Original Article

The neuroprotective effect of immune serum of adeno-associated virus vaccine containing Aβ1-15 gene on amyloid toxicity

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Abstract

Objective: The aim of this study was to explore the effect of adeno-associated virus (AAV) serotype 2 vector vaccine containing amyloid-β peptide (Aβ) 1-15 gene fragment (AAV-Aβ15) immunized mice sera on counteracting Aβ1-42 peptide toxicity towards a primary culture cortical neurons. **Materials and Methods:** BALB/c mice were vaccinated via the intramuscular immunization route with AAV-Aβ15. The anti-Aβ antibody titer of immunized mice sera was quantified by sandwich Enzyme-Linked ImmunoSorbent Assay. The toxicity of Aβ1-42 peptide on neurons was assessed by morphology with an inverse microscopy and cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay. **Results:** AAV-Aβ15 could induce an Aβ-specific immunoglobulin G (IgG) humoral immune response in /c mice the anti-Aβ antibodies were detectable at 1 month after immunization, significantly increased at 2 and 4 months after immunization, and the immunized sera could attenuate cytotoxicity of Aβ1-42 peptide on primary culture cortical neurons. **Conclusions:** The immune serum of AAV-Aβ15 could play a neuroprotective effect against Aβ1-42 peptide toxicity, which would be beneficial for Alzheimer's disease patients.

Key Words

Alzheimer's disease, amyloid-beta, immunotherapy, vaccine

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Ann Indian Acad Neurol 2013;16:603-8

Introduction

Alzheimer's disease (AD) is a progressive neurodegenerative disorder characterized clinically by loss of memory and the cognitive functions. Symptoms of AD initially manifest as mild forgetfulness; however, lapse into mental confusion and agitation, followed by severe dementia, and death, which severely affected the life quality of aged people.[1] The neuropathological hallmarks of AD are intracellular neurofibrillary tangles made of hyperphosphorylated microtubule associated protein, extracellular senile plaques composed of the amyloid-β peptide (Aβ) surrounded by dystrophic neurites with different numbers of activated microglia and reactive astrocytes.[2] However, the primary cause of AD has not been elucidated clearly and no satisfied therapy for the AD to date. There are many theories about the pathomechanism of AD. For example, the "cholinergic hypothesis" has been postulated to explain the molecular mechanism of AD, which states loss of cholinergic markers and decline in acetylcholine neurotransmitter leading to impairment of cognitive and memory functions. Cholinesterase inhibitors, such as donepezil, rivastigmine, and galantamine, provide only symptomatic relief from the cognitive impairment but do not stop the progressive mental and behavioral decline.[3]

In the past years, significant progress has been made in the understanding of the pathogenic mechanism of AD, and new therapeutic targets have become available that should allow the underlying disease process to be tackled directly. In this respect, the "amyloid cascade hypothesis" has become the dominant theory in the field.[4] It is believed that Aβ accumulation in senile plaques plays a primary role in progression of AD, which initiates pathological processes including oxidative stress, disrupted calcium homoeostasis, chronic inflammation, generation of

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DOI: 10.4103/0972-2327.120489

radicals, induction of apoptosis leads to cognitive impairment, and dementia in the end of life. Thus, therapies targeted at the clearance of Aβ or the prevention of its deposition and/or aggregation may be beneficial for treatment of AD. Various approaches to prevent aggregation of Aβ have been investigated. These methods include inhibition of secretase functions to reduce the amount of Aβ, inhibit amyloid fibrillation, and enhance the clearance of Aβ by small molecules, but none of the aforementioned treatments has produced satisfactory curative effects.[5] Immunotherapy may be one of the most promising strategies for prevention of Aβ deposition.[6] Aβ1-42 peptide active immunotherapy has been shown to elicit the high titer of anti-Aβ antibody, decrease amyloid burden in the brain and improve cognitive function in the transgenic mouse models.[7,8] These impressive results prompted the initiation of phase I/II clinical trials to evaluate the safety and tolerability of Aβ vaccination (AN1792). However, The phase IIa clinical trial (AN1792) was halted due to aseptic T-lymphocyte meningoencephalitis found in 6% of AD patients.[9] Thus, safer immunization modalities should focus on minimizing T-cell mediated inflammatory responses in efforts to prevent central nervous system (CNS) invasion of auto aggressive T-cells, at the same time promoting Aβ antibody-mediated clearance mechanisms.

Previous clinical investigation has revealed that B-cell-activating epitopes that elicit antibodies responses in humans and mice are located within the Aβ1-15 region while T-cell epitopes presenting in the C-terminus of Aβ are thought to be responsible for the adverse autoimmune inflammatory response. This segregation of T- and B-cell epitopes within the Aβ molecule allows the opportunity to induce anti-Aβ antibodies in the absence of Aβ-specific T-cell response. Moreover, some data also indicated that alternative immunogens encompassing the N-terminal epitope of Aβ (B-cell epitopes), generated specific antibodies against Aβ and elicited B-cell-mediated humoral immune responses without a significant T-cell-mediated immune response in mice.[10] At the same time, it is particularly crucial to adopt an immunization delivery system for preventing the AD besides selecting suitable Aβ fragment as antigen.

Recently, due to its inherent versatility, gene-based vaccination technology may allow for greater precision in antigen presentation and immunomodulation that could lead to safer and more efficacious vaccines for AD.[11] Among many viruses being developed as vectors for the human gene therapy currently, adeno-associated virus (AAV) is one of the most promising tools because AAV is non-pathogenic, non-toxic with low immunogenicity, and allows long-term gene expression in many tissues, including non-dividing cells like neurons. AAV has been successfully used in a number of animal models of neurological disorders. It is thought that AAV represents a well-positioned platform on which to build an Aβ-directed AD vaccine.[12]

Based on the above background, in the current study, we constructed an AAV serotype 2 vector vaccine containing Aβ1-15 gene fragment (AAV-Aβ15) and determined if immunization with the AAV vaccine would result in an adequate humoral immune response in mice. Furthermore, we explored the effect of immunized sera on counteracting cytotoxicity of Aβ1-42 in the primary culture cortical neurons.

Materials and Methods

Construction of AAV plasmid pSNAV-Aβ15

The open reading frame of the human Aβ1-15 gene was chemically synthesized with the codons optimized for expression in mammalian cells (Bioyong Technologies Inc.), and then cloned into an immunization vector pSNAV2.0 between the strong enhancer/promoter of the cytomegalovirus (CMV) immediate early genes and the bovine growth hormone polyadenylation signals (BGH polyA). The constructed plasmid pSNAV-Aβ15 also contains two packaging signal inverted terminal repeats of AAV and a neomycin resistant gene (NEO). The plasmid pSNAV-Aβ15 was amplified in DH5 α-cells and purified using a plasmid preparation kit (Qiagen Company). The presence of the Aβ1-15 gene was confirmed by sequencing. The diagrams of the base plasmid pSNAV2.0 and the constructed plasmid pSNAV-Aβ15 are shown in Figure 1.

Production of AAV vaccine AAV-Aβ15

AAV serotype 2 vector vaccine containing Aβ1-15 gene fragment (AAV-Aβ15) was prepared by the standard methods. Briefly, AAV-Aβ15 was generated by pSNAV-Aβ15 plasmid transfection with helper plasmids in human embryo kidney (HEK) 293 T-cells. Forty-eight hours after transfection, the cells were harvested and lysed in phosphate-buffered saline (PBS) by freeze thawing repeat 3 times. The titer of AAV-Aβ15 was determined by dot-blot with digoxin labeled CMV probe. The purity of AAV-Aβ15 was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis(SDS-PAGE).

Animal immunization

BALB/c mice were obtained from the SLAC laboratory animal limited liability company (Shanghai, China) at the age of 2 months. BALB/c mice were vaccinated via the intramuscular immunization route with PBS vehicle, AAV-lac and AAV-Aβ15. Each group consisted of 8 mice, 50 μl of AAV vector containing 5×10^{10} particles was injected into the right gastrocnemius muscle of each mouse. Blood was collected from the lateral tail vein at 1, 2, and 4 months after intramuscular injection. The blood was allowed to clot, and then placed at 4 overnight to facilitate separation of the serum from the clot. The clots were removed, and the serum centrifuged at 10,000 × g for 10 min to pellet any remaining

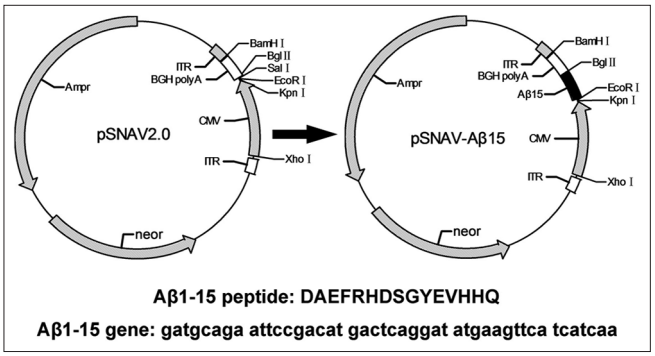

Figure 1: Schematic representation of newly constructed vector pSNAV-amyloid-β peptide 15. The plasmid pSNAV2.0 reprsented an empty vector control contained the cytomegalovirus immediate early genes and the bovine growth hormone polyadenylation signals

blood cells and debris. The clarified serum was transferred to a fresh tube and stored at 20°C until analyzed by Enzyme-Linked ImmunoSorbent Assay (ELISA). All animal housing and experimental procedures were performed in compliance with the guidelines of the Medical Experimental Animal Administrative Committee of Shanghai.

ELISA analysis for the anti-Aβ antibody in serum

Anti-Aβ antibody titer was quantified by sandwich ELISA. Ninety-six-well microtiter plates were coated with 100 μl of 2 μg/ml synthetic human Aβ1-42 peptide (Genemed Synthesis, Inc.) in 50 mM carbonate buffer (pH 9.6) overnight at 4°C. The wells were washed four times with the PBS containing 0.05% Tween 20 (PBS-T), and blocked with 200 μl of blocking buffer (5% goat serum and 1% bovine serum albumin in PBS-T) for 4 h at room temperature. Mouse serum was prepared in the PBS at initial dilution of 1:16 and subsequent two-fold dilutions were made. All samples were run in duplicate and simultaneously incubated at 37°C for 1 h followed by washing 6 times with PBS-T. Plates were blocked a second time with blocking buffer for 30 min at 37°C followed by washing 5 times, 100 μl of HRP-conjugated goat anti-mouse IgG diluted to 1:10,000 in PBS were added to the wells and incubated for 1 h at 37°C. Plates were then washed 6 times with PBS-T and developed with 100 μl of 3, 3, 5, 5-tetramethylbenzidine solution. The coloring reaction was stopped with 100 μl of 2 M sulphuric acid. Plates were read spectrophotometrically at 450 nm in a microplate reader. Antibody concentrations were calculated using a calibration curve generated with known concentrations of an anti-Aβ1-42 monoclonal antibody, 8G7 (Enzo Life Sciences International, Inc.).

Primary cortical neuronal culture and neutralization test by serum anti-Aβ antibody

Cortical neurons were obtained from the cortex of embryonic day 15 mouse according to the procedures described previously with modifications. In brief, after removal of the blood vessels and pia mater, freshly dissected cortical tissues were sectioned into approximately 1-2 mm^3 fragments and digested with 0.125% trypsin for 20 min at 37°C. The digestion was terminated by the addition of Dulbecco's modified Eagle's medium(DMEM) (Gibco, Invitrogen Corporation) containing 10% fetal calf serum (Gibco, Invitrogen Corporation). The treated tissue was then dispersed mechanically, and the cells were seeded at a density of 8×10^5 cells/ml on poly-l-lysine (0.1 mg/ml)-coated dishes in DMEM with 10% fetal calf serum. The cells were incubated in a humidified incubator (5% CO_2 and 95% air) at 37°C. After 24 h *in vitro*, the culture medium was replaced with Neurobasal medium™ containing 2% B 27 supplement (Gibco, Invitrogen Corporation). All experiments were performed using the 9-10 day old cultures. The prevention of Aβ neurotoxicity was measured as follows: 2 μM Aβ1-42 peptide was incubated for a week at 37 to produce fibrils, and then incubated with the sera of immunized animals 1 month after the intramuscular injection with AAV-Aβ15 at dilutions of 1:10 or 1:100 for 24 h. The reaction mixtures were added to the wells containing primary cultures of neurons and incubated at 37 for 2 days. Cell viability was assessed by measuring cellular redox activity with 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT).

Statistical analysis

All results are expressed as mean ± SD. The significance of the differences among multiple groups was analyzed using one-way ANOVA. The difference was considered statistically significant when $P < 0.05$.

Results

Detection of purity and titer of AAV-Aβ15

Capsid of AAV-2 is composed of three kinds of proteins: VP1, VP2, and VP3, with a molecular weight of 87, 72, and 62 kD, respectively. The ratio of VP1, VP2, and VP3 in AAV-2 capsid is 1:1:10. Therefore, three bands with specific patterns could be seen when AAV-2 virus was analyzed by SDS-PAGE. Analysis of the AAV-Aβ15 purity by coomassie brilliant blue stained SDS acrylamide gel electrophoresis is shown in Figure 2. Three clear bands representing AAV capsid proteins VP1, VP2, and VP3 could be seen on lane 2, and extremely low background was seen on lane 2, which demonstrated that contaminants had almost been removed from AAV-Aβ15. The purity of AAV-Aβ15 was estimated to be more than 95%. AAV genome containing particles was determined by the dot-blot method using digoxigenin-labeled CMV probe. The physical titer of the purified AAV-Aβ15 is estimated to be about 1.5×10^{12} particles/ml.

Generation of anti-Aβ antibody response in BALB/c mice

Sera samples from the vaccinated mice were analyzed for the titer of anti-Aβ antibody by ELISA using synthesized Aβ1-42 peptide. The anti-Aβ antibodies were detectable in the AAV-Aβ15 vaccinated mice 1 month after immunization, the antibody titer further a significantly increased 2 months after immunization, and kept elevated at least 4 months after immunization as shown in Figure 3. No anti-Aβ antibody was detected in either the AAV-lac or the PBS vehicle groups.

Partial neutralization of serum anti-Aβ antibody

Sera from AAV-Aβ15 vaccinated mice exhibited a partial protective effect in preventing the Aβ1-42 mediated neurotoxicity toward primary cultures of cortical neurons. The morphology of the neurons was evaluated under inverse microscopy. The cellular profile of untreated control neurons is smooth and with extensive neurite processes [Figure 4a].

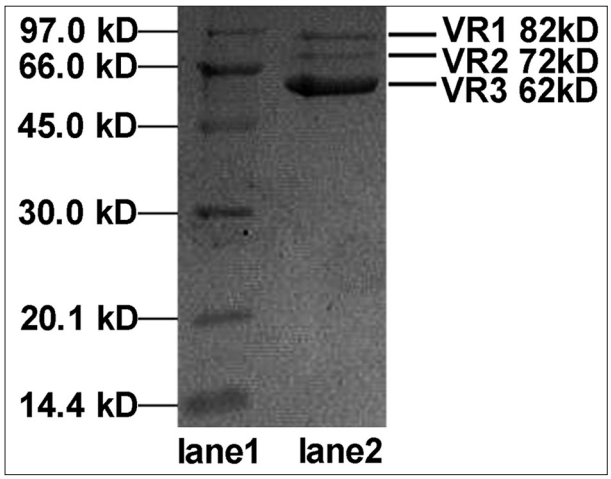

Figure 2: Detection of the purity of adeno-associated virus-amyloid-β peptide 15 by SDS-PAGE. Lane 1: Low molecular weight protein marker; Lane 2: Sample

Neurons that were treated with the Aβ1-42 alone were shrunken perikaryon with the loss of neurite processes, even though some of the cells had a cytolytic death [Figure 4b]. The neurons of diluted serum (1:10) from AAV-Aβ15 group retained a phenotype similar to untreated control cells [Figure 4c]. The neurons of diluted serum (1:100) from the AAV-Aβ15 group also displayed shrinkage, loss of neurites [Figure 4d]. The viability of cortical neurons was evaluated by MTT assay. The survival rate of neurons decreased to 69.5 ± 9.2% after Aβ1-42 treatment alone. The neuronal survival rate that was treated by diluted serum (1:10) from AAV-Aβ15 significantly increased to 80.4% ±9.9%, which showed that the toxicity of Aβ1-42 on neurons was decreased. However, the diluted serum (1:100) group did not show any significant effect [Figure 5]. These results indicated that AAV-Aβ15 vaccination could elicit "therapeutic" antibody titer within 1 month.

Discussion

Amyloid-beta immunotherapy has received considerable attention as a promising approach for reducing the level of Aβ in the central nervous system of AD patients. Based on the results obtained in animal and the human clinical studies, Aβ vaccine therapy is a principal strategy for AD treatment, but yet active peptide immunotherapy or passive immunotherapy has some problems to be solved before clinical application.[13] Newly developed gene-based vaccination was effective in reducing Aβ plaque on the model of mice and aged monkeys,[14,15] gene-based vaccination will be a promising therapy for AD in the future. A successful gene-based vaccine for AD depends on a number of key factors, such as the dose, route, delivery method, and vaccination schedule employed. As for as, Aβ gene-based vaccination is concerned, the same crucial problem is to select optimal immunogens and gene vector.

In order to avoid the adverse events observed in the human AN1792 clinical trial, the novel immunogens should be designed to target Aβ-specific B-cell epitopes and avoid Aβ-specific T-cell epitopes. Numerous studies have reported that the T-cell epitope is mapped within the Aβ15-42 fragment while the B-cell epitope is located within the Aβ1-15 region in humans, monkeys, and mice, so we selected the Aβ1-15 as immunogen.[16] What's more, almost all shorter Aβ N-terminal fragments have been shown to elicit appropriate humor immune antibodies. These antibodies could recognize and remove the toxic soluble or the aggregated forms of the Aβ1-42, clean senile plaques, improve the ethology of APP mice.[17] Meanwhile immunizing with Aβ1-15 may be less likely to be recognized as a self-antigen and therefore, safer than full-length Aβ as an immunogen. In summary, the experimental data obtained with the Aβ1-15 fragment showed that this sequence could be an ideal immunogenic peptide of vaccine against AD.

Recently viral vector has been extensively applied in gene vaccines. AAV, a member of the genus Dependovirus of the subfamily Parvovirinae within the Parvoviridae family is being developed as a vector for the gene therapy in the central nervous system. AAV has several features that make it useful as a vehicle for immunotherapy. First of all, AAV particles are icosahedral in symmetry and are relatively resistant to heat, low pH, some detergents, and solvents, which makes them particularly stable

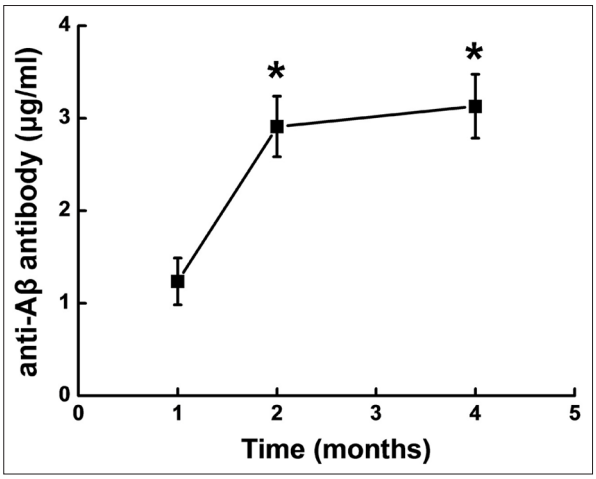

Figure 3: Serum anti- amyloid-β peptide antibody levels determined by Enzyme-Linked ImmunoSorbent Assay in immunized mice. Values given are mean ± SD. *$P < 0.05$ versus 1 month group

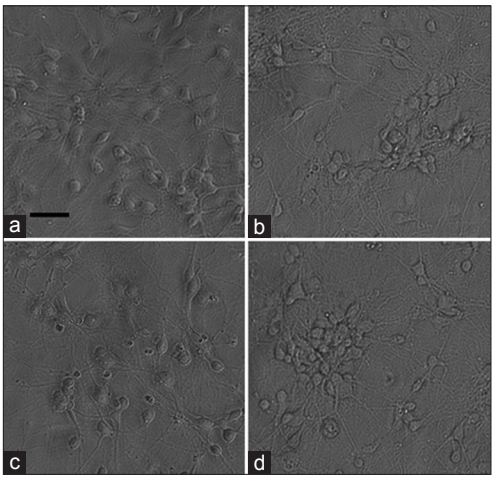

Figure 4: Photomicrographs showing the neurotoxic effects of amyloid-β peptide (Aβ1-42) on the morphology of neurons and the protection afforded by serum anti-Aβ antibody. Scale bar = 50 μm [Figure (a) Control; (b) Aβ1-42; (c) Aβ1-42 + anti-Aβ antibody (1:10); (d) Aβ1-42 + anti-Aβ antibody (1:100)]

compared with the most other virus vectors.[18] Secondly, AAV is safe since wild AAV is not pathogenic to human or other species and AAV DNA normally does not integrate into the cellular genome, rather, it remains in the episome.[19] About 50-90% of the population is seropositive for AAV and presence of the virus has been detected in the human genital tract.[20,21] However, there has been no conclusive evidence of any association of AAV with disease or pathology so far. Furthermore, AAV vector could sustain long-term expression of a protein in large animal models after a single administration in skeletal muscle. It is reported that after intramuscular injection of AAV vectors in non-human primates, a rise in the level of vector-expressed secreted proteins is seen in the serum during the first 2-3 months the protein is still highly expressed 6-8 months post-injection.[22] After intramuscular injection of AAV vectors in mouse, a gradually increase in transgene expression is also detected at the first 60 days, and the expression sustained without a significant diminution

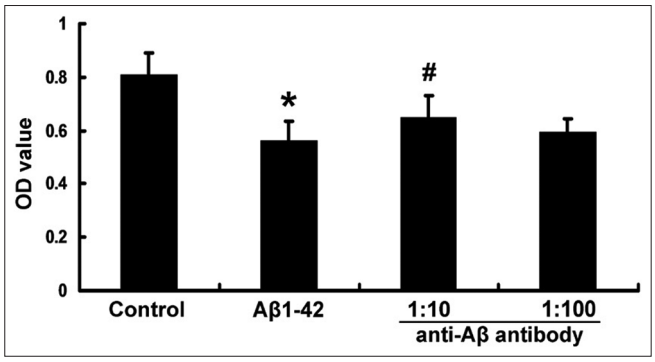

Figure 5: Effects of sera from vaccinated mice with adeno-associated virus-amyloid-β peptide (Aβ15) on the viability of primary neurons induced by Aβ1-42. The MTT assay was used to estimate the cell survival. Values given are mean ± SD **P* < 0.05 versus control group. #*P* < 0.05 versus Aβ1-42 group

over 240 days.[23] In the end, AAV has been shown to be highly efficient in infecting neuronal cells.[24] Therefore, we think that AAV is a desirable candidate for treating the degenerative diseases of the central nervous system, such as Alzheimer's disease, Parkinson's disease, Huntington's disease etc.

In the present study, we took advantage of the merits of short Aβ immunogen and AAV vector, constructed a new AAV-Aβ15 gene vaccine. Our findings revealed that intramuscular injection with the AAV-Aβ15 could induce the generation of the IgG antibodies against Aβ1-42 peptide in BALB/c mice the IgG antibodies could be detected at 1 month post-injection, gradually increased in the first 2 months, and then remained constant without significant diminution until 4 months. Furthermore, the IgG antibodies could recognize the full-length β-peptide 1-42 and play an anti-aggregating role as antibody raised against Aβ1-42 peptide, thereby protected the neuronal viability against Aβ induced damage. The above data provide preliminary experimental basis for the protective effect of the vaccine AAV-Aβ15, in our later further research, we will clarify the type of protective antibodies, observe the immunization effect of the AD transgenic mice and take measures to avoid adverse effects as much as possible.

Acknowledgment

We thank Che-jiang Wang for his efforts in data management and his technical support. Ling-Yun Liu, Yuan-Yuan Ma,these authors contributed equally to the work.

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How to cite this article: Liu L, Ma Y, Yang T, Li X, Li W. The neuroprotective effect of immune serum of adeno-associated virus vaccine containing Aβ1-15 gene on amyloid toxicity. Ann Indian Acad Neurol 2013;16:603-8.
Received: 28-09-12, **Revised:** 22-02-13, **Accepted:** 11-03-13

Source of Support: This research was suppored by foundation of Shanghai municipal health bureau (2008/048),
Conflict of Interest: Nil