

Effect of topiramate on apoptosis-related protein expression of hippocampus in model rats with Alzheimers disease

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Abstract. – OBJECTIVES: Alzheimer's disease is an age-related neurodegenerative disease and a synaptic function defect disease, the clinical symptoms are mainly progressive cognitive impairment, affecting patient's social function. Aim of this report was to investigate the effect of topiramate on apoptosis-related protein expression (Bcl-2, Survivin, Fas, Bax and Caspase-3) in the hippocampus of a rat model with Alzheimers Disease (AD).

MATERIALS AND METHODS: Thirty-six adult Wistar rats were randomly divided into a control group, a model group and a test group. A dose of amyloid beta-protein 1-40 (A β 1-40) was injected into the hippocampus of the rats in the model and test groups, and the control rats are injected with same amount of saline. After AD model was successfully established, the rats in each group were administrated with an i.p. injection of topiramate (20 mg/kg/d) for 30 days. The effect of topiramate on the apoptosis-related protein levels in hippocampus neurons was studied by immunohistochemistry.

RESULTS: The number of Bcl-2 and Survivin positive cells and optical density in hippocampus of rats in test group was more than those of rats in model groups, but less than those of rats in control group ($p < 0.01$); Fas, Bax and Caspase-3 positive cells and optical density in hippocampus of rats in test group was less than those of rats in the model group, but more than those of rats in control group ($p < 0.01$).

CONCLUSIONS: Alzheimer's disease can induce apoptosis of hippocampus neurons in rats. Topiramate can prevent apoptosis of hippocampus neurons, at least in part, by increasing expression of Bcl-2 and Survivin and decreasing expression of Fas, Bax and Caspase-3.

Key Words:

Alzheimer's disease, Topiramate, Bcl-2, Survivin, Fas, Bax, Caspase-3.

Introduction

It is estimated that there are 240 million patients with dementia all over the world, while

Alzheimer's disease (AD) accounts for the vast majority of these cases. The incidence of AD has exceeded that of stroke, cardiovascular disease and cancer among the people aged over 60. Therefore, AD has become a major public health problem. But what is the specific pathogenesis of AD? How to early diagnosis and take further prevention? How to treat these patients and improve the quality of their lives and ease the economic and psychological burden of the family and society? Therefore, AD has become a hot topic of research¹.

Alzheimer's disease is an age-related neurodegenerative disease and a synaptic function defect disease²⁻⁵, the clinical symptoms are mainly progressive cognitive impairment, affecting patient's social function. The main pathology change is in the cortex and hippocampus, which is the memory and learning center. The main pathological characteristics of AD are neuronal degeneration loss, a large number of senile plaques (SP) and neurofibrillary tangles (NFT)¹, accompanied by diffuse cerebral atrophy, other features include lipid peroxidation, protein oxidation and the direct toxic effects of free radicals, the interaction of inflammation, abnormal calcium deposition, metal ions and beta-amyloid⁶⁻⁸. Previous research has shown that among these pathological changes, the main pathological change is neuron loss, which is the main cause of clinical symptoms. To explore the specific reason of neurons loss and find effective measures to protect the neuron is the basis of this study.

At present, most hypotheses of Alzheimer's progression state that free radicals and apoptosis can only partly explain the pathological process of AD and patient's symptoms, and cannot fully elucidate the pathogenesis of AD. Most scholars believe that AD is a multi-cause disease related to many factors including genetic, age, abnormal immune function, endocrine, infection, poisoning, environment, gender, adverse cerebral circu-

lation, energy metabolic disorders, occupation and education levels⁹. It is currently accepted that neuronal loss from the cerebral cortex and hippocampus is the main reason behind the symptoms¹⁰, the neuronal loss due to cell apoptosis. Apoptosis is programmed cell death, and is caused by various factors, and closely related to the occurrence.

The specific mechanisms of neuronal apoptosis in AD may be related to abnormal neuronal cell cycle, apoptosis related gene expression disorders and free radical damage. At the same time, the abnormal protein deposition and neuron axon and dendrite degradation of cerebral cortex and hippocampus will eventually yet lead to neuronal apoptosis. At present apoptosis mainly happens in two ways: one is the mitochondrial pathway (also known as intracellular pathway) (Bcl-2, Bax, Cyt C, Caspase-9, Caspase-3); and the second is the extracellular pathway (Fas, FasL, FRADD, Caspase-8, Caspase-3). Caspase-3 is common to both pathways, and is the executor of apoptosis. At the same time, there are anti-apoptotic mechanisms, one of which is via the protein Survivin. In this study, our purpose is to specify the role of specific path by establishing an AD animal model, and detecting the key molecular protein expression of each pathway.

At present, there is no cure for AD, the most common used drugs are central nervous system cholinesterase inhibitors and NMDA N-methyl-D-aspartate antagonists. These drugs may ease some of the symptoms in patients who are mildly affected, but there's no effective treatment for patients with severe forms of the disease¹¹⁻¹³. Furthermore, cholinesterase inhibitors and NMDA antagonists are expensive and have many side effects, while other drugs have no exact curative effect. We will explore the treatment drugs from restraining the AD neuron apoptosis pathway.

Topiramate (TPM) is a widely applied antiepileptic drug at present, which controls various types of epileptic seizures and plays a protective role in nerve cell apoptosis. It has also been found in clinical work that, topiramate can protect cognitive function in patients with chronic epilepsy. Whether topiramate can control the apoptosis induced by AD and therapy AD will need to be further explored.

We will set up establish a classic rat model of AD and apply antiepileptic drug topiramate to intervene, to apply classic immunohistochemical method to detect the key molecular expression of hippocampus neuron apoptosis related path: Bcl-

2, Survivin, Fas, Bax and Caspase 3 protein, and identify topiramate plays a therapeutic role mainly through multi-channel antagonism apoptosis.

Materials and Methods

Adult Wistar rats (male, 235 g-350 g), were provided by the animal center of Hubei College of Chinese Medicine. All animals were treated in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory and approved by the Animal Ethic Committee of Yangtze University Clinic Medical College [(Project #0312)]. All rats underwent preliminary screening with Morris water maze test. Rats with congenital dementia and improper swimming style were eliminated. β -amyloid ($A\beta$)₁₋₄₀ and paraformaldehyde were provided by Sigma company (St. Louis, MO, USA) and rabbit anti Bcl-2, Survivin, Fas, Bax and Caspase-3 antibodies were purchased from Jingmei Biotech Co. Ltd., Shanghai, China. Goat anti-rabbit antibody and 3,3'-diaminobenzidine (DAB) colorant are provided by Kangwei company, Jiangsu, China. The topiramate injection was purchased from Ligand company Ltd (La Jolla, CA, USA).

Incubation of $A\beta$ ₁₋₄₀

$A\beta$ ₁₋₄₀ was diluted to 2 g/L with sterile saline, and incubated for one week at 37°C.

Rat Model Making and Processing

Twelve qualified control group rats were injected with 5 μ l of normal saline into the bilateral intra-hippocampus (Bregma points as 0 points, AP-3.0 mm, ML2.0 mm, DV-2.9 mm). 5 μ l of condensed state $A\beta$ ₁₋₄₀ was injected into the bilateral intra-hippocampus of model and test groups (Injection site is the same as the control group). One week after the injection, Morris water maze tests were conducted. The value that exceeded 95% mean incubation period of the control groups determined the success of the model for dementia. The dementia rats are randomly divided into model and test group (12 rats per group). The rats in the model group are injected with normal saline for 30 days by intraperitoneal injection. The test group were injected with topiramate (20 mg/kg/d) for 30 days by intraperitoneal injection.

Morris Water Maze Test

The water depth was 25 cm (1 cm above the platform) and water temperature is controlled at

22- 26°C. The maze was divided into four quadrants, and the platform is placed in the third quadrant. The test was divided into two steps. The first step was to conduct positioning test for five days, where each rat was trained eight times in one day and each time by a different entry point and practice for two minutes. When the rat found the platform by itself within two minutes, it is left on the platform for 30 seconds. If the rat can't find and climb up platform by itself within two minutes, it was placed on the platform for 30 seconds. 30 seconds rest was given between each practice. The mean time (escape latency) the rats spend in finding the platform will be tested in order to evaluate their cognitive function. The second step is to conduct a space exploring test. Six days later, the platform was removed and all rats will be placed into water from the same entry point. To record the search time and search distance of rat across the original platform quadrant in 120s (the percentage of swimming distance of rats in the original platform quadrant accounting for the total distance correction is used to eliminate the differences in swimming speed of each rat), testing its ability of finding the original site from memory.

Drawing Materials and Sliced

One week later, all rats in all three groups are deeply anesthetized with a 10% chloral hydrate (300 mg/kg i.p). A 150 ml dose of normal saline is quickly injected, followed by 4% paraformaldehyde (500 ml). The rats were then decapitated and the whole brain tissue (including the hippocampus) was removed and placed into a 4% paraformaldehyde. Two to four hours later, the brains were removed from the paraformaldehyde and placed into 20% sucrose, followed by routine dehydration, embedding, and slicing (4 µm-thick).

Immunohistochemistry

With Streptomyces avidin-peroxidase method links method, twenty slices were taken from each rat in order to test for Bcl-2, Survivin, Fas, Bax and Caspase-3. Control experiments were conducted with normal rabbit serum and phosphate buffered saline (PBS) instead fo the primary antibody.

Image Analysis

Four slices from each rat were analyzed, by use of a PIPS-2011 color pathology graphic analysis and management system. All of the brain slices were analyzed under the same mag-

nification factor (×200) and light intensity. The first step was to analyze the Bcl-2, Survivin, Fas, Bax and Caspase-3 positive cells in hippocampus; then, detect the average optical density of the positive cells. The degree of immune reaction is indicated by optical density value, the darker it is, the larger the value is.

Statistical Analysis

Data is indicated by mean ± standard deviation (±SD). The comparisons of quadrant search time, quadrant search arrange, the average positive cell number and optical density of positive cells variance was analysed by pair wise comparisons and *q* test. *p* < 0.05 was considered statistically significant.

Results

Comparison of Bcl-2 positive cells and optical density in the hippocampus. The Bcl-2 positive cells and optical density in the hippocampus of rats in test group are significantly higher than those of rats in model group, but less than those of rats in control group (*p* < 0.01) (Table I). Bcl-2 positive cells and optical density in the hippocampus of rats in the model group are significantly less than those of rats in the control group (*p* < 0.01).

Comparison of Survivin positive cells and optical density in the hippocampus. The Survivin positive cells and optical density in the hippocampus of rats in test group are significantly higher than those of rats in model group, but less than those of rats in control group (*p* < 0.01) (Table II). Survivin positive cells and optical density in the hippocampus of rats in the model group are significantly less than those of rats in the control group (*p* < 0.01) (Table II).

Comparison of Bax cells and optical density in the hippocampus. The Bax positive cells and

Table I. Comparison of Bcl-2 positive cells and optical density in each group ($\bar{x} \pm s$).

Group	Positive cells	Optical density
Test group	52.45 ± 10.26 ^{a,b}	0.472 ± 0.047 ^{a,b}
Model group	37.03 ± 8.62 ^c	0.315 ± 0.035 ^c
Control group	68.36 ± 12.73	0.567 ± 0.061

Note: In comparison with control group: ^a*p* < 0.01, in comparison with model group: ^b*p* < 0.01; in comparison with control group; ^c*p* < 0.01.

Table II. Comparison of Survinin positive cells and optical density in each group ($\bar{x} \pm s$).

Group	Positive cells	Optical density
Test group	52.45 \pm 10.26 ^{ab}	0.472 \pm 0.047 ^{ab}
Model group	37.03 \pm 8.62 ^c	0.315 \pm 0.035 ^c
Control group	68.36 \pm 12.73	0.567 \pm 0.06

Note: In comparison with control group: ^a $p < 0.01$, in comparison with model group: ^b $p < 0.01$; in comparison with control group; ^c $p < 0.01$.

optical density in the hippocampus of rats in test group are significantly less than those of rats in model group, but higher than those of rats in control group ($p < 0.01$) (Table III). Bax positive cells and optical density in the hippocampus of rats in the model group are significantly higher than those of rats in the control group ($p < 0.01$) (Table III).

Comparison of Fas cells and optical density in the hippocampus. The Fas positive cells and optical density in the hippocampus of rats in test group are significantly less than those of rats in model group, but higher than those of rats in control group ($p < 0.01$) (Table IV). Fas positive cells and optical density in the hippocampus of rats in the model group are significantly higher than those of rats in the control group ($p < 0.01$) (Table IV).

Comparison of Caspase-3 cells and optical density in the hippocampus. The Caspase-3 positive cells and optical density in the hippocampus of rats in test group are significantly less than those of rats in model group, but higher than those of rats in control group ($p < 0.01$) (Table V). Caspase-3 positive cells and optical density in the hippocampus of rats in the model group are significantly higher than those of rats in the control group ($p < 0.01$) (Table V).

Table III. Comparison of Bax positive cells and optical density in each group ($\bar{x} \pm s$).

Group	Positive cells	Optical density
Test group	29.49 \pm 9.28 ^{ab}	0.372 \pm 0.041 ^{ab}
Model group	41.26 \pm 12.14 ^c	0.426 \pm 0.053 ^c
Control group	17.45 \pm 7.53	0.297 \pm 0.028

Note: In comparison with control group: ^a $p < 0.01$, in comparison with model group: ^b $p < 0.01$; in comparison with control group; ^c $p < 0.01$.

Table IV. Comparison of Fas positive cells and optical density in each group ($\bar{x} \pm s$).

Group	Positive cells	Optical density
Test group	59.82 \pm 12.87 ^{ab}	0.386 \pm 0.054 ^{ab}
Model group	76.54 \pm 16.37 ^c	0.568 \pm 0.072 ^c
Control group	43.29 \pm 9.63	0.282 \pm 0.035

Note: In comparison with control group: ^a $p < 0.01$, in comparison with model group: ^b $p < 0.01$; in comparison with control group; ^c $p < 0.01$.

In all control experimental when substituting normal rabbit serum and PBS for level 1 antibody and incubating slices, the results are negative.

Discussion

Alzheimer's disease is a neurodegenerative diseases characterized by progressive cognitive decline and is common in patients aged over 60. The incidence of AD gradually increases with age. The main clinical manifestations are progressive memory disorders, cognitive disorders and neuropsychiatric disorders. The pathology of the disease is characterized by senile plaques and neurofibrillary tangles in the cerebral cortex, and is accompanied by synapses decrease in the brain regions of learning and memory. Beta-amyloid is the core components of senile plaques and the abnormal metabolism and deposition of beta-amyloid are key factors in the formation and development of AD¹⁴. Accordingly, bilateral hippocampal directional injection of beta-amyloid is a commonly used method for preparing AD model¹⁵. The exact etiology of AD is unclear before Alois Alzheimer systematically and clearly described the typical clinical manifestations and

Table V. Comparison of Caspase-3 positive cells and optical density in each group ($\bar{x} \pm s$).

Group	Positive cells	Optical density
Test group	78.16 \pm 13.22 ^{ab}	0.342 \pm 0.047 ^{ab}
Model group	92.63 \pm 17.56 ^c	0.526 \pm 0.053 ^c
Control group	54.69 \pm 10.57	0.263 \pm 0.032

Note: In comparison with control group: ^a $p < 0.01$, in comparison with model group: ^b $p < 0.01$; in comparison with control group; ^c $p < 0.01$.

pathological features of AD in 1906. Since the 1990s, there have been scholars studying the relationship between apoptosis and AD^{16,17}. A growing number of studies have shown that abnormal neuronal apoptosis is closely associated with the occurrence and development of AD, and the loss of neurons in the cerebral cortex and hippocampus is more apparent¹⁸. Studies have shown that the cycle change of abnormal neuronal cell occurs in the early stage of AD¹⁹, even before the appearance of senile plaques and neurofibrillary tangles²⁰.

At present, apoptosis has two main pathways: one is mitochondrial pathway (also known as intracellular pathways): BID, Bax, Cyt C, Caspase-9, Caspase-3; the other is the extracellular pathway: Fas, FasL, FRADD, Caspase-8, Caspase-3, Caspase-3 is common to both two pathways, and the executor of apoptosis. At the same time, there are antiapoptotic mechanism, under the regulation of the Survivin protein. The neuronal apoptosis mechanisms of AD are related to the following factors: firstly, amyloid beta protein (A β) can activate the NF-kappa B expression in neurons, resulting in the occurrence of AD^{21,22}; Secondly, the mitochondrial respiratory function and oxidative phosphorylation is reduced. The change of mitochondrial function includes damaged electron transfer coupling and decreased membrane potential (its changes are earlier than nuclear pyknosis) and the destruction of the cell membrane integrity, eventually leading to neuronal apoptosis in patients with AD²³⁻²⁵; thirdly, abnormal cell cycle, amyloid levels rise, leading to neuronal apoptosis²⁶⁻²⁸, especially the histone metabolic abnormalities plays an important role in AD neuronal apoptosis.

There are no good therapeutic measures for AD. Currently the applied drugs can only ease some of the symptoms in mild patients, which are expensive and have side effects. It is, therefore, difficult for the patients to take them for a long time; thus, we need to continuously explore new drugs. Because histone metabolism plays an important role in neuronal apoptosis of AD, the current research focuses more attention on this field. Histone metabolism is regulated by histone deacetylase and histone acetyltransferase²⁹⁻³¹. Currently, histone deacetylase inhibitors have been approved by the U.S. Food and Drug Administration as a medicine for cutaneous lymphoma. Meanwhile many studies have found that they can also be used to treat asthma, cardiac hypertrophy and degenerative diseases of

nervous system³¹⁻⁴⁰. More and more evidence suggests that classic HDAC inhibitors have potential treatment benefits in model of AD. For example, trichostatin A has been shown to improve memory contents of a mouse model of AD⁴¹. Similarly, hydroxamic acid has been shown to improve the memory content in transgenic mice model of AD⁴². Currently, Topiramate (TPM) is a widely used antiepileptic drug, which has potential histone deacetylase inhibitor effects. Not only can it control various types of seizures, but also it has a protective effect on neuronal apoptosis⁴³, and it can reduce the behavioral defects and neuropathological changes of APPswe/PS1dE9 transgenic mice⁴⁴, Topiramate also has a controlling role in behavioral disorders of AD patients in clinical practice⁴⁵. Whether it can inhibit apoptosis induced by AD has not been reported at present. So, we have established a rat model of AD and applied topiramate to intervene and observe its effect on Bcl-2, Survivin, Fas, Bax, Caspase-3 of hippocampal neurons, understood its protective effect on neuronal apoptosis and have provided a new basis for the treatment of AD.

In our experiments, we discovered that the expression of apoptosis related proteins increases significantly in our AD model rats, and the expression of anti-apoptosis related proteins reduces significantly in the hippocampal neuron of our AD model. It mainly manifested that the expression of Fas, Bax and Caspase-3 in model group hippocampal neurons were significantly higher than those of the control group, while the expression of the Bcl-2 and Survivin in model group hippocampal neurons was lower than that of the control group, which further confirms that the neuronal apoptosis is one of the pathogenesis mechanisms of AD. Neuronal apoptotic mechanisms of AD are not very clear, but studies have shown that the intracellular domain of amyloid precursor protein (APP) can influence the expression of some genes, thereby, inducing neuron specific apoptosis⁴⁶. Because we applied bilateral hippocampal directional injection of A β to make our AD model, we can further speculate that the neuronal apoptosis of AD is primarily caused by the expression of A β antiapoptotic genes such as Bcl-2 and Survivin and the increased expression of apoptosis promoting genes such as Fas, Bax and Caspase-3. If it regulate the expression of one gene or entire gene expression in apoptosis gene and the pathways also requires further confirmation in the next experiments.

Topiramate is an antispasmodic, it performs extensive neuroprotection mainly by regulating anti-apoptotic factors in the central nervous system hypoxic-ischemic disease⁴⁷. We have found in our experiments that the expression of Fas, Bax and Caspase-3 in test group hippocampal neurons are significantly less than that of the model group, while the expression of the Bcl-2 and Survivin in test group hippocampal neurons are higher than that of model group; this shows that topiramate can antagonize neuronal apoptosis and improve learning ability and memory function of AD patients. It could, therefore, have possible application as a therapeutic agent to treat AD. The anti-apoptotic mechanisms of topiramate in AD are quite different from the traditional anti- γ -aminobutyric acid. We speculate that it mainly plays a role through the following mechanisms: (1) topiramate is a histone deacetylase inhibitor which can inhibit histone deacetylase and play a protective role by inhibiting the expression of pro-apoptotic genes⁴⁸; it also can directly inhibit the beta-amyloid and indirectly inhibit neuronal apoptosis; (2) topiramate plays an anti-apoptotic role by adjusting voltage-gated calcium channels and glutamate receptors⁴⁹; (3) topiramate plays an anti-apoptotic role by directly inhibiting the synthesis of APP. Topiramate can antagonize apoptosis in many ways. Not only can it affect the mitochondrial pathway of apoptosis, but also it can inhibit the extracellular pathway and plays a role by promoting the expression of Survivin protein. However, we also found in our experiments that although topiramate can play an anti-apoptotic role in the AD model, there is statistical difference in Fas, Bax, Caspase-3, Bcl-2 and Survivin compared with the control group. This suggests that Topiramate can't completely antagonize apoptosis as there are other neuron apoptosis pathways at play in AD. We need to find a drug which is compatible with topiramate. Further analysis of the pharmacology of topiramate in AD is required to fully understand its role in AD treatment.

Conclusions

Alzheimer's disease can induce apoptosis of hippocampus neurons in rats; Topiramate can prevent apoptosis of hippocampus neurons, at least in part, by increasing expression of Bcl-2 and Survivin and decreasing expression of Fas, Bax and Caspase-3.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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