. Scientific, Inc. 800 242-5560, France) and photographed under fixed conditions (the distance, the light and the zoom). The results photos were analysed with scion image® release Alpha 4.0.3.2. software for windows 8 which performs bands detection and conversion to peaks. Area under each peak was calculated in square pixels and used for quantification. Gene expression levels were determined by calculating the ratio between the square pixel value of the target gene in relation to the internal housekeeping control gene (B-actin). Minus RT controls permitted to rule out genomic contamination. Similarly, no products were detected when the RT-PCR step was carried out with no added RNA, indicating that all reagents were free target sequence contamination.

Histological examination

The brain tissue was fixed in 10% formalin for one week, washed in running tap water for 24 hour and dehydrated in an ascending series of ethanol (50–90%), followed by absolute alcohol. The samples were cleared in xylene and immersed in a mixture of xylene and paraffin at 60°C. The tissue was then transferred to pure paraffin wax of the melting point 58°C and then mounted in blocks and left at 4°C. The paraffin blocks were sectioned on a microtome at a thickness of 5 μ m and mounted on clean glass slides and left in the oven at 40°C to dryness. The slides were deparaffinized in xylene and then immersed in descending series of ethanol (90–50%). The ordinary haematoxylin and eosin stain were used to stain the slides.

Transmission electron microscope

Small pieces of the hippocampus were cut into 2–3 mm³ and immediately fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 at 4°C for 2–3 hour. Specimens were post fixed in 1% osmium tetroxide in sodium cacodylate buffer,

dehydrated in an ascending series of ethanol and embedded in Spurr's resin. Semithin sections were stained with toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate. Three to five random micrographs for each thin section were examined with a Jeol 1011 TEM at 80 KV, micrographs were taken and analyzed carefully for fine structural changes.

Statistical analysis

The results were expressed as means \pm S.D. Statistical analysis was performed by using GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA). The efficacy of training in the holeboard apparatus was assessed by the Two-way ANOVA, followed by the Tukey Multiple Comparison Test. The efficacy of behavior after BCCAO in the holeboard apparatus was assessed by the Two-way ANOVA, followed by the Tukey Multiple Comparison Test. The central level of acetylcholine, norepinephrine and dopamine were analyzed by One-way ANOVA, followed by the Tukey Multiple Comparison Test. P value of 0.05 or less was considered significant.

RESULTS

Effect of DHEA and donepezil treatment on the central level of acetylcholine, norepinephrine and dopamine

Fig. 2a-2c showed that bilateral common carotid artery occlusion (BCCAO) significantly ($p < 0.05$) reduced the central level of acetylcholine, norepinephrine and dopamine as compared with the control group. While DHEA treatment after BCCAO produced a significant increase ($p < 0.05$) in the central level of acetylcholine, norepinephrine and dopamine as compared with the rats exposed to BCCAO. Also, donepezil after BCCAO treatment increased significantly ($p < 0.05$) the level of the above-mentioned chemical transmitters as compared with the rats exposed to BCCAO. However donepezil in comparison to DHEA treatment after BCCAO increased central level of acetylcholine significantly ($p < 0.05$) and decreased norepinephrine significantly ($p < 0.05$).

Effect of DHEA and donepezil treatment on time in holeboard memory test

Fig. 3A showed that the time needed to collect the pellets from the holes of the holeboard memory showed a significant decrease ($p < 0.05$) on the 7th day as compared with the training sessions of the 1st, 2nd, 3rd and 4th days in the four experimental groups. On the R1 the time increased significantly ($p < 0.05$) after BCCAO as compared with the control rats. DHEA treatment and donepezil treatment significantly ($p < 0.05$) reduced the time needed to collect the pellets as compared with the non-treated BCCAO rats on R1, R2, R3 and R4. Although, time needed to collect the food pellets in both groups is reduced but still significantly ($p < 0.05$) higher than the control rats. F (degree of freedom) for interaction value was 201.6.

Effect of DHEA and donepezil treatment on the latency in holeboard memory test

Fig. 3B showed that the time to visit the first hole (latency) is significantly shortened ($p < 0.05$) in the four groups with the best time on the 4th and 7th days. On R1 the latency in the BCCAO was significantly increased ($p < 0.05$) as compared with the control rats. DHEA treated rats showed a significant reduction in latency on R1, R2 and R3 as compared with donepezil treatment and BCCAO rats. On R4 the latency was

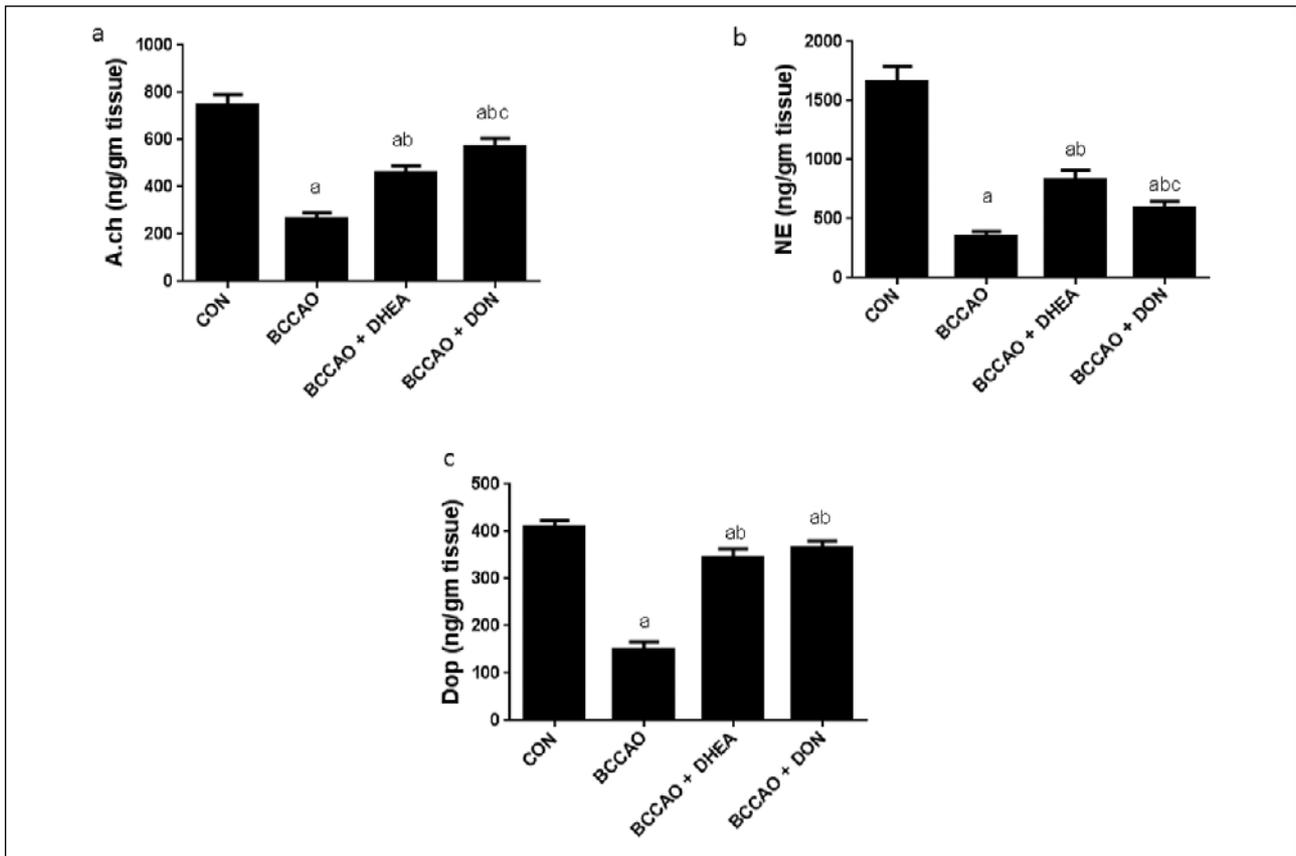


Fig. 2. The central level of acetylcholine, norepinephrine and dopamine (ng/ mg tissue) in the hippocampus. All data were expressed as mean \pm S.D., (n=12). ^a $p < 0.05$ as compared with the control group; ^b $p < 0.05$ as compared with the BCCAO non treated group; ^c $p < 0.05$ as compared with the BCCAO + DHEA treated group.

significantly reduced ($p < 0.05$) in DHEA and donepezil post BCCAO as compared with BCCAO rats, but still significantly higher than the control rats. F (degree of freedom) for interaction value was 86.11.

Effect of DHEA and donepezil treatment on the working memory (calculated as the number of food rewarded visits divided by the number of visits and revisits to the baited set of holes) in holeboard memory test

Fig. 3C showed that the working memory increased significantly ($p < 0.05$) in the four groups on 7th days as compared with results on the previous days. Post BCCAO the working memory significantly decreased in R1 as compared with the control group. Treatment with DHEA and donepezil produced a significant ($p < 0.05$) increase in working memory on R1, R2, R3 and R4 as compared with BCCAO non-treated rats. The working memory was insignificantly ($p > 0.05$) changed in donepezil treatment when compared with DHEA treated rats. F (degree of freedom) for interaction value was 101.7.

Effect of DHEA and donepezil treatment on the reference memory (calculated as the number of visits and revisits to the baited set of holes divided by the number of visits and revisits to all holes) in holeboard memory test

Fig. 3D showed that the reference memory increased significantly ($p < 0.05$) in the four groups on 7th days as compared with results on the previous days. Post BCCAO the reference

memory significantly decreased in R1 as compared to the control group. Treatment with DHEA and donepezil produced a significant ($p < 0.05$) increase in reference memory on R1, R2, R3 and R4 as compared with BCCAO nontreated rats. The reference memory was insignificantly ($p > 0.05$) changed in donepezil treatment when compared with DHEA treated rats. F (degree of freedom) for interaction value was 284.3.

Effect of DHEA and donepezil treatment on the brain derived neurotrophic factor (BDNF) expression and BDNF protein in the hippocampus

Fig. 4a and 4b showed that the BDNF expression in the hippocampus was significantly ($p < 0.05$) reduced in response to BCCAO as compared to the control rats. DHEA treatment and donepezil treatment significantly ($p < 0.05$) increased the expression of the hippocampal BDNF expression as compared with non-treated BCCAO rats. Fig. 4c showed that BCCAO resulted in significant ($p < 0.05$) decrease in BDNF content in the hippocampus as compared with the control group. DHEA and donepezil significantly increased significantly ($p < 0.05$) the hippocampus BDNF as compared with BCCAO, but still significantly ($p < 0.05$) lower than the control rats.

Correlation between brain derived neurotrophic factor expression and central acetylcholine level

There was a positive correlation between BDNF expression and central acetylcholine level in the hippocampus ($r = 0.8934$) and ($p < 0.0001$) (Fig. 4).

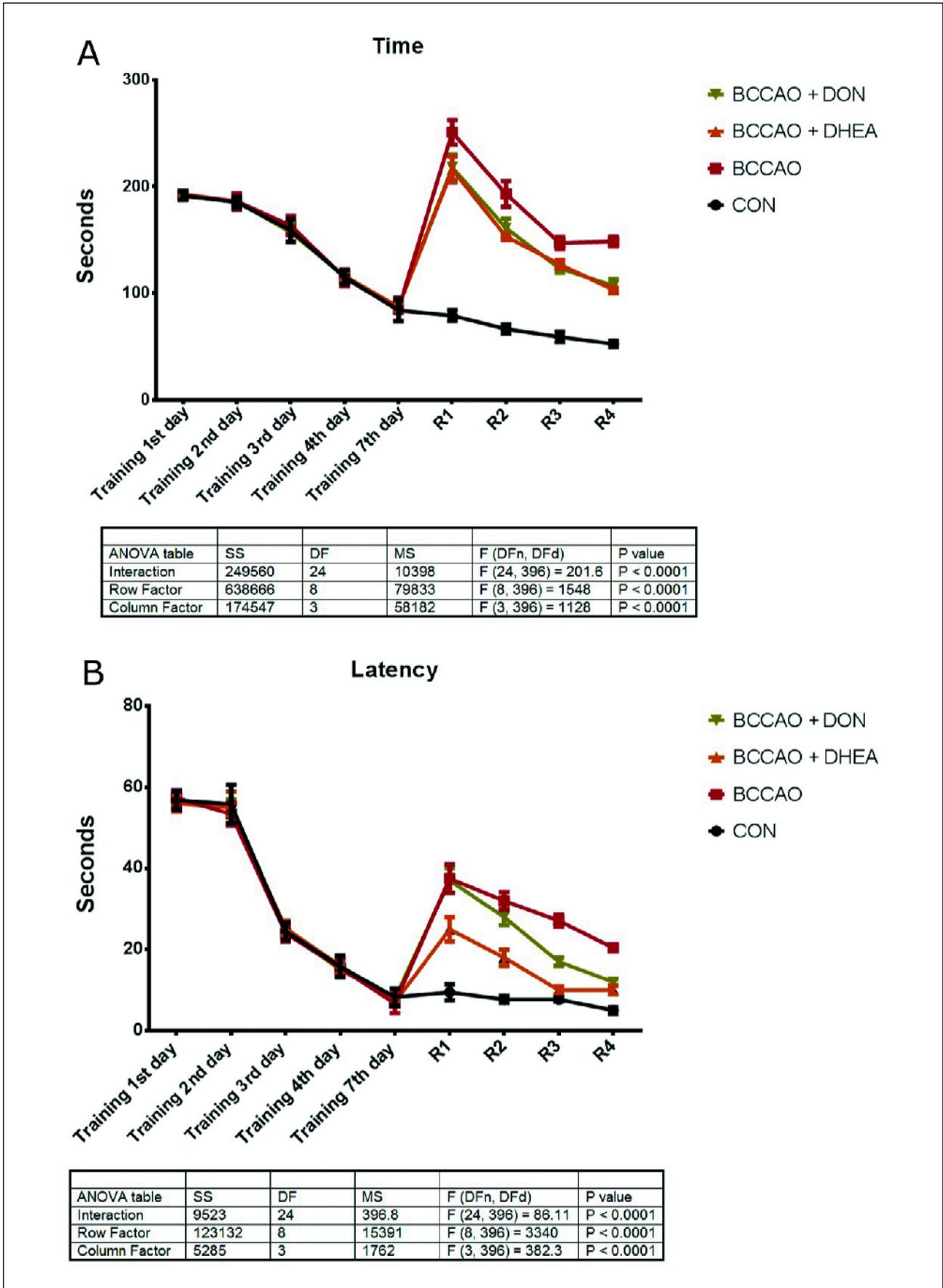


Fig. 3. (A): Time 5 min or less (the time needed to collect all food pellets) in the experimental groups on the 1st, 2nd, 4th, 7th, R1, R2, R3 and R4 days of training and retest. (B): Latency: the time that elapse between trial start and visit to the first hole parameter in the holeboard memory test in the experimental groups on the 1st, 2nd, 4th, 7th, R1, R2, R3 and R4 days of training and retest.

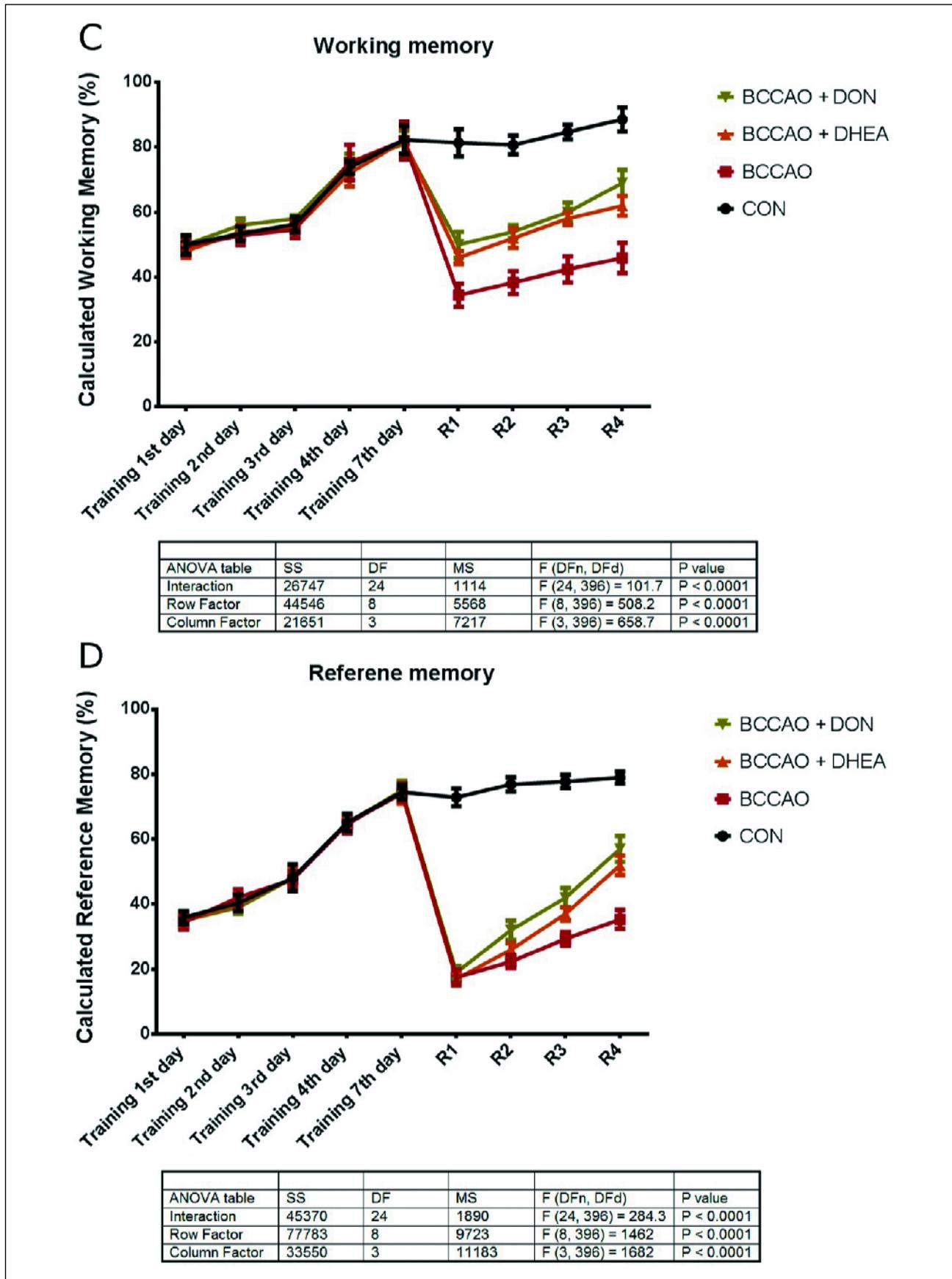


Fig. 3. (C): Calculated working memory number of food rewarded visits divided by number of visits and revisits to the baited set of holes '100 in the experimental groups on the 1st, 2nd, 4th, 7th, R1, R2, R3 and R4 days of training and retest. *(D):* Calculated reference memory number of visits and revisits to the baited set of holes divided by number of visits and revisits to all holes '100 in the experimental groups on the 1st, 2nd, 4th, 7th, R1, R2, R3 and R4 days of training and retest.

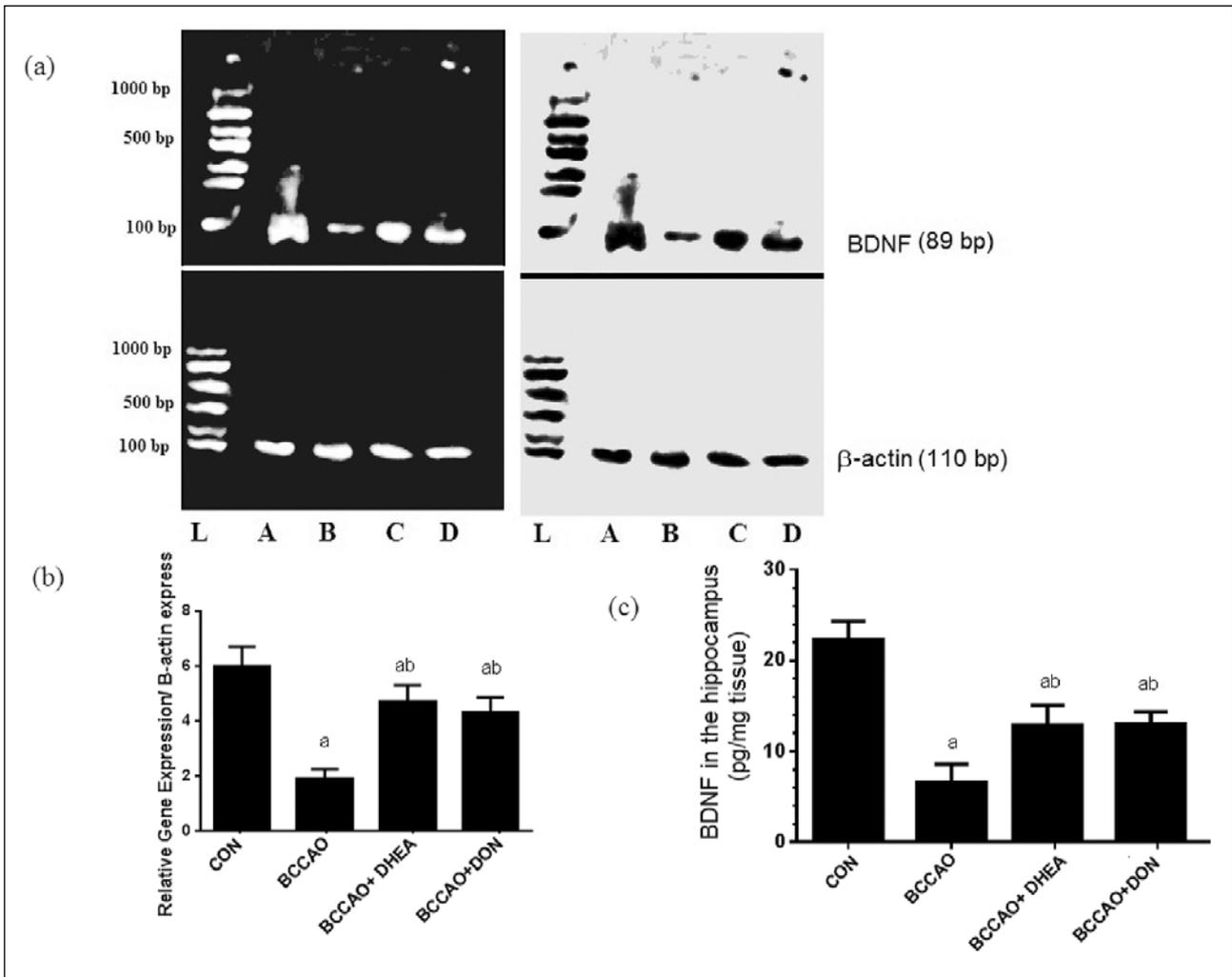


Fig. 4. (a): Semiquantitative reverse transcriptase PCR products of hippocampal mRNA of BDNF and β -actin (housekeeping gene). The RT-PCR products, obtained from all groups was separated by 2% agarose gel electrophoresis containing 100 ng/ml ethidium bromide. L - Ladder, A - control group, B - BCCAO non treated rats, C - BCCAO + DHEA treatment, D - BCCAO + donepezil treatment, $p < 0.05$ as compared with the control group. (b): relative BDNF expression to housekeeping (β -actin) gene expression in the hippocampus, $p < 0.05$ as compared with the BCCAO non treated group. (c): BDNF protein level in the hippocampus, $p < 0.05$ as compared with the BCCAO non treated group.

All data were expressed as mean \pm S.D. (n=12). ^a $p < 0.05$ as compared with the control group; ^b $p < 0.05$ as compared with the BCCAO non treated group; ^c $p < 0.05$ as compared with the BCCAO + DHEA treated group.

Results of histopathological examination

1. Haematoxylin and eosin staining:

Fig. 6 showed histopathological findings of the cerebral cortices of the control and experimental rats (original magnification $\times 400$). Fig. 6A section from a control rat (group A) showed intact neurons and well-preserved cell density. Neuronal cells showed vesicular nuclei with prominent nucleoli. Neutrophils and neuroglial cells are numerous in comparison with nerve cells. Fig. 6B section from the BCCAO rats showed neuronal loss with ischemic changes (cytoplasmic eosinophilia, pyknotic nuclei, pancellular necrosis, indicative of cerebral infarction) and severe neuropilar microvacuolation. Fig. 6C BCCAO + DHEA treated rats showed more or less intact neurons with intact chromatin. Fig. 6D BCCAO + DON treated rats showed the same ischemic changes but less than the BCCAO rats.

2. Transmission electron microscope

The observed ultrastructure of the control rat hippocampus (Fig. 7a) showed myelinated (M) and unmyelinated (U) neurons with normal features. Thick myelin is also formed. BCCAO rats showed that there are many vacuoles on the myelin sheath of axons with loss of myelin from some axons. Axonal shrinkage is seen in some axons. Some nerve cells showed axonal degeneration (absence of myelin). Multiple vacuoles are seen in the neuropils. (Fig. 7b). Treated rats with DHEA and DON after BCCAO showed the same changes but less than the BCCAO rats with preservation of the myelin with less vacuoles (Fig. 7c and 7d).

DISCUSSION

The present study demonstrated that DHEA treatment resulted in: (i) significant increase in working and reference

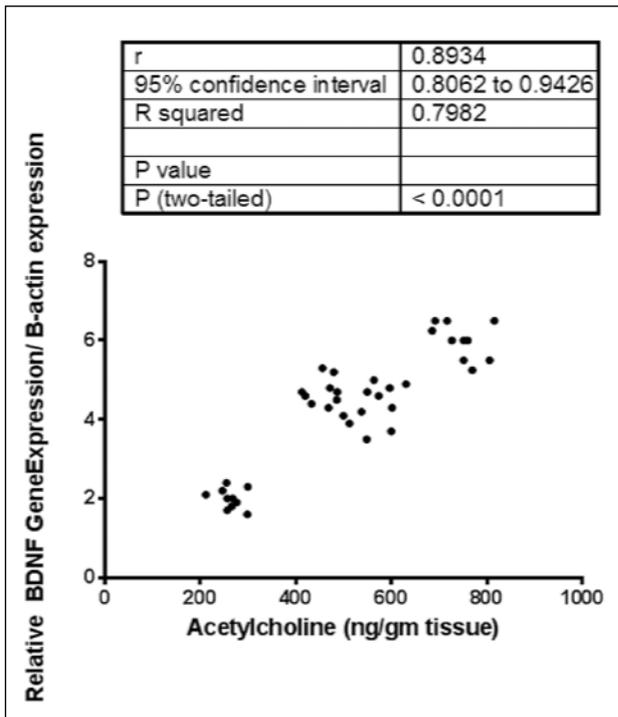


Fig. 5. Positive correlation between BDNF expression in the hippocampus and central acetylcholine level.

memories in the rat model of vascular dementia, (ii) significant increase in the levels of acetylcholine, norepinephrine and dopamine and (iii) significant increase in the hippocampal expression of BDNF. To the best of our knowledge, this is the first study that addressed the effect of DHEA on BDNF expression in a rat model of vascular dementia that is induced through BCCAO.

In the present study, we had been using bilateral common carotid artery occlusion to produce a rat model of vascular dementia. Permanent, bilateral occlusion of the common carotid arteries (2VO) in rats has been documented as a procedure to study the effects of chronic cerebral hypoperfusion on cognitive dysfunction and neurodegenerative processes. The hypothesis that chronic cerebral hypoperfusion contributes to the progression of dementia was proposed long ago (2, 27-29) Thus, it has been firmly established that experimental cerebral hypoperfusion compromises spatial learning in rats. The holeboard memory test was previously used by Stasiak *et al.* who assessed the working and reference memory in a rat model of vascular dementia (30).

In the current research we used the DHEA (dissolved in 5% DMSO in saline) orally in a dose of 250 mg/kg body weight/day. This dose was previously used by Aly *et al.* (31) to explore the neuroprotective effects of DHEA in a rat model of dementia that was induced by intraperitoneal administration of AICl. Also, we used donepezil 3 mg/kg/ day; i.p. as described by McLean *et al.*

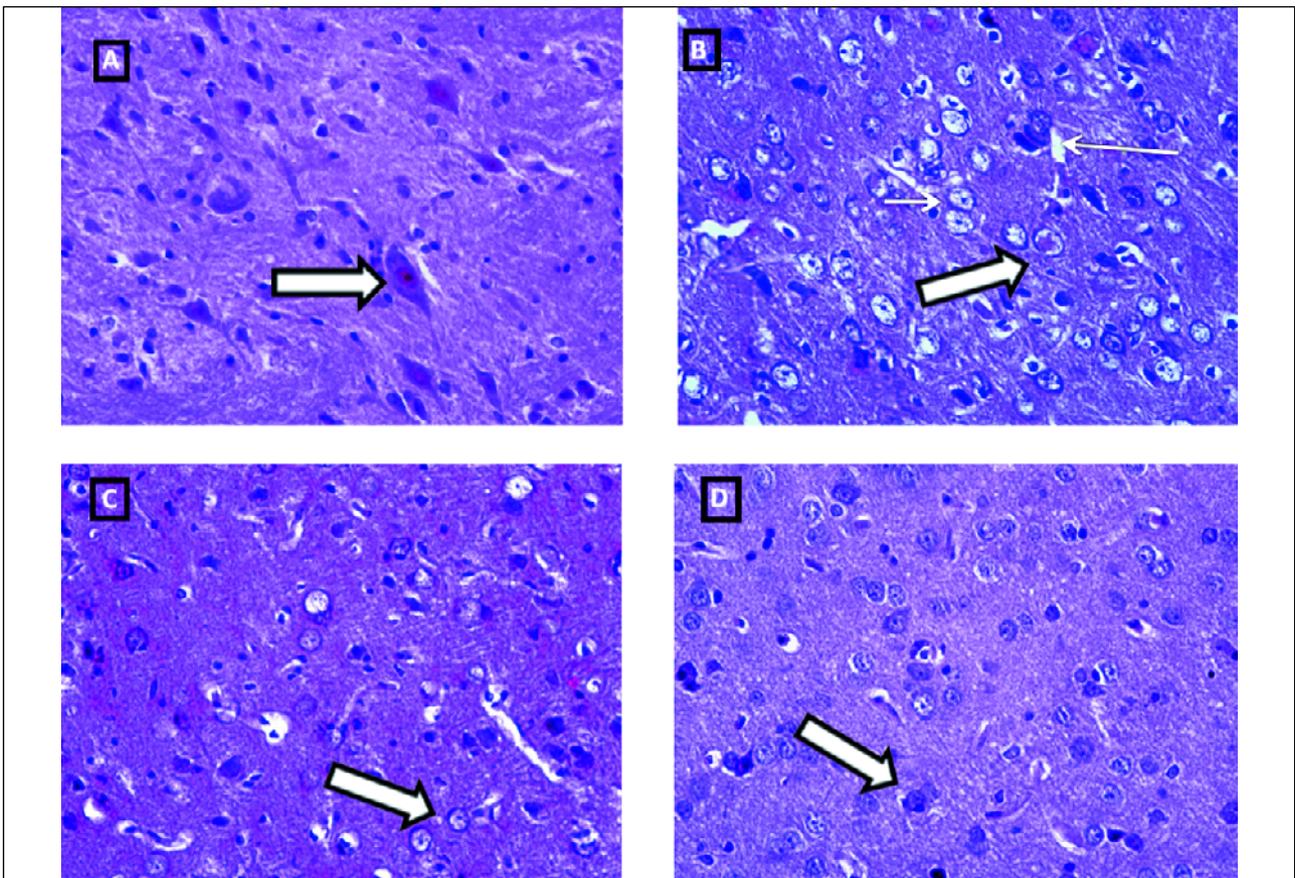


Fig. 6. Histopathological findings of the cerebral cortices of the control and experimental rats (H and E, original magnification $\times 400$). (A): section from a control rat (group A) shows intact neurons and well-preserved cell density. (B): section from the BCCAO rats shows neuronal loss with ischemic changes (cytoplasmic eosinophilia, pyknotic nuclei, pancellular necrosis, indicative of cerebral infarction and severe neuropilar microvacuolation). (C): BCCAO + DHEA treated rats shows more or less intact neurons with intact chromatin. (D): 6D. BCCAO + DON treated rats shows the same ischemic changes but less than the BCCAO rats.

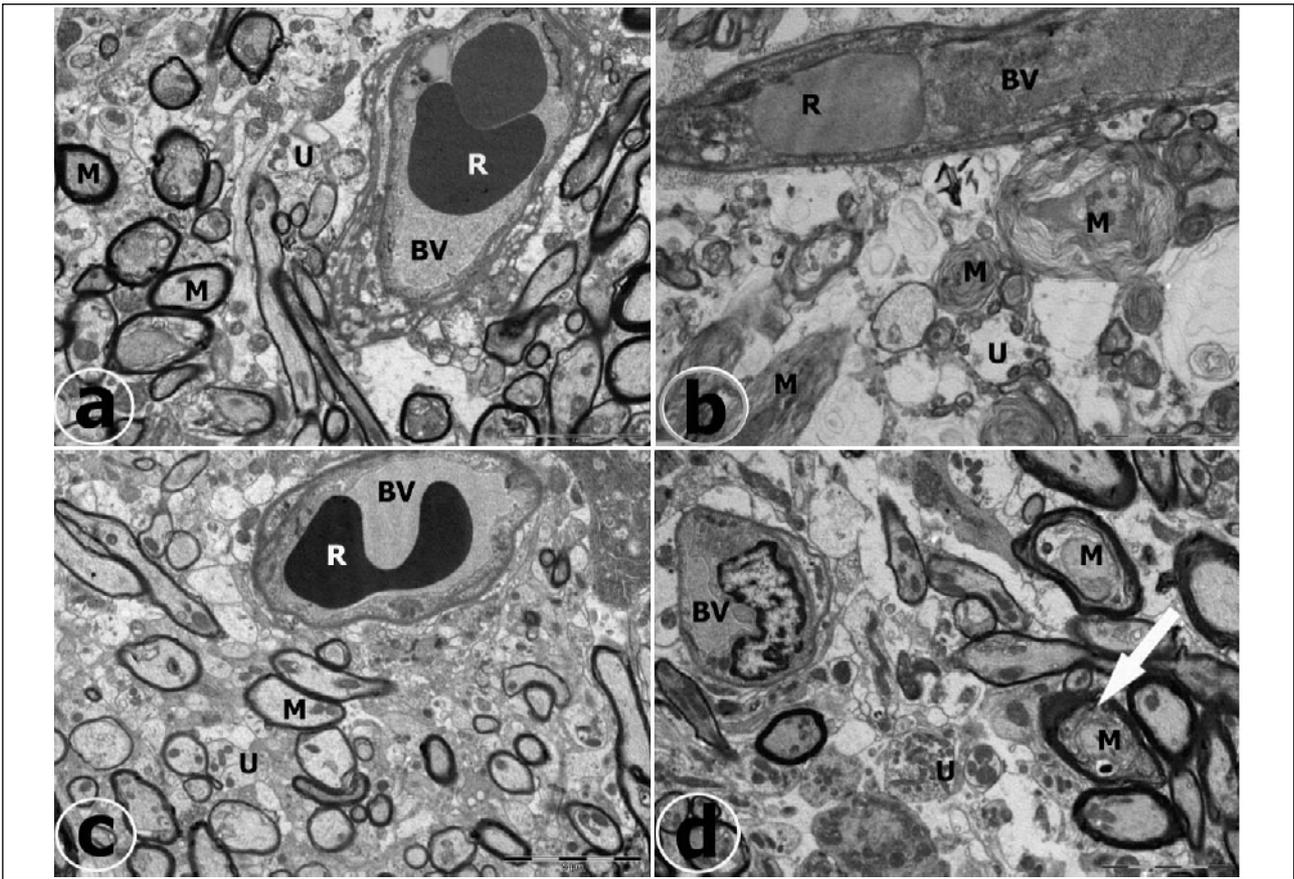


Fig. 7. Transmission electron microscope of the hippocampus. The observed ultrastructure of the control rat hippocampus. (a): shows myelinated (M) and unmyelinated (U) neurons with normal features. BCCAO rats shows that there are many vacuoles on the myelin sheath of axons with loss of myelin from some axons. Axonal shrinkage is seen in some axons (b): (c): and (d): treated rats with DHEA and DON after BCCAO shows the same changes but less than the BCCAO rats.

who concluded that donepezil improves cognition in two cognitive domains and in two distinct models of cognitive impairment in female rats (32).

Our results showed that BCCAO produced a significant decreased the central level of acetylcholine, norepinephrine and dopamine in the hippocampus as compared with the control group. These data were in accord with previous studies that reported that cognition deficits and memory loss after BCCAO may be partly due to cholinergic dysfunction in the brain. These results were consistent with previous studies (33). These neurochemical data indicated that BCCAO leads to progressive and time-dependent neuronal damage, cellular necrosis and loss of myelin from myelinated neurons as detected from histopathological examination (*Figs. 6B and 7b*). On the other hand, treatment with DHEA increased the central level of the above-mentioned neurotransmitters. Our results were in agreement with previous studies that showed that DHEA administration produced a significant decrease in the brain acetylcholine esterase activity associated with a significant increase in the brain acetylcholine level in AI-intoxicated ovariectomized rats (31). It has been demonstrated that DHEA significantly increases the acetylcholine release in the hippocampus (34). Thus, the promoting effect of DHEA on acetylcholine release in the hippocampus may be one mechanism for its memory enhancing effect (35). Regarding the central level of noradrenaline and dopamine, they increased significantly in response to DHEA treatment. This could be attributed by the antioxidant effects of DHEA that protects the

hippocampal neuronal cells from oxidative stress induced damage caused by different factors as hypoxia and ischemia (36). This was in parallel with the histopathological findings in *Figs. 6C and 7c*.

Furthermore, donepezil treatment post BCCAO increased the central level of acetylcholine considerably as compared with DHEA, while the level of noradrenaline was significantly ($p < 0.05$) reduced as compared with DHEA treated rats. Dopamine was insignificantly changed in the donepezil treated versus the DHEA treated. Donepezil is one of the three currently approved acetylcholine esterase inhibitors for treating AD symptoms delaying the decline in cognitive function (37).

Prior the BCCAO, in order to eliminate other causes of random memory disorders, rats underwent an initial holeboard memory test, according to Lanert and Hoyer (25). Only the animals, which positively passed this first behavioral evaluation, were entered into further experiments. As it is shown in *Figs. 3-6* days training on the holeboard apparatus significantly increased short- and long-term memory, designated respectively as working (WM) and reference (RM) memory, and decreased time parameters, i.e. the total time and latency. The results obtained on the 7th day allowed us to choose "intelligent" rats with a large capacity for learning. As measured by subsequent retests, BCCAO resulted in a dramatic increase in time parameters and decline of memory function (*Fig. 3A-3D*). These disturbances may be in part a result of brain ischemia and neuronal degeneration caused by surgery.

Additionally, from *Fig. 3A-3D* the time and latency were significantly shortened in DHEA, and donepezil treated rats with significant improvement in the both working and reference memories as compared with BCCAO rats. It is well established, that cerebral cholinergic transmission plays a leading role in the modulation of cognitive processes. This could be attributed by the elevation of the central neurotransmitters in the hippocampus of the treated rats. DHEA actions are mediated in part *via* activation of androgen receptors (AR), which are localized in many brain areas, including regions important for learning and memory such as the hippocampus and amygdala. Advantageous actions of androgens in the brain include stimulation of neuronal differentiation, maintenance of neuronal morphology density and promotion of synaptic density (38). Also, *in vitro* studies suggested that excitatory neurosteroids DHEA, DHEAS at physiological concentrations participate in the inhibition of cortical neuronal degeneration elicited by staurosporine and glutamate (39). For example, studies of the hippocampus in male rats show a significant decrease in the density of spine synapses following gonadectomy (GDX), an effect reversed by replacement with either testosterone or DHT (40).

Brain-derived neurotrophic factor (BDNF), broadly and abundantly expressed in the mammalian brain, is a member of the neurotrophin family. Stress or corticosterone administration reduces BDNF mRNA expression in the brain (41, 42). BDNF also represents a crucial signaling molecule in adaptive brain plasticity after stroke (43-45). Interventions that improve recovery of function are most often associated with increased BDNF levels in perilesional areas (34). Conversely, attenuating BDNF levels or its effects following cerebral ischemia reduces neuroplastic changes or recovery of function either spontaneous or induced by rehabilitation (46-48). Several factors affected the BDNF expression like exercise that induces enhancement of the BDNF expression and release in the brain as well as in the peripheral tissues (49). While stress-induced modifications of basal glutamatergic transmission and synaptic plasticity (50).

Interestingly, the BDNF expression and protein content were significantly reduced in BCCAO group as compared to the control group. Ischemia as well as hypoxia affected the expression of BDNF in the rat cortex. In agreement with our results Tian *et al.*, (49) concluded that BDNF expression decreased in response to prolonged hypoxia in the cerebral cortex. Furthermore, the blockade of endogenous BDNF activity exacerbates the effects of cerebral ischemia (51, 52). In contrary to our results, Bejot *et al.*, (53) reported that BDNF expression increased significantly after ischemic stroke. In addition, they determined that non neuronal cells could produce substantial amounts of BDNF after ischemic stroke.

On the other hand DHEA and donepezil treatment significantly increased BDNF expression and protein content in the hippocampus after BCCAO. Several previous studies investigated the effects of DHEA on BDNF expression. A Single i.p. injections of either DHEA (25 mg/kg) or DHEAS (50 mg/kg) into adult male Sprague-Dawley rats changed regional brain concentrations of BDNF during the 300 minutes of the experiment (54). Rats that received DHEA had decreased BDNF content in the hippocampus, no change in BDNF content in the amygdala, and increased BDNF in the hypothalamus compared to sham rats that received sesame oil. Rats that received DHEAS had decreased BDNF 30 minutes after injection and increased BDNF 180 minutes after injection in the hippocampus, biphasic increases in BDNF in the amygdala, and decreased BDNF in the hypothalamus (54). Further, previous data showed that DHEA and DHEAS have different effects and suggest that they may operate through different mechanisms. Important actions of DHEA in the central nervous system (CNS) were initially inferred from observations that DHEA and DHEAS were

synthesized *de novo* in the brain. Also, the brain concentrations were higher than plasma concentrations and brain concentrations remained high after adrenalectomy and gonadectomy of rats (55).

Indeed, they have been termed “neurosteroids” for this reason (56, 57). DHEA and DHEAS were among the first neurosteroids identified in rat brains (58). DHEA(S) promotes neurogenesis and neuronal survival. Male Lister hooded rats implanted with 200–250 mg DHEA pellets had increased neurogenesis in the dentate gyrus compared to animals who received paraffin pellets (59). A possible mechanism by which DHEA(S) could promote neurogenesis and neuronal survival is by affecting concentrations of brain-derived neurotrophic factor (BDNF), a member of the nerve growth factor family that plays a role in central nervous system development and plasticity. Furthermore, BDNF expression could be related to acetylcholine esterase inhibition. Inhibition of acetylcholine esterase led to increased BDNF expression (60). Moreover, our results showed that donepezil treatment increased BDNF expression that is in agreement with the previous study by Leyhe *et al.* (60) Donepezil is well identified to produce neurogenesis in the adult hippocampus neurons (61). This was in accord with our results that showed a positive correlation between BDNF expression and central acetylcholine level in the hippocampus.

Conclusion

Surgical bilateral common carotid artery in rats impaired the working and reference memories, decreased the central level of acetylcholine, norepinephrine and dopamine as well as suppressed the expression of BDNF in the rat hippocampus. Treatment of the rat model of vascular dementia by DHEA improved the memory functions, increased the central level of acetylcholine and catecholamine and upregulated the expression of BDNF. Despite many of the experimental data suggest a beneficial effect for DHEA supplementation to produce protective effects in an animal model of vascular dementia and Alzheimer’s disease, clinical trials in vascular dementia patients are still required to further validate many of the laboratory findings. The use of DHEA as a possible alternative treatment still requires further investigation, especially as part of a treatment protocol in clinical trials.

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