Neuropathological Changes in Brain Cortex and Hippocampus in a Rat Model of Alzheimer’s Disease

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ABSTRACT

Background: Alzheimer’s disease (AD) is a neurodegenerative disorder with progressive loss of cognitive abilities and memory loss. The aim of this study was to compare neuropathological changes in hippocampus and brain cortex in a rat model of AD.

Methods: Adult male Albino Wistar rats (weighing 250-300 g) were used for behavioral and histopathological studies. The rats were randomly assigned to three groups: control, sham and β-amyloid (Aβ) injection. For behavioral analysis, Y-maze and shuttle box were used, respectively at 14 and 16 days post-lesion. For histological studies, Nissl, modified Bielschowsky and modified Congo red staining were performed. The lesion was induced by injection of 4 µL of Aβ(1-40) into the hippocampal fissure.

Results: In the present study, Aβ(1-40) injection into hippocampus could decrease the behavioral indexes and the number of CA1 neurons in hippocampus. Aβ injection CA1 caused Aβ deposition in the hippocampus and less than in cortex. We observed the loss of neurons in the hippocampus and cerebral cortex and certain subcortical regions. Y-maze test and single-trial passive avoidance test showed reduced memory retention in AD group.

Conclusion: We found a significant decreased acquisition of passive avoidance and alternation behavior responses in AD group compared to control and sham group (P<0.0001). Compacted amyloid cores were present in the cerebral cortex, hippocampus and white matter, whereas, scattered amyloid cores were seen in cortex and hippocampus of AD group. Also, reduced neuronal density was indicated in AD group. Iran. Biomed. J. 15 (1 & 2): 51-58, 2011

Keywords: Alzheimer's disease (AD), Hippocampus, β-amyloid (Aβ), Neuropathological changes

INTRODUCTION

Alzheimer’s disease (AD) is a complex, chronic and devastating disease affecting a high percentage of the population over 65 years of age [1]. Oxidative stress may underlie the progressive neurodegeneration characteristic of AD [2]. The neurodegenerative process in AD may involve β-amyloid (Aβ) toxicity. Neurotoxicity of Aβ can be demonstrated in vitro, and appears to involve oxidative stress [3]. Aging, the major risk factor for AD [4], leads to loss of free radical scavenging ability by endogenous mechanisms [5]. Free radical-mediated damage to neuronal membrane components also is implicated in aging, as well as the etiology of AD [6]. Most cases occur sporadically, although a small proportion is inherited as an autosomal dominant disorder. Several gene loci are involved in familial cases, including the amyloid precursor protein gene on chromosome 21, the presenilin-1 gene on chromosome 14 and the presenilin-2 gene on chromosome 1. There is an increased incidence of sporadic Alzheimer's disease in individuals with ApoE e4 genotype on chromosome 19. Females are affected almost twice as frequently as males [7].

AD is associated with cholinergic neuronal loss [8] and characterized by deficits in memory and
cognition that are associated with significant losses of presynaptic cholinergic function in the brain, particularly the nucleus basalis [9, 10]. The major microscopic abnormalities of AD, which form the basis of the histologic diagnosis, are neuritic plaques and neuro-fibrillary tangles. There is progressive and eventually severe neuronal loss and reactive gliosis in the same regions that bear the burden of plaques and tangles. Neuritic plaques are focal, spherical collections of diluted, tortuous, neuritic processes often around a central amyloid core, which may be surrounded by a clear halo [11].

Aβ has been shown to provoke neuronal death, decreased synaptic plasticity, aberrant sprouting of growing axons, chronic inflammation and hyperphosphorylation of tau [12]. Deposition of Aβ is an early and critical event in the pathogenesis of AD [13], first forming in temporal cortical regions including the hippocampus [14], a region implicated in memory formation [15]. It was proposed that Aβ aggregates to form neurotoxic plaques, which lead to neurodegeneration accompanied by dementia. The present experiment was to evaluate the effects of injection of Aβ intrahippocampal on memory tasks in adult male rats measured by behavioral tests (Y-maze and passive avoidance) and histopathological examination. Inhibitory avoidance (also called passive avoidance) is acquired in only one trial, where the subject is punished for doing something 52 Nobakht et al. Iran. Biomed. J., January & April 2011

MATERIALS AND METHODS

Animals. Thirty adult male Albino Wistar rats (weighing 250-300 g, 8-10 weeks old), obtained from the Animal Center of Iran Medical College, were used in this experiment. The rats were randomly assigned to three groups with ten animals in each group: A) Control group age-matched rat, B) Sham-lesioned group, injected with deionized water and C) Experimental-lesioned group, injected with Aβ (1-40). All animal procedures were performed in accordance with the Guide to the Iranian Council for Use and Care of Animals and were approved by the Animal Research Ethical Committee of Tehran University of Medical sciences.

Animal surgery. Synthetic Aβ-40 amyloid protein (Sigma, CA, USA) was dissolved in deionized water at a concentration of 2 mmol/μl and then aliquot and stored at -70°C before use. Rats were anesthetized with intraperitoneal injection of ketamine and xylazine (60 and 20 mg/kg body weight, respectively, Alfasan, Woerden, Holland). Animals were placed in the stereotaxic frames (Stoelting Co. USA), the skull exposed and disinfected with betadine. An incision was made into the scalp and the cranium drilled to a depth of 2.6 mm. The place was localized at 2.0 mm medial-lateral and 3.8 mm posterior-anterior to bregma, following by Paxinous and Watson atlas (1986). In AD group, 4 µl solution of Aβ40 amyloid protein was bilaterally injected into each hippocampus with a 10 μl Hamilton syringe over a period of 12 min. The infusion was made over 10 minutes, retained 2 minutes, and retarded 2 minutes to allow for complete diffusion of drug [1]. The needle was slowly withdrawn after injection. Sham group underwent the same procedures except that only deionized water was injected.

Y-maze task. Y-maze test was initiated 14 day after Aβ injection. All testing was carried out from 3 p.m. to 6 p.m. The Y-maze was a Y-shaped plexiglas holding cage with a 40 cm length, 30 cm height and 15 cm width. Rats were allowed to freely discover the maze for 8 min observation period. Air circulation equipment in continuous operation provided masking noise of 40 dB. Alternation was defined as successive entries into the three arms on overlapping triplet sets. The alternation percentage was calculated as the ratio of actual to possible alternations (defined as the total number of arm entries minus two) [19].

Single-trial passive avoidance test. This test was performed 16 days after surgery. The apparatus (Behin Paya Tajhiz Co., Tehran) consisted of light

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and dark compartments of equal size connected by a small central guillotine door. Electric shocks were given to the rats in the dark compartment with the grid floor. All rats were adapted to the apparatus on the first and second days of testing for 5 min. On the third day, the rats individually entered the light compartment and remained there for 2 min, then the guillotine door was opened and the rat entered the dark compartment and received a 2 s, 1 mA electric footshock. For retention, 24 hours later, each rat was again placed in the light compartment. The latency to step through the dark compartment (maximum 600 s) was measured and recorded as the index for the passive avoidance behavior. The behavioral observations were carried out between 12:00 and 15:00 [19].

**Histological procedure.** After the behavioral test, animals were killed 12 weeks after surgery and brains were removed and dissected into hippocampal and cortex blocks. After the behavioral test, all rats were anesthetized with ketamine and xylazine and perfused with 4% paraformaldehyde in 0.1 mol/L phosphate buffer solution (pH 7.4). Then, the brains were removed and fixed in the similar solution for 24 h. Following routine processing in paraffin, serial coronal sections of the brain were cut at 8 µm thickness in a rotary microtome (Leitz, 1512, Germany). The parts of each rat brain section were stained with modified Bielschowsky, Nissl staining, and modified Congo red staining. Modified Bielschowsky gives a good compromise between sensitivity for plaques and tangles can be used as a single stain for diagnosis of AD. Briefly, sections were deparaffinized through xylene and alcohols into tap water before being placed into fresh 20% silver nitrate solution for 20 min. After washing thoroughly with distilled water, slides were immersed in 20% silver nitrate solution titrated with fresh sodium hydroxide and evaporated ammonia (200 ml of 28% ammonium hydroxide by leaving in an open beaker for 20 min in a fume cupboard). After 15 min, slides were washed with ammonia water before being individually developed with 100 ml of developer (20 ml of formaldehyde, 100 ml distilled water, 20 µl concentrated nitric acid, and 0.5 g citric acid) and then added to 50 ml of titrated silver nitrate solution. Slides were then rinsed in tap water, fixed in 5% sodium thiosulfate, and dehydrated through alcohols and xylene. Congo red staining is an accepted histochemical marker for the β-pleated-sheet structure of amyloid. Sections were deparaffinized through xylene and alcohols into tap water. Afterwards, slides were immersed in alkaline sodium chloride. After 20 min, they were immersed in alkaline Congo red solution for 20 min and then differentiated with alcoholic potassium. After this, slides were counterstained with alum hematoxylin and dehydrated through alcohols and xylene. All of the cerebral cortex and molecular layer cells in the CA1 region were counted on Nissl stained under a light microscope at 40× magnification. Four contiguous 0.936 mm² areas in cortex and 0.26 mm² areas in hippocampus were counted. Measurement of the area was performed using the Olysys BioReport software imaging system (Olympus Corporation, Tokyo, Japan) [20] and neuritic plaques were counted on modified Bielschowsky stained separately in cortical regions and hippocampus at 100× magnification.

**TUNEL procedures.** Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick-end labeling (TUNEL) staining for apoptotic neurons was performed according to the manufacturer's instruction [21]. Cell death was detected in situ in resected tissues by enzymatic labeling of DNA strand breaks using a TUNEL assay according to the manufacturer's instructions. Paraffin-embedded tissue were deparaffinized in xylene and rehydrated with ethanol series (absolute, 95%, 90%, 80%, 70%, diluted in double distilled water). After this stage and all subsequent stages, washing with PBS was performed. The sections were treated with proteinase K (20 µg/ml in 10 mM Tris/HCl, pH 7.6) for 30 minutes, the endogenous peroxidase activity was blocked by using 0.3% H₂O₂ in methanol for 30 minutes. Then, the sections were prepared with permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 15 minutes. The labeling of apoptosis was induced by TdT, which catalyses deoxyribonucleotide, and fluorescein conjugated dUTP. During this step, slides were coverslipped and incubated in a humidity chamber at 37°C for 60 minutes. Then, the sections were washed and incubated with a convertor-POD at 37°C in a humidity chamber for 30 minutes. After a wash, color was developed by incubating sections with a diaminobenzidine substrate solution for 15 minutes. After a final wash, sections were viewed by light microscopy and only those cells with positive TUNEL staining and of apoptotic morphology were considered apoptotic [22].
Statistical analysis. All results were performed as mean ± S.E.M. for the behavioral tests including passive avoidance test and Y-maze task and Neuronal counts in cerebral cortex and CA1, one-way analysis of variance (ANOVA) was used. Normality and homogeneity of the data were confirmed before ANOVA analysis. In all analyses, a $P$ value of <0.05 was considered statistically significant.

RESULTS

Y-maze task. For Y-maze task, short-term spatial memory performance was examined in this study. The mean scores of alternation behavior for sham, control, and AD group were 80.56, 86.7, and 46.2%, respectively. AD group showed a significant reduction in alternation behavior as compared to control and sham group ($P<0.0001$, Fig. 1). There was no significant difference between sham and control groups.

Single-trial passive avoidance test. The performance of control and AD group rats for passive avoidance test was shown by initial and step-through latency. The AD group rats indicated significant damage in retention in passive avoidance test. The mean of acquisition in passive avoidance test showed a non-significant decrease in sham group compared to control group. This result exhibited significantly decreased acquisition to passive avoidance response in AD group compared to control and sham group ($P<0.0001$, Fig. 2).

Histological procedure. Two types of plaque were seen in AD cerebral cortices, diffuse plaques and compacted amyloid cores. In this study by Bielschowsky silver staining, diffuse plaques were detected in the cerebral cortex and hippocampus (Fig. 3). These diffuse cerebral plaques ranged in size smaller than 210 µm. In this study, the number of plaques in hippocampus was more than cortex and white matter. Also, neuronal population reduction in hippocampus was observed comparing to cortex. The perivascular amyloid deposits were seen clearly in cortex, hippocampus and white matter. Dystrophic neurites were not seen associated with diffuse plaques. Diffuse plaques were not found with reactive neurites or glial cells. Compacted amyloid cores were present in the cerebral cortex, hippocampus and white matter. scattered amyloid cores were seen by Congo red and Bielschowsky silver stain in AD cortex and hippocampus. Rare amyloid angiopathy was detected in the cerebrum, hippocampus and white matter in AD group (Fig. 3). Congo red staining showed evidence of some birefringent material indicating the presence of fibrillar structures. Congo red staining slides showed typical green birefringence under polarized light in AD group (Fig. 4). Nissl-stained neuronal densities indicated AD group (Fig. 5) had consistent reduction of neuron densities 55.6% in the CA1 and 25.9% in cortex. The number of plaques in the hippocampus were present 11 ± 2 diffuse plaques and 3 ± 1 cored plaques per 100× field and the neuritic plaques in cerebral cortex showed numerous 7 ± 2 diffuse plaques and 2 ± 1 cored plaques per 100× field (Table 1).

TUNEL procedure. TUNEL staining was used to show apoptosis (Fig. 6). Brain tissue sections from AD cases showed comprehensive cellular
DNA fragmentation revealed by TUNEL. This method detected primarily double-stranded DNA breaks via TdT. In AD group in CA1 region of hippocampus and cortex, TUNEL-positive cells were observed.

**DISCUSSION**

In this study, we demonstrated diffuse plaques and compacted amyloid cores by modified Bielschowsky silver staining in the cerebral cortex and hippocampus. Also, we showed the increased number of plaques and decreased neuronal population in hippocampus compared to cortex and white matter by modified Congo red staining that showed typical green birefringence under polarized light in AD group. For evaluation of behavioral changes in small animals such as rats and mice, the following methods have been used: (1) passive avoidance response, (2) active avoidance response, and (3) the maze test [9]. In this study, passive avoidance response and maze test were used to appraise the preservation of memory after Aβ effects. The AD groups that received Aβ, showed

| Table 1. Neuronal counts in cerebral cortex and CA1. |
|-----------------|-----------------|-----------------|-----------------|
| **Group**       | **Region**      | **Neurons per mm²** | **Average of neurons per field** | **Std. deviation** |
| AD              | CA1             | 6.35**          | 165.0           | 3.830           |
|                 | Cortex          | 6.35**          | 165.0           | 3.830           |
| control         | CA1             | 6.250           | 162.6           | 0.843           |
|                 | Cortex          | 6.250           | 162.6           | 0.843           |
| Sham            | CA1             | 6.250           | 162.6           | 0.843           |
|                 | Cortex          | 6.250           | 162.6           | 0.843           |

*P<0.0001, AD group compared with control and sham groups; **P>0.05, control group compared with sham group.
significant decrease in behavior scores in comparison with the control and sham group in Y-maze test and passive avoidance response. This study similar to other studies in this field confirmed that deposition of Aβ in AD brains impairs learning and memory [1, 8, 12]. The passive avoidance procedure is a quick and simple task to administer [23]. Therefore, this procedure is widely used to measure cognitive alterations after drug administration, lesions, and behavioral manipulations [24]. The passive avoidance test has been widely used to evaluate rodent working memory ability in association with cortical and hippocampal functions [25]. In this study, the latency periods of passive avoidance response were evaluated in these rats and the retention of memory was significantly disturbed in AD group. The neurotransmitters in the central nervous system have important roles in normal functioning and behavior of the adult individual. They interact with each other in complex networks in the process of learning and memory, in which acetylcholine is proposed to have a central role. 

Fig. 4. Tissue obtained with Congo red staining from AD group (40×). (A) amyloid deposits in CA1 region; (B) typical green birefringence amyloid deposits under polarized light and (C) control group (40×).

Fig. 5. Nissl staining AD group. (A) CA1 Nissl staining AD group (40×): (1) cell death and (2) apoptotic body; (B) control group (40×).

Fig. 6. Cell death detection in situ by terminal deoxynucleotidyl transferase mediated dUTP nick end labeling (TUNEL). Cells with positive TUNEL staining (brown) and exhibiting apoptotic morphology were considered apoptotic. (A1) AD group Tunel positive in cortex; (A2) apoptosis; (B) control group Tunel negative in cortex; (C1) AD group Tunel positive in CA1; (C2) apoptosis; (D) control group Tunel negative in CA1 (40×).
role. Acetylcholinesterase is an enzyme responsible for hydrolyzing and so deactivating acetylcholine in the body. Alterations in this enzyme level are indicative of impairment of cholinergic function [26]. Several studies have reported that infusion of Aβ peptide (1-40) into the rat cerebral ventricle induces learning impairment, neuronal and morphological degeneration and alteration of enzyme markers such as acetylcholine esterase and choline acetyltransferase; all of which are well-known characteristics of AD [27]. The hippocampus and the cerebral cortex are the key structures of memory formation; Aβ injection into hippocampus can deficit in both synaptic transmission and plasticity [28, 29, 30] and neuronal dysfunction [31].

In the present study, cerebral plaques of the diffuse type were present only in AD group. The cerebral lesions observed in cases of AD are definable as diffuse plaques because they appear to lack dystrophic neurites and that is dispersed rather than compacted into a core. An alternative view could be that diffuse plaques are a separate plaque type which does not evolve further in the cerebral cortex. We did not detect morphologic changes in any of the cells or processes in areas of cortex containing abundant diffuse plaques. Injection of Aβ protein is widely used in AD model preparation. Aβ proteinosis is an important structure of senile plaques and is thought to be the main reason for the loss of neurons and the resulting memory disability [32].

In present survey in the AD group, it was shown that Aβ to be caused DNA damage in neurons that finally degenerate and die. Apoptotic and necrotic changes that make neuronal cell death occur in AD brains. Multiple laboratories have reported evidence for apoptosis, or programmed cell death, in pathological tissues obtained from the brains of AD patients [33]. Although TUNEL is not a definitive marker of apoptosis, it still allows identification of dying cells in situ [32, 33]. Lassmann et al. [34] also reported that TUNEL-positive cells were positioned in brain regions containing extracellular Aβ deposition. In this regard, although there has been some debate on whether amyloid deposition correlates with the degree of dementia [35], recent evidence indicates that the severity of dementia can be correlated with the Aβ load [36]. This does not, however, imply that other factors such as cytoskeletal abnormalities are not also involved in the disease process [37].

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