HUMAN PLASMA GELSOLIN GENE DELIVERED VIA AAV-8 RETARDS MEMORY LOSS IN APP/PS1 MICE

BY

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A THESIS PRESENTED TO THE GRADUATE SCHOOL
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To my son, Alex Chen Wang

ACKNOWLEDGMENTS

I would like to thank my parents, Zhongxin and Shuyun Wang, for all of their love and support. I also need to thank my wife, Xiuhua Chen; and my son Alex for their inspiration, love, and support.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

AAV	Associate Adeno Virus
Aβ	amyloid beta
Ach	acetylcholine
AD	Alzheimer’s disease
APP	Amyloid Precursor Protein
BBB	blood brain barrier
CNS	central nervous system
CSF	cerebrospinal fluid
ELISA	Enzyme Linked Immunosorbent Assay
FAD	familial Alzheimer’s disease
HRP	Horseradish peroxidase
IDE	insulin-degrading enzyme
LOD	limit of detection
LOQ	limit of quantitation
NFTs	neurofibrillary tangles
NMDA	*N*-methyl *D*-aspartate
PS	presenilin
RAWM	radial arm water maze
MWM	morris water maze
RAGE	receptor for advanced glycation end

Abstract of Thesis Presented to the Graduate School
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Alzheimer's disease (AD) is the most common form of dementia. It is progressive and unrecovered. Memory loss is the earliest and important syndrome. AD is characterized by a progressive accumulation of extracellular amyloid plaques, and intracellular neurofibrillary tangles (NFTs) in the brain. Evidence suggests that the increased production or decreased clearance of amyloid β (Aβ) which is the main constituent of amyloid plaques triggers a cascade that ultimately leads to Alzheimer's pathology, making it a promising target for the treatment of AD. One strategy targeting Aβ that has shown promise is increasing its clearance by binding Aβ peripherally, either by immunization or treatment with agents that bind Aβ creating a peripheral sink that shifts the equilibrium of free Aβ from the central nervous system (CNS) to the periphery, then reduce Aβ burden in the brain and increase the clearance of Aβ.

One such agent is a protein named plasma gelsolin, which has 6 subunits. Evidence shows that gelsolin can bind to Aβ to prevent Aβ deposits and disassemble Aβ fibrils. Furthermore, even delivery of the plasmid of the gene for plasma gelsolin, which can be expressed peripherally, in a mouse model of Alzheimer's disease can

decrease Aβ burden in the brain. But there still are some questions around the treatments of AD. For example, in 2008, more than 100 clinical trials of AD failed, including the treatments targeting Aβ. And we notice that most treatments need multiple dosing because the traditional medicines are not reproducible by themselves.

In order to found an ideal treatment method for one dose life long term treatment, we develop a new gene therapy method for AD by focusing on the promising target Aβ. We packaged the coding sequence for human plasma gelsolin into an AAV-8 (Associate Adeno Virus) vector. Then we peripherally delivered, via the tail vein, this vector into APP/PS1 mice, a kind of AD mouse model.

After behavior training, we find that peripheral expression of plasma gelsolin retards the memory loss in treated mice compare with control mice. We found that treatment reduced Aβ burden in the hippocampus. Our results suggest that the reduced memory loss in treated mice may be due to reduced Aβ burden in the brain. These results show that we may have developed a new method for one dose life long term treatment for AD which has been characterised with Aβ burden in the brain.

CHAPTER 1
INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia. It is estimated that there are 5.3 million Americans have AD in 2010. [8] And this number is still increasing. The number of Americans impacted by AD is estimated more than 10 million in 2050. This increased numbers of AD patients can be attributed to the aging of the population. [1, 2] Alzheimer's disease would impact a lot on a person's life quality. [3] Besides, as the disease progresses, independence decreases, this would place a huge financial problem on social and family caregivers. Total payments for AD are expected to be $172 billion in 2010 based on 2004 data. [4, 5, 6, 7] So it is important to find an efficacious treatment for AD to ease the social and family burden of the disease.

Dementia

"Dementia" is taken from Latin meaning "madness". [9] It is characterized by the loss in memory and cognitive abilities which is caused by many different diseases that would damage brain cells. The syndrome of dementia is the loss of abilities of language, perception, judgment and memory.

Discovery of Alzheimer's Disease

In 1901, German psychiatrist Alois Alzheimer met a patient named Mrs. Auguste Deter who had behavioral problems, including a loss of short-term memory. Alzheimer followed her case until she died and he was the first scientist who reported the case publicly. [10] The disease was first described as a distinctive disease by Emil Kraepelin. [11,12] Since then Alzheimer's disease has been recognized as the most common form of dementia in the world.

Current Therapies

Currently only four drugs in two groups have been approved by the FDA for the treatment of AD. Three are acetylcholinesterase inhibitors (donepezil, rivastigmine, and galantamine) and the other one is an NMDA receptor antagonist (memantine). The activity of the cholinergic neurons is reduced in AD. [13] Acetylcholinesterase inhibitors can be used to decrease the catabolism of acetylcholine (ACh), which is lost in AD due to the death of cholinergic neurons. [14] Increasing the level of acetylcholine can improve the memory and learning abilities. [15] Memantine is a noncompetitive NMDA receptor antagonist which is used to block NMDA receptors and inhibit their overstimulation by glutamate whose elevated levels may lead to neuronal dysfunction. [16] Both treatment have just shown modest improvements in Alzheimer's disease patients with behavioural problems, but they are far from an ideal treatment. They only delay the progression of AD and are not a cure. [17, 18]

Cause of Alzheimer's Disease

Senile plaques are a characterization of AD. In 1983, Allsop identified that a 40-42 amino acid peptide named amyloid beta Aβ is the main element. [19] This peptide come from a larger protein named amyloid precursor protein (APP) after APP had been cleaved by secretases. [20] The discovery that all of the autosomal dominant familial AD can be attributed to mutations in one of three genes: amyloid precursor protein (APP) and presenilins 1 and 2. [21, 22, 23, 24] Most mutations increase the production of Aβ42. Some of the mutations only alter the ratio between Aβ42 and the other major form Aβ40 without increasing Aβ42 levels. [25, 26] This suggests that presenilin mutations can cause disease even if they lower the total amount of Aβ. All these results led to the amyloid cascade hypothesis proposed by Hardy and Allsop, which suggested that the

mismetabolism of APP triggers AD pathogenesis. And formation of neuritic plaques would induce a reduction of neurotransmitters, and then intrigue the death of neurons and dementia, it means that increased concentration and deposition of Aβ in the brain may be a cause of AD. [27] Although autosomal dominant familial AD is less than 0.1% of all Alzheimer's cases, every autosomal dominant familial AD involves the Aβ oligomerization and precipitation. [28] Since 1991, the amyloid cascade hypothesis turn to be the fundamental cause of the disease. [29,30] Further support evidence that transgenic mice that express a mutant form of the human APP gene develop amyloid plaques and Alzheimer's-like brain pathology with spatial learning deficits. [31,32,33,34]

Amyloid Precursor Protein

Human Amyloid Precursor Protein (APP) is a transmembrane protein. It is expressed in many different types of cells. [35] Scientists have identified ten isoforms of APP, which are created by alternative splicing. [36] There are three major isoforms, C, B, and A which have 695, 751, and 770 amino acids, seperately. The C isoform is primarily present in neurons. [35]

The primary function of APP is not known, though evidence suggests it may play roles as regulator of synapse formation and neural plasticity. [37, 38] Besides, the roles of APP in cell signaling, long-term potentiation, and cell adhesion have been proposed and supported by limited research. [39] Although the function of APP is not clear, APP knockout mice showed relatively minor phenotypic effects of impaired long-term potentiation and memory loss comparing with transgenic mice of upregulated APP expression. [40, 41]

Amyloid Beta

Amyloid beta (Aβ) is formed after cleavage of the amyloid precursor protein (APP). There are three cleavage sites identified on APP. The proteases for the cleavage are known as α-, β- and γ-secretases. Aβ protein is generated by β and γ secretases cleavage. First, β-secretase cut APP on the extracellular site, and then γ-secretase cut the remaining part on the transmembrane site, the fragment between the β- and γ-secretases cutting sites is Aβ. The most common isoforms of Aβ are Aβ40 and Aβ42. The Aβ40 form is more common, but Aβ42 is the more important to oligomerzation and fibril formation and associated with the disease. [42] Evidence showed that autosomal dominant familial AD increased the relative amounts of Aβ42 by comparing with Aβ40. [43,44,45,46] Amyloid β is first released as a soluble monomer with α-helical secondary structure, then structure changes to β-sheet structure as soluble oligomers. The soluble oligomers aggregate to protofibrils and fibrils, finally come together to make up the plaques. [29]

Amyloid Cascade Hypothesis

One of the major questions in AD research is whether Aβ is the cause of the pathogenic process. The hypothesis that Aβ is central to the pathogenesis of AD is known as the amyloid cascade hypothesis which was proposed in 1991 by John Hardy and David Allsop. [29] This hypothesis suggests that the mismetabolism of APP would induce AD due to increased Aβ level, specifically Aβ42, increased Aβ polymerized to oligomers, and then oligomers aggregate to fibrils and deposit as plaques. The plaques would active of microglia and astrocytes, make them release pro-inflammatory cytokines, finally induce neuron injury and lead to dementia. [47, 48, 49]

The evidence support the amyloid cascade hypothesis is that every form of familial Alzheimer's disease (FAD) involves mutations of either APP or the enzymes that cleave APP, make an increase level of Aβ42. [50] And transgenic mice expressing mutations of APP and PS1 would increase Aβ levels and amyloid plaques. [51] Large amounts of Aβ have been shown to be neurotoxic to cells via a variety of ways. [52, 53, 54, 55, 56, 57, 58] In normal metabolism, Aβ are degraded by amyloid degrading-enzymes, which include the insulin-degrading enzyme (IDE) and neprilysin. Extracellular Aβ can be cleared and exported via binding of A2M and APOE to LRP, and degrade through the lysosomal pathway. Aβ can also be removed from the brain and into the periphery by LRP mediated endocytosis, and then degraded peripherally. Endosomal dysfunction is one of pathologies of sporadic AD and abnormally enlarged endosomes may induce Aβ deposition. [59] These data suggest that increased level of Aβ can initiate AD.

But there are some research not support amyloid cascade hypothesis. [60, 61] They point out that Aβ can be toxic in vitro; but it did not show in animal models, the in vitro toxicity may due to artifact cause. They showed that neurofibrillary tangles (NFT) and neuron numbers may be better parameters than amyloid beta. [62] And there are some evidences that soluble Aβ42 oligomer had higher correlation with memory loss than Aβ deposits may due to Aβ plays as an antioxidant or a neurotrophin. [63, 64, 65, 66, 67, 68, 69, 70] Although there are some evidences argue the amyloid cascade hypothesis, the majority of the evidence suggests that Aβ is the initiating key factor in the development of Alzheimer's disease.

Sink-Hypothesis

The observations that immunization of Aβ could reduce the Aβ burden in mice and prevent memory loss make Aβ as a promising target to treat AD. [71, 72, 73] Although the clinical trial has failed due to the reason that the 6% patients developed meningoencephalitis, the following studies showed that antibody producers get significantly improved memory ability. [74, 75]

"Sink theory" had been proposed to explain how immunization works, the hypothesis is shifting the equilibrium of free Aβ from the CNS to the periphery supported by the finding that less than 0.1% of antibodies in the serum across the blood brain barrier (BBB). [76] And the support evidence for the sink theory come from the studies of many different Aβ binding agents were administered peripherally. Soluble truncated RAGE protein (receptor for advanced glycation end) which can bind Aβ treated PD-hAPP mice showed significant increase in plasma Aβ level along with a decreased Aβ level in brain. [77] Similar results have been shown by treating APP/PS mice with the Aβ binding agents GM1 and plasma gelsolin. [78, 79] Our study focused on the effects on the relationship of the memory loss and amyloid deposition by plasma gelsolin gene expression.

Gelsolin

Gelsolin is an 82-kD, with six subunits, actin-binding protein which play a key role on actin polymerization and actin filament depolymerization. [80, 81] Gelsolin can be divided into two groups by location, which are intracellular (cytoplasmic gelsolin) and extracellular (plasma gelsolin). [82] Gelsolin's major function is that work as an actin-binding protein for its polymerization and actin filaments depolymerization. [83] The gelsolin function is regulated by Ca^{2+} and phosphoinositides. [84] Intracellular gelsolin is in

the cell, whereas the plasma gelsolin is a secreted protein with a signal peptide. [85] Both kinds of gelsolin come from same gene but splicing on different sites, also, their structure are different and plasma gelsolin has 25 amino acids residue on amino-terminus compare with the intracellular gelsolin. [86] And the concentration of gelsolin in the plasma is 179 μg/ml. [87]

Several studies suggest that gelsolin involved in other events besides regulate actin polymerization which include calcium channels and *N*-methyl *D*-aspartate (NMDA) receptors, apoptosis modulation, tumor suppression, immune reactions and cell motility and endocytosis. [88, 89, 90, 91,92,93, 94, 95, 96, 97, 98, 99, 103] An important result is a gelsolin mutation would induce an aberrant cleavage of it and produce a 68kD anomal gelsolin fragment to be secreted and then deposited in the Finish type of amyloidosis. [100, 101, 102] But non-mutated gelsolin plays a different role in AD, which is emphasized below. Plasma gelsolin could against inflammatory reactions which is induced by injury. [104, 105] And plasma gelsolin can reduce the viscosity of cystic fibrosis sputum. [106] Some evidences showed that human plasma gelsolin can bind to Aβ to prevent Aβ fibrillization, and even to disassemble Aβ fibrils, suggest a possible role of gelsolin in the amyloid beta clearance. [107, 108] A fact that gelsolin level in AD patients' was significantly lower than controls suggests that decreased gelsolin concentration may be a cause in the increased amyloid beta level in AD. [109] Matsuoka reported that inject bovine plasma gelsolin can prevent Aβ deposit in the brain in younger huAPP K670N,M671L/ PS-1 M146L mouse, but they did not see the gelsolin's effects in older mice and on memory related behavior. [110] Furthermore, Hirko showed that peripheral deliver plasmid of

plasma gelsolin can decrease Aβ burden in the brain in APP/PS1 mice, but they did not find correlation between the reductions and memory related behavior. [112]

Gene Therapy

Gene therapy is a method through transfer genes into an patients to treat disease. There are three ways to deliver genes into patients, directly delivery, non-virus vector and virus vector delivery. Since most traditional treatments, directly delivery and non-virus vector delivery are short-term, multiple dosing treatments, virus vector delivery show certain advantage that it provide a long-term, one dose treatment which will be cheaper, more convenience to use in the future.

There are several virus vectors that can be used for gene delivery. The recombinant Adeno-associated viruses (rAAV) would be a good choice. rAAV does not contain any virus genes but only the therapeutic using gene so that it could not integrate into the patients genome and this character would make it safer than others. There are a few disadvantages to using rAAV, including the low DNA capacity and the difficult to produce. But the production problem has recently been solved by Amsterdam Molecular Therapeutics. [111] Compare to most other virus, most patients who had been treated by rAAV would not be induced an immune reaction to block and clearance the virus so that the treatments would be successful for the disease. The focus of our study is on the effects of peripherally deliver AAV-8 packaged human plasma gelsolin gene expression on Aβ burden in the brain and memory related behavior on AD animal model APP/PS1 mice.

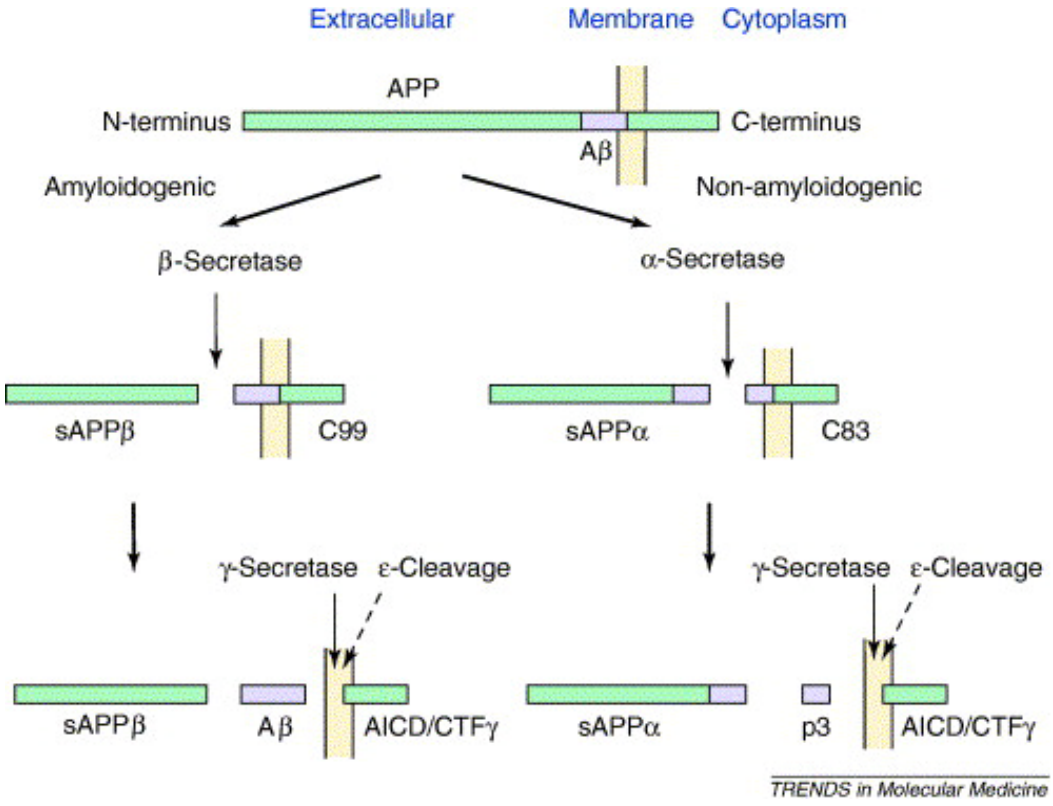


Figure 1-1. Mechanism of APP cleavage to amyloid beta.

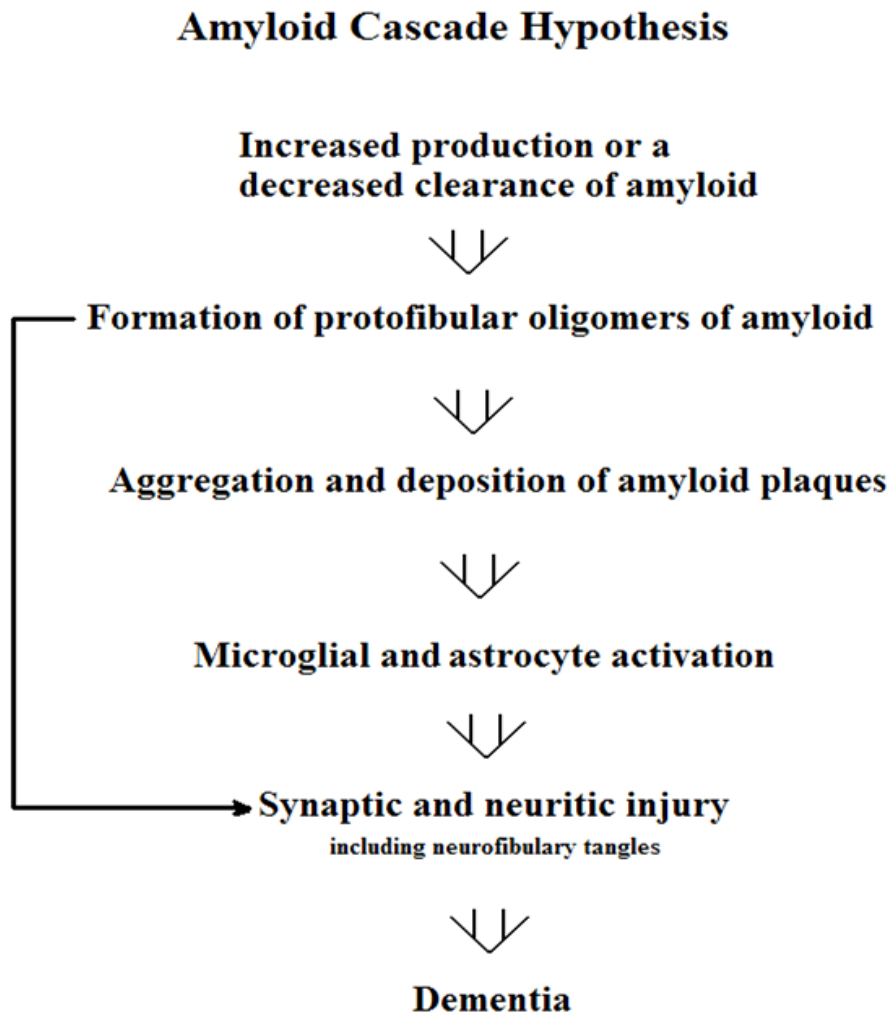


Figure 1-2. Hypothesis of amyloid cascade theory.

CHAPTER 2
MATERIALS AND METHODS

Reagents

Except noted, all chemicals were purchased from Fisher Scientific. Human Aβ42 colorimetric ELISA kits were purchased from Invitrogen (catalog #: KHB3441). Molecular biology reagents and enzymes were purchased from New England Biolabs. The PCR primers were ordered from Sigma Genosys. PVDF membranes were purchased from Bio-Rad Laboratories. His-tagged Human Plasma Gelsolin was purchased from Cytoskeleton. Phosphate Buffered Saline (PBS) was purchased from Mediatech. Gelsolin Antibody (C-18) was purchased from Santa Cruz Biotechnology. His-Tag Polyclonal Antibody was purchased from Cell Signaling Technology. Donkey Anti-Goat IgG (H&L) Horseradish peroxidase (HRP) Polyclonal Antibody was purchased from GenScript. Chitosan and O-Phenylenediamine dihydrochloride tablet set were purchased from Sigma-Aldrich.

Subcloning Vectors

Plasma gelsolin plasmid, pPGLE, was kindly provided by Dr. Hisakazu Fujita. [113] The sequence of plasma gelsolin was removed and then inserted into a pGFP plasmid backbone which is described by Dr. Hirko. [112] Plasmids were propagated in SURE cells and purified. AAV-8 vector packaging purified plasmid is provided by Dr. Zolotukhin. [114]

Large Scale Plasmid Preparation

SURE cells were transformed with the human plasma gelsolin plasmid, using a Bio-Rad electroporator. Transformed bacteria were grown for an hour in 1mL of LB medium at 37°C, followed by plating on LB agar plates containing ampicillin (50mg/L), and then grown overnight at 37°C. Several colonies were selected for screening, and

each was grown overnight in 5 mL of ampicillin-containing LB medium. Plasmids were purified from the cultures, using Qiagen mini plasmid prep kits. Plasmids were then subjected to a BglII digest to confirm. (Figure 2-1)

After we collected the sample of plasmids, we had to pick up the correct clone for large-scale preps since recombined plasmids may lose insert DNA sequence. In order to find the correct clone which still keeps the human plasma gelsolin sequence, we used BglII digest plasmid then run agarose gel to separate the DNA strend, from the results we can pick up the correct plasmids for use. First we placed a comb in the agarose apparatus. Then we prepared a 1% agarose solution in TBE buffer and poured it into the tray. After the gel was set, we added TBE buffer and removed the comb. Mixed the samples with loading dye then loaded samples into the wells 30 ul per well. Also, we loaded lader as a marker. Closed the lid and turned on the power, increased the voltage to 80 V, bubbles should be seen at the electrodes and the bromophenol blue tracking dye should enter the gel. Run the gel until the fastest moving dye has run 3/4 of the gel. Turn off the power. Removed the gel and stain it with ethidium bromide solution (1ug/ml) for 10 min. Drained off the ethidium bromide solution. We rinsed the gel. Finally placed the gel on a UV transilluminator and took a picture. (Figure. 2-1) From picture, we can see sample 3, 4 have two more DNA bands than sample 1, 2 because plasmids sample 3, 4 still keep the human plasma gelsolin DNA sequence.

For large-scale preparations, picked a single colony and inoculate a starter culture of 10 ml ampicillin containing LB medium for 8 h at 37°C with 300 rpm shaking. This was used to inoculate 2 L of ampicillin containing LB medium. We grew the bacteria at 37°C for 12h with 300 rpm shaking again. Harvest the bacterial cells by centrifugation at

6000 x *g* for 15 min at 4°C. Then we used Qiagen Plasmid Mega Kits to purify the plasmid DNA. Resuspend the bacterial pellet in 125 ml of Buffer P1. Added 125 ml of Buffer P2, mixed thoroughly by vigorously inverting 4–6 times, and incubated at room temperature for 5 min. Added 125 ml of chilled Buffer P3, mixed immediately and thoroughly by vigorously inverting 4–6 times, and incubated on ice for 30 min. Centrifuged at 20,000 x *g* for 30 min at 4°C. We collected supernatant. Centrifuge the supernatant again at 20,000 x *g* for 15 min at 4°C. We collected supernatant. Equilibrate a QIAGEN-tip 10000 by applying 75 ml Buffer QBT, and allowed the column to empty by gravity. Added the supernatant into the QIAGEN-tip and allowed it to enter the resin by gravity. We washed the QIAGEN-tip with 600 ml Buffer QC. Eluted DNA with 100 ml Buffer QF. Precipitate DNA by adding 70 ml isopropanol to the eluted DNA. Mixed and centrifuge immediately at 15,000 x *g* for 30 min at 4°C. We decanted the supernatant. Washed DNA pellet with 10 ml 70% ethanol, and centrifuge at 15,000 x *g* for 10 min. Decanted the supernatant. Air-dry the pellet for 10–20 min, and redissolve the DNA in 100ul water. Concentration and purity of the samples were determined by UV absorbance at 260/280 nm.

Cell Culture

Human embryonic kidney (HEK) 293 cells were cultured in DMEM with 10% fetal bovine serum (FBS), 100 units/mL penicillin and 100 µg/mL streptomycin (Gibco, Invitrogen, CA) in a 5% CO2 incubator at 37°C.

Animals and Procedures

All procedures were performed with approval of the University of Florida's Institutional Animal Use and Care Committee. Double-transgenic mice expressing both of mutant $APP695_{K594N,M595L}$ and mutant presenilin-$1_{\delta E9}$ ($APP/PS1_{\delta E9}$ mice) were

supplied by the Jackson Laboratories. [115] Gelsolin transgene expression was accomplished by injecting 10^{11} rAAV8 viral genomes per mouse via the tail vein. Because the Aβ deposit will be formed starting around 3 months old for the APP/PS1 mice, the n=16 APP/PS1 male mice were divided into 3 groups: control group: no treatment; young age treatment group: treatment at 4 months old; older age treatment group: treatment at 6 months old. The Morris Water Maze test was used to evaluate memory. The mice were trained 4 one minute trials per day for 9 days, then a probe test for memory evaluation was performed. The Radial Arm Water Maze test was used to train the mice in the same way as the Morris Water Maze, except probe evaluation was done daily. And the n=24 APP/PS1 female mice were divided into 2 groups: control group: no treatment; treatment group: treatment at 4 months old. The Radial Arm Water Maze test was used to train the mice. During the period, all blood samples were collected from mice arteria caudilis by using plasma collect tube and kept in ice. Then the tubes were centrifuged at 1000*g for 5 minutes, the blood cell and plasma were separated by gel in the tube, blood cells below the gel, plasma above the gel.

Finally, animals were anesthetized with isoflourane and perfused with phosphate-buffered saline (PBS). The brains were excised and divided into two halves. One hemi-brain was frozen in liquid nitrogen and stored at -80 °C for analysis by ELISA. The whole hemi-brains from male mice were analysis by ELISA, but we just picked up hippocampi of the hemi-brains to do ELISA analysis from female mice. The remaining hemi-brain was fixed for 48 hours in a 4% paraformaldehyde in PBS solution and then cryoprotected in 30% sucrose PBS solution. We then performed brain slicing and Thioflavin-S staining to obtain Aβ deposits.

Western Blot

For western blot analysis of plasma samples 4μL of plasma was diluted in 21 μL of distilled water and then mixed with 25 μL of 2X Laemmli sample buffer with 5% β-mercaptoethanol, boiled for 5 minutes, and then loaded onto a precast SDS 10% PAGE Tris-HCl gel. Gels were run using a Biorad power supply set at 100 V for one hour.

Separated proteins were then transferred to a PVDF membrane at 75V for 2 hours on ice. Membranes were incubated overnight in a blocking solution (5% nonfat dry milk and 0.05% Tween 20 in PBS) at 4°C. Primary antibodies were then added monoclonal anti-gelsolin antibody from CDBR lab in UF was used at 1:1000 dilution and incubated at room temperature for 2 hours. Membranes were washed three times in PBS with 0.05% Tween 20, and incubated with horseradish peroxidase (HRP)-conjugated anti-mouse antibody [Amersham (Piscataway, NJ) at 1:5000] in blocking solution for 1 hr at room temperature. Following three more washes, they were incubated with substrate [electrochemiluminescence (ECL), Amersham(Piscataway, NJ)] for 1 min and exposed (Kodak, Rochester, NY).

Enzyme Linked Immunosorbent Assay for Aβ

For enzyme linked immunosorbent assay (ELISA) Biosource colorimetric immunoassay kits were used for both β amyloids 1-40 and 1-42. Frozen hippocampi were homogenized in 10μl/mg carbonate buffer (100mM sodium carbonate, 50mM NaCl, protease inhibitors, pH 11.5) and centrifuged at 20,000 × g for 25min by using a Polytron homogenizer (Brinkmann Instruments). The carbonate-soluble supernatant was collected as soluble Aβ, and the pellet was re-homogenized with 350μl of guanidine buffer (5 M guanidine, 50mM Tris, protease inhibitors, pH 8.0). The homogenate was centrifuged at 20,000 × g for 25 min, and the guanidine extract was collected as

unsoluble Aβ. [116] Aβ quantification was performed by Invitrogen Inc. produced Human Aβ42 colorimetric ELISA kit. Samples were then incubated on a shaking platform at room temperature for two hours in the wells provided with the Biosource kit with an equal volume of primary antibody solution. Samples were then washed four times, and incubated in HRP solution for one half hour. Samples were washed four times again and then incubated for a half hour with HRP substrate, in a box to protect the samples from light. Stop solution was then added and absorbance at 450 nm was measured using a Dynex Technologies MRX microplate reader. Concentrations of Amyloid β were determined from standards provided with the kit.

Sandwich ELISA for Gelsolin

Centrifuge the blood sample (5min). Coated ELISA plate (96 well plate) with his-tag Polyclonal antibody (1μg/μl; 100μL/well). Sealed the plate and incubated overnight at 4°C. Deposed samples and then washed plate with PBS-T 3 times. We blocked plate with BSA (100 μL /well) at room temperature for 1 hour. Disposed solution and then washed plate with PBS-T 3 times. We added PBS as control groups and the standard preparation of 5ng, 10ng, 20ng, 40ng and 80ng, and waited at room temperature for 1 hour. Disposed solution and then washed plate with PBS-T 3 times. We added gelsolin antibody (1μg/μL; 100μL/well),and waited at room temperature for 1 hour, then prepared substrate solution (20mL) of phosphate-citrate buffer with urea hydrogen peroxide tablets. Disposed solution samples and then washed plate with PBS-T 3 times. Donkey anti-goat IgG (H&L) horseradish peroxidase (HRP) polyclonal antibody (0.2μg/μL; 100μL/well) was added. Then we waited at room temperature for 1 hour. Added substrate of 1, 2-phenylenediamine dihydrochoride into the substrate solution of

phosphate-citrate buffer with urea hydrogen peroxide tablets (step 5) and mixed them. Developed color using the mixed solution as a substrate (100µL /well) and incubated at room temperature for 15-30 min. Stopped reaction by addition of 4N H2S04 (50µL/well). Finally, we recorded the absorbance at 490 nm on a plate reader within 30 minutes of stopping the reaction.

New Method of ELISA for Gelsolin

Centrifuge the blood sample (5min). Coated ELISA plate (96 well plate) with sample (1µL/well), blank as control groups and the standard preparation of 1ng, 2ng, 5ng, 10ng, 20ng and 50ng. We added TBS until 100 µL. Sealed the plate and incubate overnight at 4°C. Disposed of samples and then washed plate with PBS-T 3 times. Block plate with BSA (100µL/well) at room temperature for 1 hour. Disposed solution and then washed plate with PBS-T 3 times. Then we add gelsolin antibody (1µg/µL; 100µL/well), and wait at room temperature for 1 hour. Substrate solution (20mL) of phosphate-citrate buffer with urea hydrogen peroxide tablets was prepared. Disposed solution samples and then washed plate with PBS-T 3 times. Donkey anti-goat IgG (H&L) horseradish peroxidase (HRP) polyclonal antibody (0.2µg/µL; 100µL/well) was added, then wait at room temperature for 1 hour. Then we add substrate of 1, 2-phenylenediamine dihydrochoride into the substrate solution of phosphate-citrate buffer with urea hydrogen peroxide tablets (step 5) and mixed them. Developed color using the mixed solution as a substrate (100µL/well) and incubated at room temperature for 15-30 min. Stoppped reaction by addition of 4N H_2S0_4 (50µL/well). The absorbance at 490 nm on a plate reader within 30 minutes of stopping the reaction was recorded.

Histochemistry

Coronal sections (50 μm thick) were cut from the hemi-brains on a sliding microtome with a freezing stage. Four sections, six sections apart each, were mounted on slides for thioflavine S staining. Sections were allowed to dry on the slides for 15 minutes. The slides were then placed in deionized water for five minutes. They were then placed in filtered Mayer's Hematoxylin for five minutes. Next, the slides were rinsed under running water for five minutes, followed by a five minute rinse in deionized water. The slides were then placed in a 1% thioflavine S solution (in dH2O, filtered, Sigma) for five minutes. The slides were differentiated in 70% ethanol for five minutes, given short rinses in deionized water followed by PBS, and cover slipped with glycerol gelatin (Sigma).

Thiazine Red Staining

Free floating sections were stained for 30 minutes at room temperature in 0.01% thiazine red in PBS solution. Sections were washed 3 times in PBS for 5 minutes each time, then mounted and cover slipped using glycerol gelatin.

Image Analysis

For percent amyloid burden measurements (both dense cored and diffuse) sections were analyzed in a blinded manner using the NIH Image J software. Regions of interest (ROI) were created encompassing both the hippocampus and neocortex of digital micrographs of each stained section. The ROI's area was measured in pixels2. The number of plaques stained, plaque sizes (in pixels2), and total stained areas in the hippocampus and cortex (in pixels2) were determined by thresholding segmentation. Total stained areas were divided by total area, and then multiplied by 100% to give the percent amyloid burden.

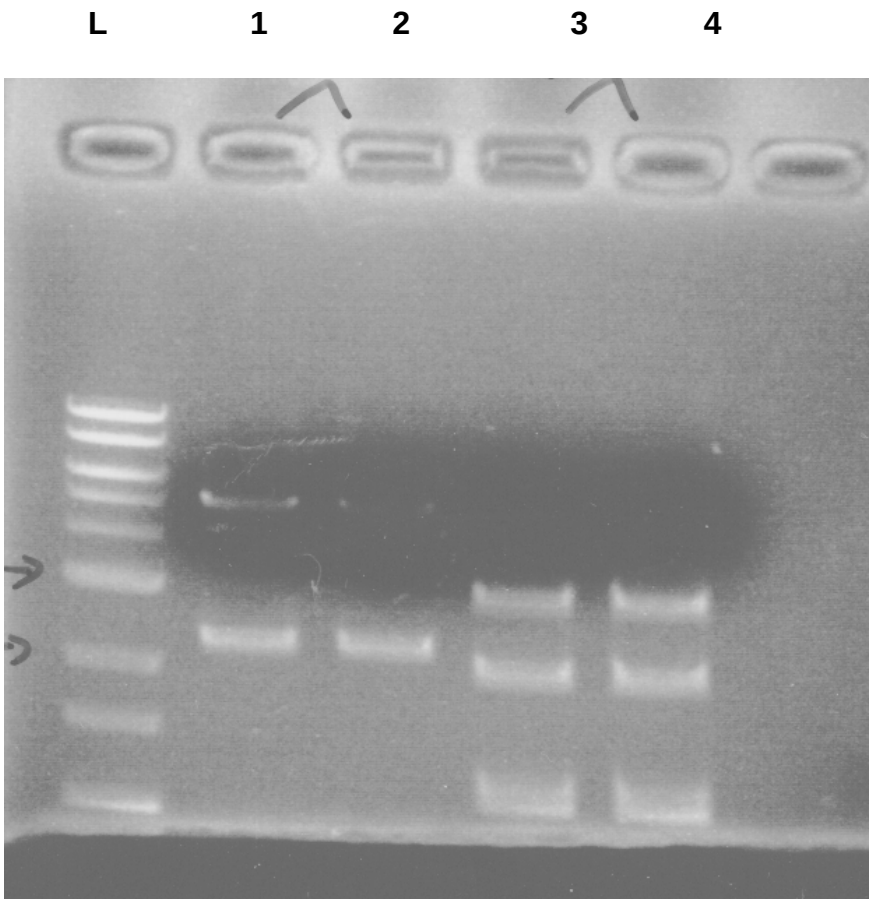


Figure 2-1. HindIII and BglII digest plasmids. L (Laddar), 1, 2 (sample 1, 2), 3, 4 (sample 3, 4). From picture, we can see sample 3, 4 have two more DNA bands than sample 1, 2 because plasmids sample 3, 4 still keep the human plasma gelsolin DNA sequence.

CHAPTER 3
AAV-8 VECTOR WITH HUMAN PLASMA GELSOLIN DELIVERY AND EFFECT IN APP/PS1 MALE MICE BEHAVIOR

Gene Therapy and Behavior Training

Gene therapy is a new method to use specific sequences of DNA or RNA to treat, cure, or ultimately prevent disease. There are still many hurdles to overcome before it could be used in the future as a mature technic. The major hurdle is degradation of DNA or RNA due to endogenous nucleases. The other challenge is the ability of the cells to transcript and translates genes. Another one to overcome is the period that the cells can keep the gene expression on a reasonable level for cure. Our target is the amyloid β that accumulates and deposits in the brain of patients who have Alzheimer's disease. Chauhan showed that human plasma gelsolin can bind to amyloid β, prevents it fibrillization, and even disassembles amyloid β fibrils, giving a possible role for gelsolin of amyloid β clearence. [93, 108] Matsuoka showed injections with bovine plasma gelsolin can prevent deposition of amyloid β in young huAPP K670N,M671L/ PS-1 M146L, but they failed of the testing gelsolin's effects in older mice and on memory related behavior. [110] And Hirko showed peripheral deliver plasmid of plasma gelsolin can decrease Aβ burden in the brain in APP/PS1 mice, however they did not correlate these reductions with retard memory loss. [112] Since gene therapy can be used as a treatment targeting Aβ, and all the traditional treatments are short term, multiple dose treatments, all these results triggered us to ask a question whether we can use rAAV-8 to deliver the human plasma gelsolin gene and test it in an AD mouse model as a life-long, one dose ideal treatment. In this chapter we characterize the relationship between the rAAV-8 with human plasma gelsolin gene treatment and AD model male mice memory ability.

The Morris water maze is a behavioral test used to study spatial learning and memory. It was developed by neuroscientist Richard G. Morris in 1981. [118, 119] In a standard protocol, a mouse is putting into a pool of water with an platform below the water surface. Some signs are placed around the pool. After released, the mouse swims and tries to escape from the water. The whole experiments are taped. During the experiment, the mouse would learn to find the platform, and the mouse would find the platform more rapidly than beginning of the experiment. This improvement of performance due to mouse learned and kept the memory where the hidden platform is. After training, a capable mouse would spent less time before it find the platform. Therefore we used Morris water maze to compare the difference of the memory ability between the treated mice and untreated control mice by probe trial after all mice learned where the platform is located in the pool through the learning trial.

Since standard Morris water maze just can provide one group data to be used for the learning and memory ability analysis in ten days so that the chance of random results are increased, we use the radial arm water maze instead of Morris water maze laterly. Radial arm water maze provide data which can be used for learning ability analysis and we can have one group of data for memory ability analysis each day in ten days so that the chance of random results are decreased. In a typical radial arm water maze system, six arms were placed in a pool of water which would make six swim channels. And signs were put on the walls in each arm. The standard radial arm water maze testing procedure would have four learning trials and one memory trial each day for 10 days. During the experiment, there is a submerged escape platform was put in the target arm; the target arm was randomly changed for each day experiment. And four

starting arms were randomly selected for learning trials, the number of errors during the fourth trial would be an index of learning. Each trial would be 1-min, and the number of errors of going into wrong arms before mouse found platform was recorded. A memory ability trial was then done by using the fourth arm as the starting arm. Over 1-min trial, the number of errors was recorded. Finally, we will have more data than Morris water maze to analysis the learning and memory ability. [120]

Results and Discussion

Behavior Training of Male Mice

Double-transgenic mice expressing both of mutant $APP695_{K594N,M595L}$ and mutant presenilin-$1_{\delta E9}$ ($APP/PS1_{\delta E9}$ mice) were supplied by the Jackson Laboratories. Gelsolin transgene expression was accomplished by injecting 10^{11} rAAV8 viral genomes per mouse via the tail vein. Because Aβ deposits will be formed starting around 6 months old for the APP/PS1 mice, the n=16 APP/PS1 male mice were divided into 3 groups: control group: no treatment; young age treatment group: treatment at 4 months old; older age treatment group: treatment at 6 months old. When mice were at 8 months old, Morris Water Maze test was used to evaluate memory. The mice were trained for 4 times with one minute trials each time per day for 9 days, and then a probe test for memory evaluation was performed. Morris water maze learning curve shows that all male mice shorten the latency to the target platform from around 30 seconds to around 20 seconds after nine days training, indicating that all male mice learned the target platform position. The following probe test results would not be random results. (Figure 3-1) After statistics tests were performed using SAS software based on a one-way ANOVA test, take p-value: 0.05. , results showed that **s**patial memory retention of the 4 month-old treated group was significantly different compared to the control group

because the 4 month-old treated group spend more time in target quadrant area compare with the control group. (Figure 3-2) 6 month-old treated group show similar results but not show significant difference compare with control group.

At the age of 11 and 14 months old, the radial arm water maze test was used to test memory. Training the mice occurred in the same way as the Morris water maze, except probe evaluation was done daily. (Fig.3-3) The results showed that the treated groups make less mistakes before they selected the target arm and found target compared with the control group, but failed to show significant difference base on one way ANOVA test, take p-value: 0.05.

Male Mice Results Analysis

In this chapter we demonstrate that human plasma gelsolin gene delivery via AAV-8 vector in APP/PS1 male mice will retard memory. Treated mice stayed for longer in target area than control mice in the Morris water maze probe test and also made fewer mistakes before they found the platform than control mice during the radial arm maze. We also showed that the earlier age treatment showed more efficacies at preventing memory deficits than the older age treatment in mice. At the same time, we observed that plasma gelsolin gene delivery via AAV-8 did not affect the learning ability of mice.

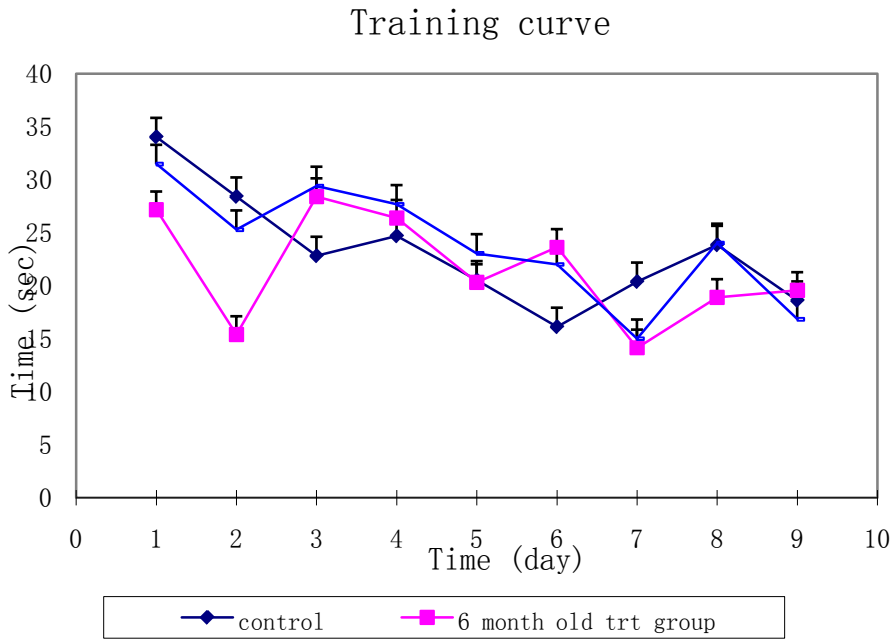


Figure 3-1. Morris Water Maze learning curve. Morris water maze learning curve showed that all male mice shorten spent time to find target platform after nine days training from around 30 seconds to around 20 seconds.

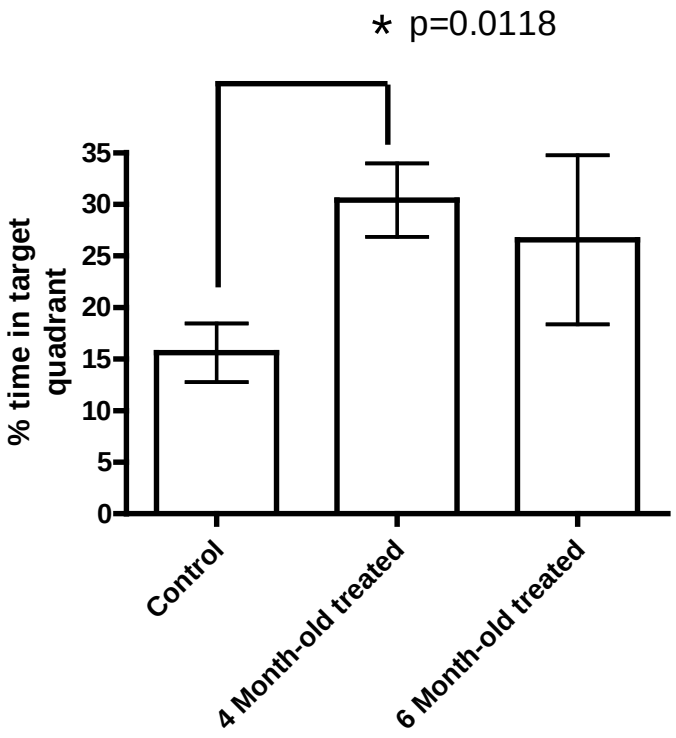
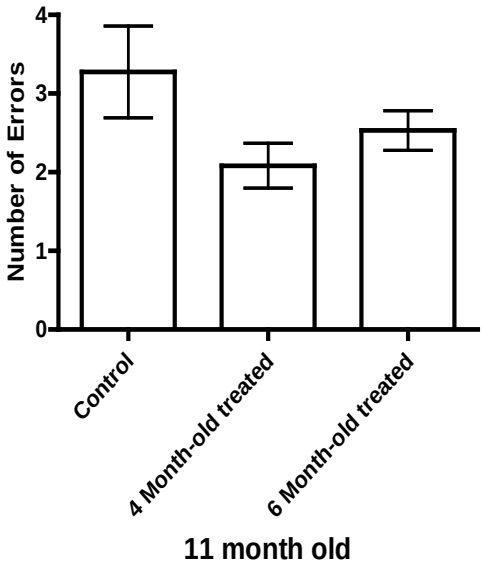
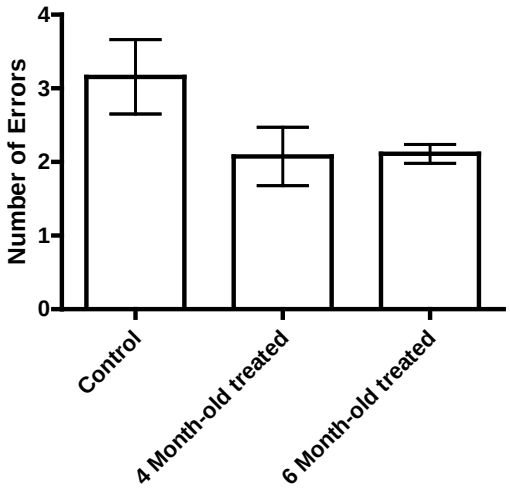


Figure 3-2. Morris Water Maze test. Morris water maze probe test of percentile of time spent in target quadrant area by trained mice. Graph showed significant difference (p value < 0.05) by comparing young age treated group and control group. The treated group spent more time on target area than control group.





14 month old

Figure 3-3. Radial Arm Water Maze test. Radial arm water maze probe test of mistake times made by trained mice. Graph showed treated group make less mistake by comparing with control group.

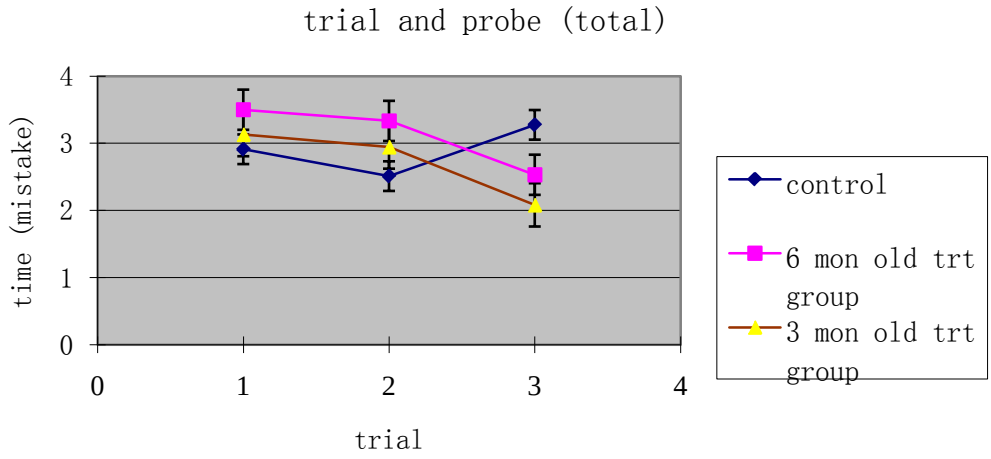


Figure 3-4. Eleven month old male mice radial arm water maze. Treated group made less mistake than control group during the probe test of the memory ability, but the learning ability did not show a difference among three group.

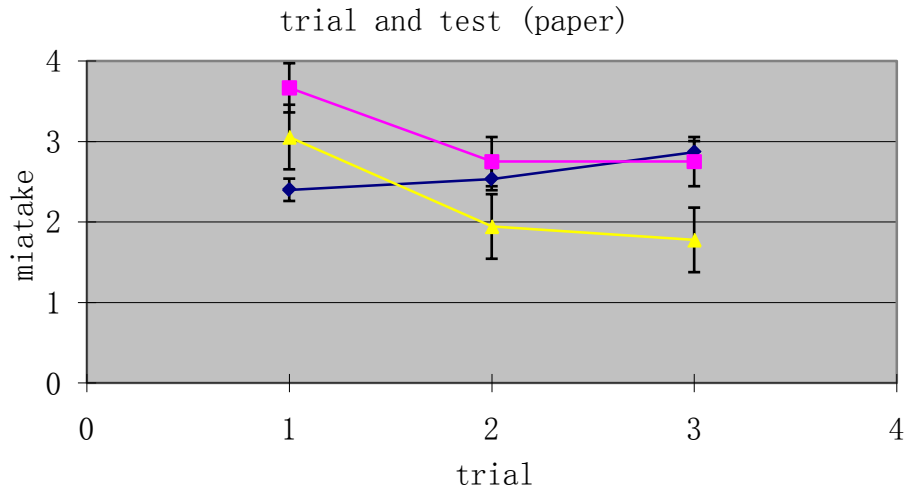


Figure 3-5. Fourteen month old male mice radial arm water maze. Three month old treated group show made less mistakes than control group and six month old treated group during the probe test of the memory ability, but the learning ability did not show a difference among three group.

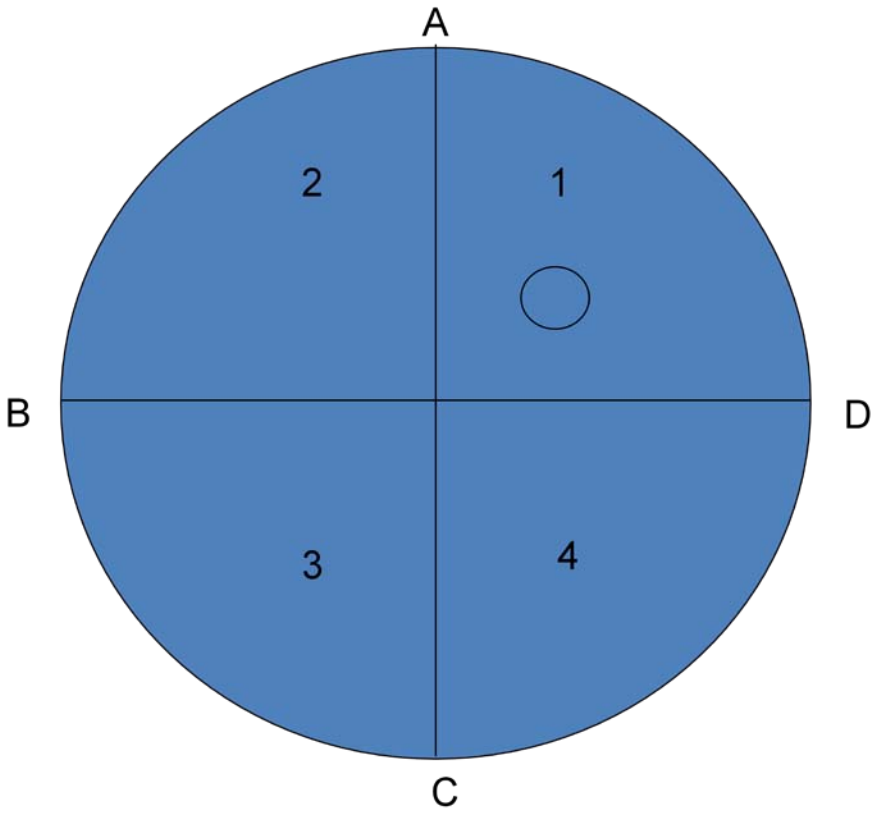


Figure 3-6. Morris water maze system.

Figure 3-7. Radial arm water maze system.

CHAPTER 4
AAV-8 VECTOR WITH HUMAN PLASMA GELSOLIN DELIVERY AND EFFECT IN APP/PS1 FEMALE MICE BEHAVIOR

Behavior Training of Female Mice

From the previous chapter's results, we noticed that the sample size of each group of male mice was too small to have enough power to do statistical analysis and the results of the younger age treatment group had a better effect of treatment than the older age treatment group and control group. Twenty four APP/PS1 female mice were purchased and divided into 2 groups, each group having 12 female mice: control group: no treatment; treatment group: treatment at 4 months old. As a positive control we included 12 same strain wild type female mice. The Radial Arm Water Maze test was used to train the mice instead of the Morris water maze. During the period, all blood samples were collected from mice arteria caudilis by using plasma collect tube and kept in ice. Then the tubes were centrifuged at 1000*g for 5 minutes, the blood cell and plasma were separated by gel in the tube, blood cells below the gel, plasma above the gel.

Results and Discussion

Same strain female APP/PS1 mice were purchased and the same treatment and procedure were used except using Radial arm water maze instead of Morris water maze in order to compare the results with male mice.

When female mice aged to seven months old, eleven months old and sixteen months old, we performed the redial arm water maze to match the male mice behavior training time points. After analysis of the data generated from seven month and eleven month radial arm water maze experiment, we found no differences of learning and memory ability. (Figure 4-1, 4-2) We analyzed the latency to the target and the number

of errors made before they found the target platform. No differences of learning ability and memory ability between the treated and control female mice were observed even when they are sixteen months old. (Figure 4-3, 4-4)

Female Mice Results Analysis

In this chapter we show that human plasma gelsolin gene delivery via AAV-8 vector in APP/PS1 female mice has no effect on the learning and memory ability of female APP/PS1 mice.

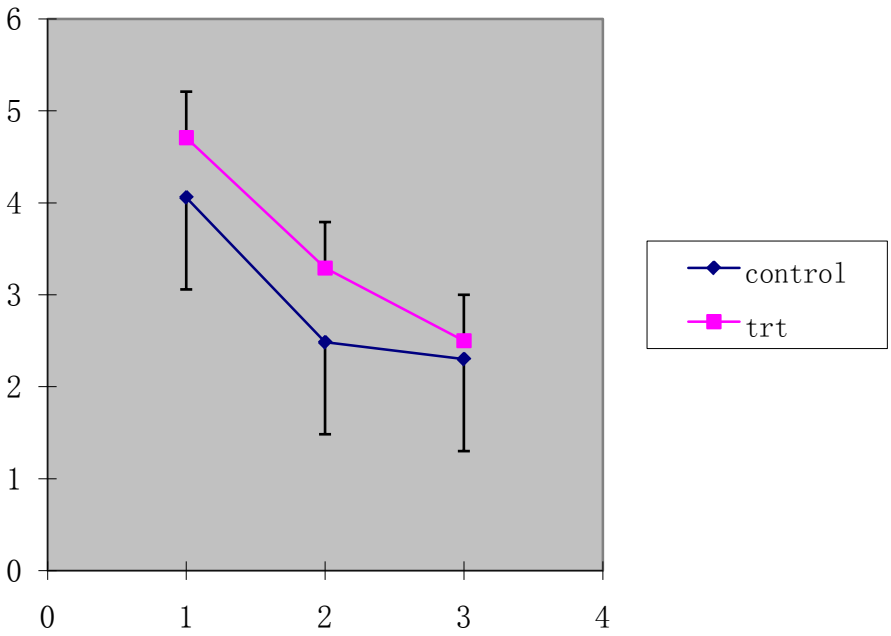


Figure 4-1. Seven month old female mice radial arm water maze.

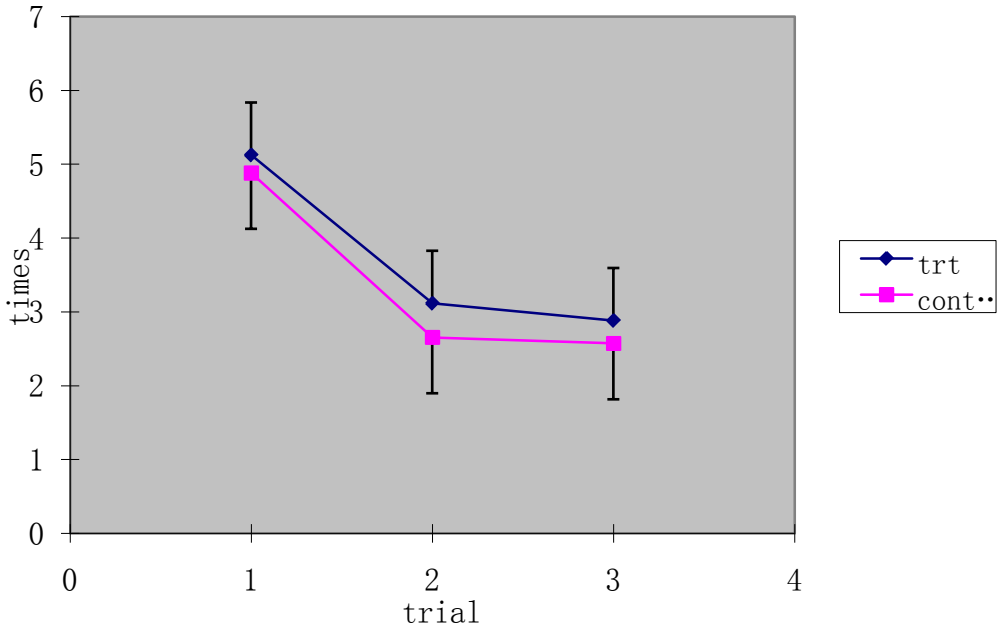


Figure 4-2. Eleven month old female mice radial arm water maze.

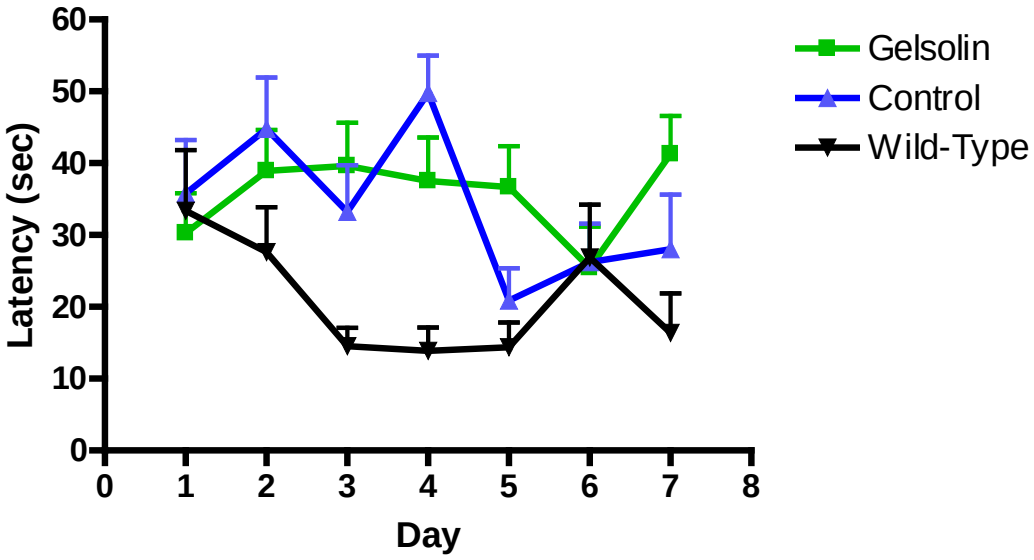


Figure 4-3. Latency time before female mice find target platform.

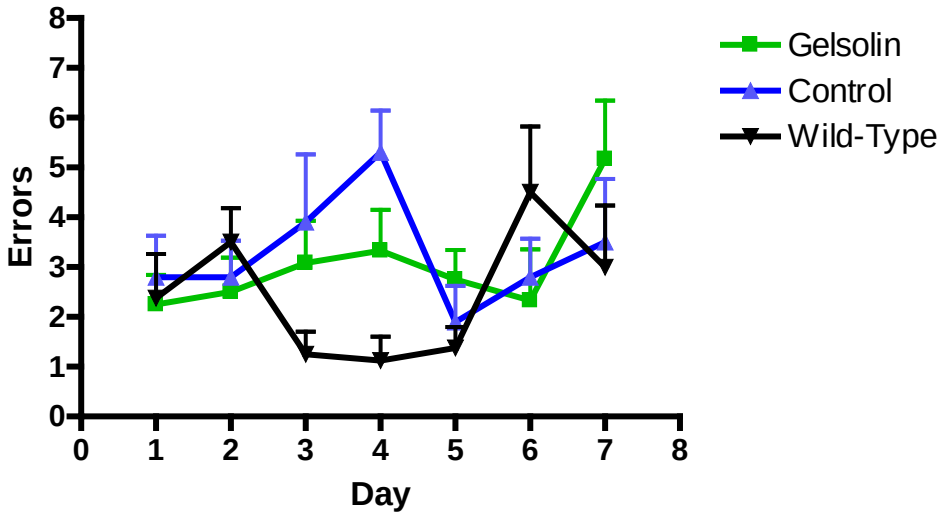


Figure 4-4. Errors made before female mice find target platform.

CHAPTER 5
AAV-8 VECTOR WITH HUMAN PLASMA GELSOLIN DELIVERY AND EFFECT OF AB BURDEN IN APP/PS1 MICE BRAIN AREA

Amyloid Burden in Brain

As shown in the previous chapters, we noticed that Aβ could be used as a promising target to treat AD and APP/PS1 mice would be a good animal model for research of AD since the mutation of APP gene and the mismetabolism of APP would intriguer AD pathogenesis due to increase the level of Aβ, finally induce the aggregation of Aβ in the brain, specifically Aβ42 [21, 22, 23, 24] and transgenic mice that express a mutant form of the human APP gene would develop amyloid plaques, and then intriguer Alzheimer's pathology with spatial learning deficits. [31,32,33,34]

Since Chauhan showed that human plasma gelsolin can bind to amyloid β and prevents fibrillization, [93, 108] Matsuoka showed injections with bovine plasma gelsolin can prevent deposition of amyloid β in young huAPP K670N,M671L/ PS-1 M146L, [110] and Aaron showed peripheral deliver plasmid of plasma gelsolin can decrease Aβ burden in the brain in APP/PS1 mice, [112] all these results give the supports that gene therapy by using human plasma gelsolin gene may be an ideal treatment for AD.

We have shown that AAV-8 with human plasma gelsolin gene delivery can retard memory loss in APP/PS1 male mice, but there still have a question of correlation between the treatment and Aβ burden in the brain. In this chapter, we will character the effect of treatment on the Aβ burden in the brain. And the sink theory may be used to explain how it works after gene expressing.

Results

Dense Cored Amyloid Deposits

Thioflavin S staining was used to examine the amyloid plaques in male mice brain. [121] Thiazine red staining was used to examine female mice. 50μm thick brain slices were sliced and stained (Fig.5-1, 5-6), and digital micrographs were taken as described previously. Images were analyzed in a blinded manner using Image J software. The area of the hippocampus and cortex, total stained area, and the number of plaques was determined. The amyloid burden was determined by dividing the total area stained by the total area of the hippocampus and cortex. A one-way ANOVA was performed for statistical analysis.

The images of the male mice brain slices shown less amyloid deposit in the brain in treated group mice, (Figure 5-1) but female mice show different results. (Figure 5-6). Then we quantify the amyloid burden level by comparing percentage area stained, amyloid plaque numbers, amyloid plaque average size and amyloid beta concentration between treated and control mice. Figure 5-2 shows the male mice amyloid plaques deposit percentage area results, we noticed that 4 month old group has less than the control group in both the hippocampus and cortex area. We then count amyloid plaque numbers and measure plaque size, we observed that the mice in 4 month old age treated group show less numbers and smaller size of plaques compared to the control group, especially in memory related hippocampus area. The mice in 6 month old treated group did not show very close level to control group. (Figure 5-3, 5-4)

After analysised of the female mice results, we find similar results except the plaques numbers for the treated group are increased compared to the control group. (Fig 5-7) Since we increased the sample size, we have more power for data analysis,

and we observe that there are significant differences of the plaques size between the treated group and control group. Also, the hippocampus, an important structure for memory and learning, shows a significant difference of percentage area between these two groups. (Fig 5-8, 5-9)

Brain Aβ Concentrations

The results from the image analysis only show a 2-D level of the amyloid burden. We use an ELISA method to quantify amyloid beta 42 concentration by using frozen half brain samples. Human Aβ42 colorimetric ELISA kits were used to measure the concentration of both soluble and insoluble fractions of amyloid β (1-42) in the hemi-brains from the mice. By comparing the analysis results, we find that the unsoluble Aβ42 concentration decreased and at the same time soluble Aβ42 concentration increased in gelsolin gene delivery mice compare with control mice. Especially in hippocampus area in the brain from the female mice, soluble Aβ42 concentration show significant difference by comparing the gelsolin gene delivery mice compare with control mice. (Figure. 5-5, 5-10)

Amyloid Burden Results Analysis

In this study, we examined the effects of peripheral human plasma gelsolin gene delivery via AAV-8 on Aβ burden in the brain of APP/PS1 mice. From the dense-cored amyloid plaque results, we observe that the treatment reduced Aβ burden in the brain, especially in the hippocampus area, which is important for memory ability. From the Aβ concentration results, we noticed that unsoluble Aβ level in the brain decreased, especially in the hippocampus area.

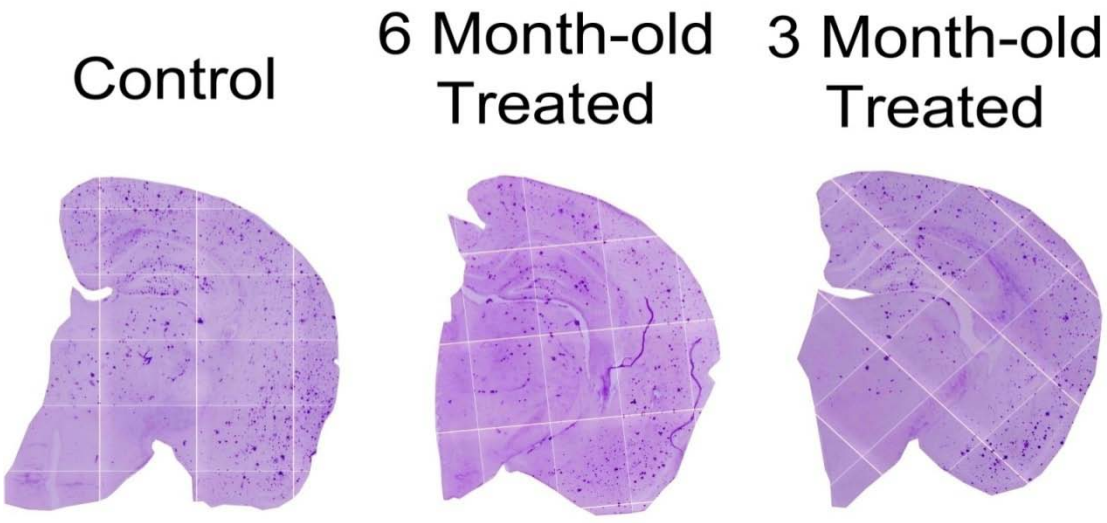
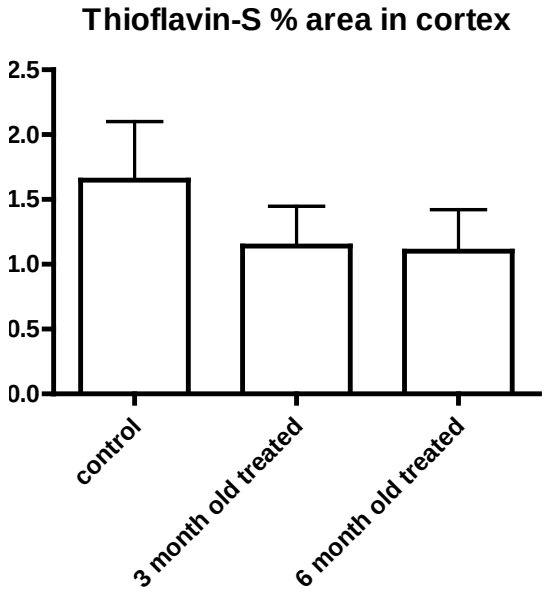


Figure 5-1. Dense-core amyloid deposits in APP/PS1 male mice. Deposits visualized by thioflavin S Staining. Negative digital micrographs of control mouse (left), 6 month-old treated mouse (middle) and 3 month-old treated mouse (right).

a.



b.

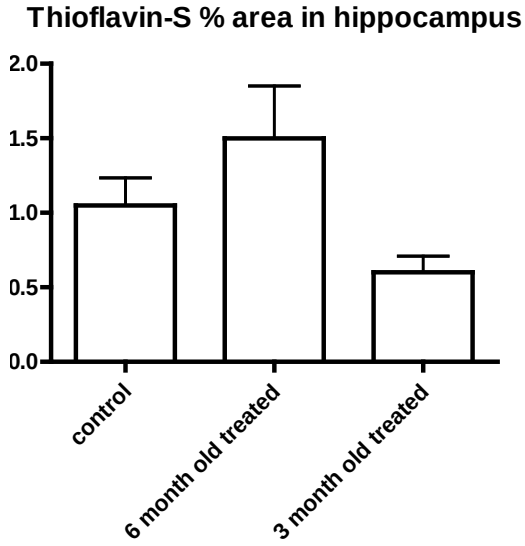
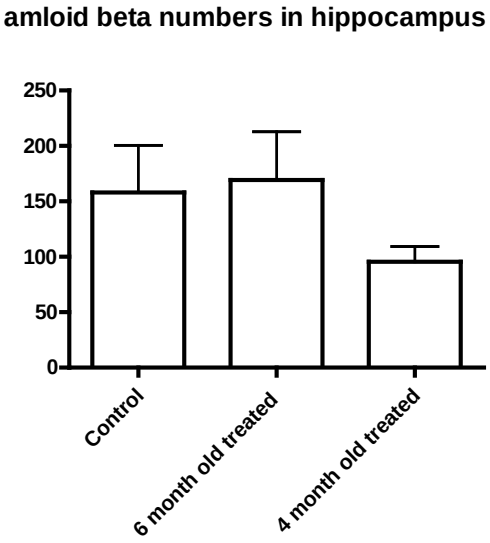


Figure 5-2. Analysis of dense-core amyloid deposits in APP/PS1 male mice. Deposits visualized by thioflavin S staining. a) Percent amyloid burden in cortex area. b) Percent amyloid burden in hippocampus area. Bars represent group means ± the standard error.

a.



b.

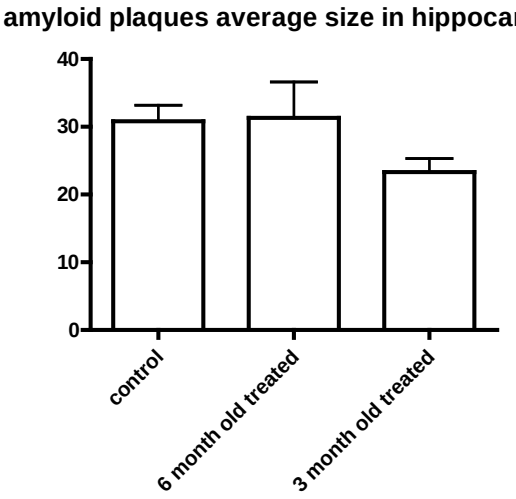
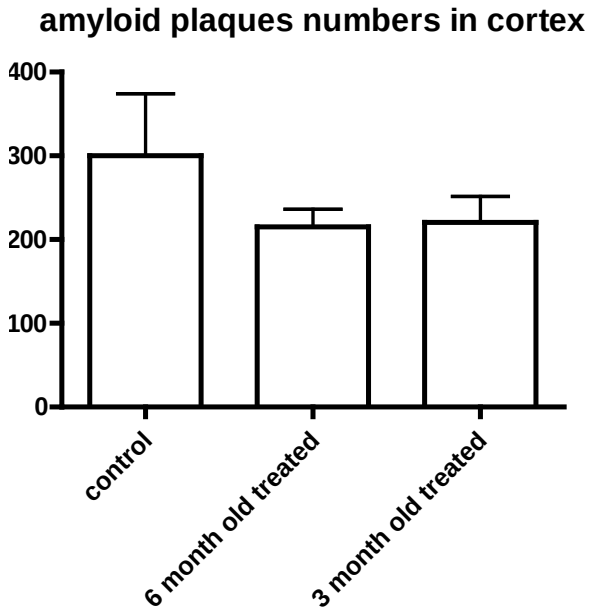


Figure 5-3. Analysis of dense-core amyloid deposits in APP/PS1 male mice hippocampus area. Deposits visualized by thioflavin S staining. a) Amyloid plaques total numbers. b) Average size of amyloid plaques. Bars represent group means ± the standard error.

a.



b.

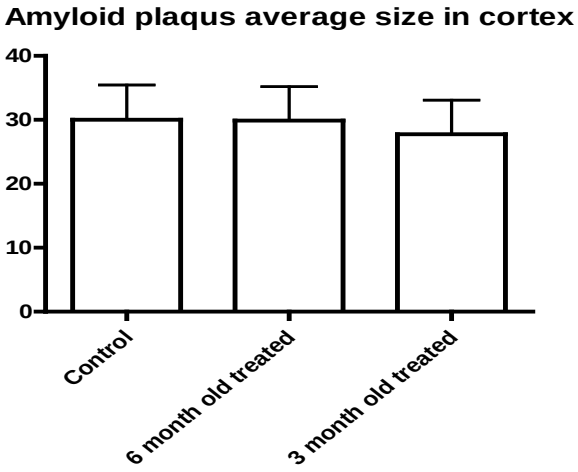
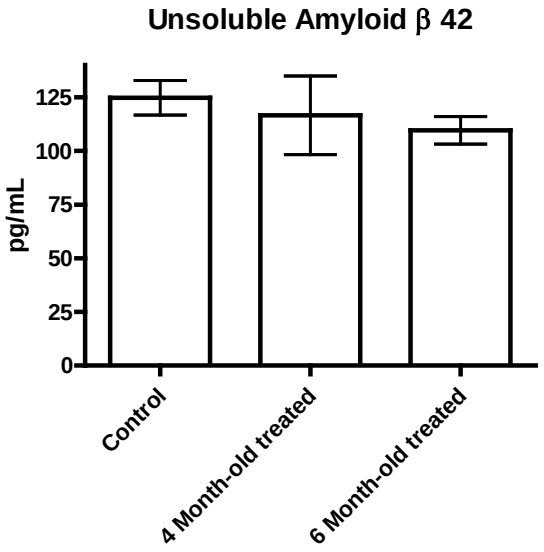


Figure 5-4. Analysis of dense-core amyloid deposits in APP/PS1 male mice cortex area. Deposits visualized by thioflavin S staining. a) Amyloid plaques total numbers. b) Average size of amyloid plaques. Bars represent group means ± the standard error.

a.



b.

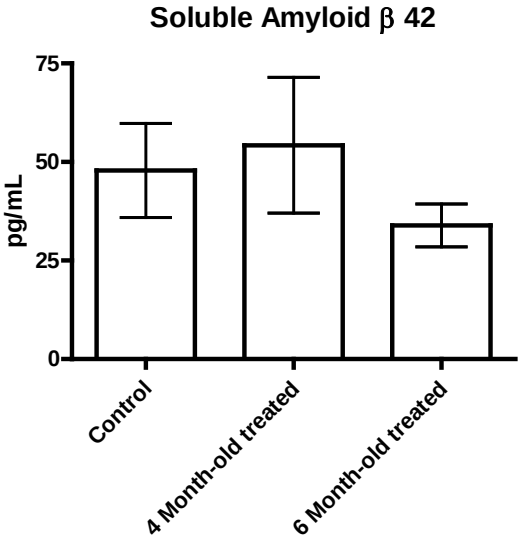


Figure 5-5. ELISA analysis of the Aβ42 concentration in the male mice brain section. Unsoluble Aβ42 concentration is lower and the soluble Aβ42 concentration is higher in the mice which were treated by gelsolin gene delivery than control mice.

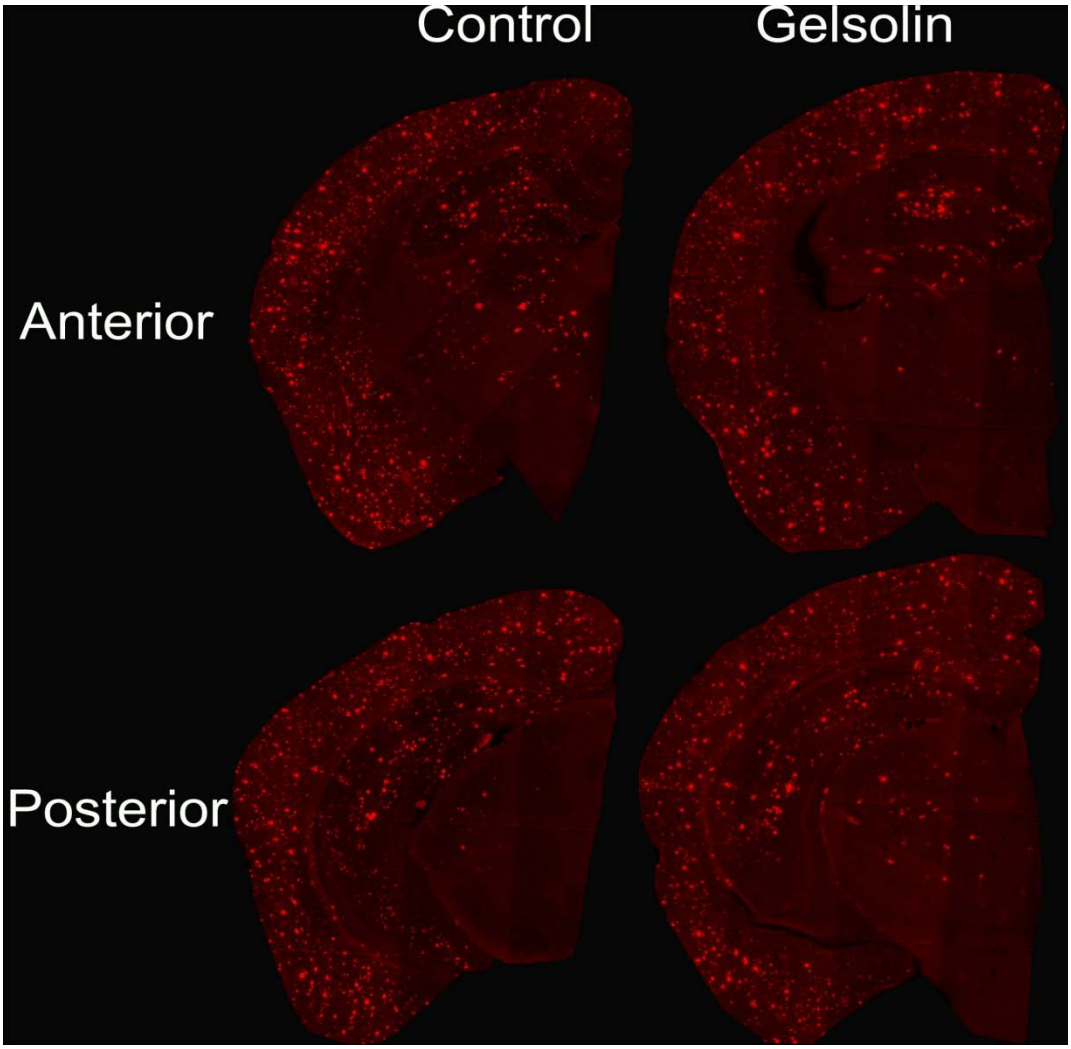
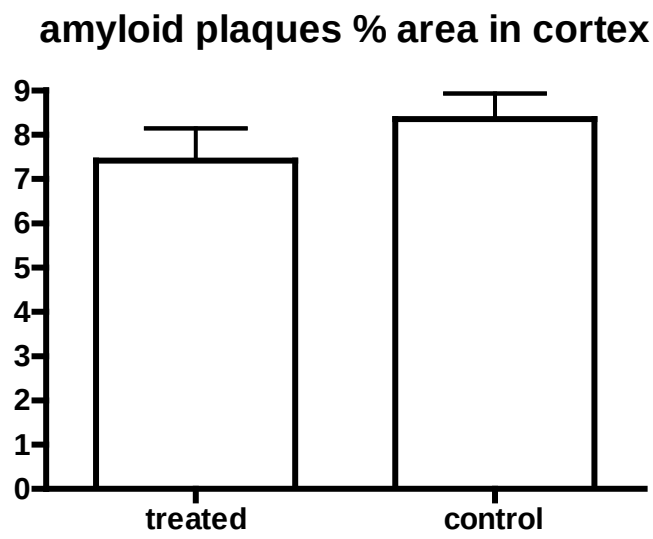


Figure 5-6. Dense-core amyloid deposits in APP/PS1 female mice. Deposits visualized by Thiazine red Staining. Digital micrographs of control mouse (left), treated mouse (right).

a.



b.

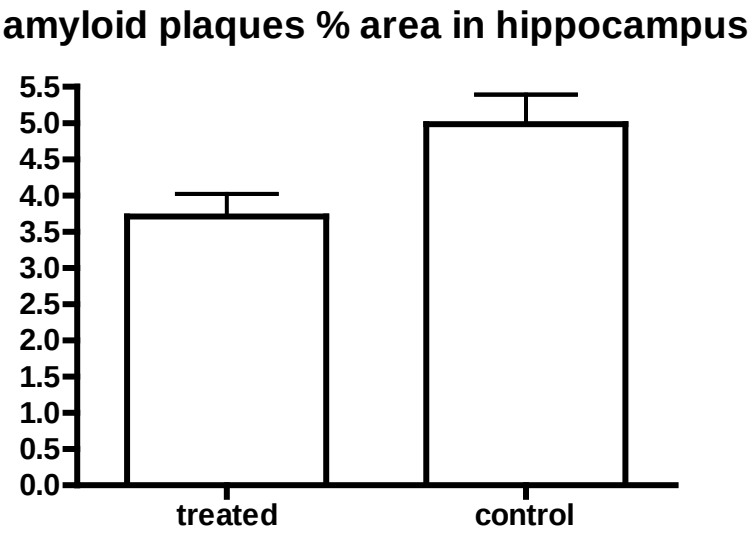
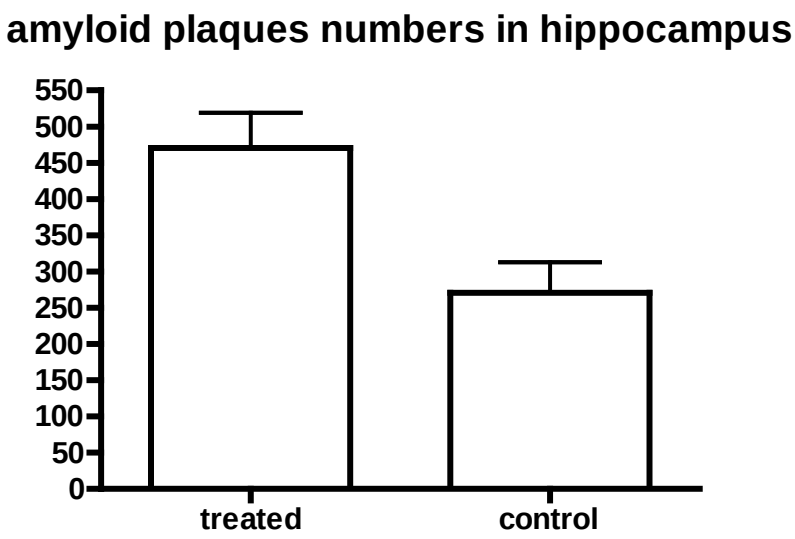


Figure 5-7. Analysis of dense-core amyloid deposits in APP/PS1 female mice. Deposits visualized by Thiazine red staining. a) Percent amyloid burden in cortex area. b) Percent amyloid burden in hippocampus area. Bars represent group means ± the standard error.

a.



b.

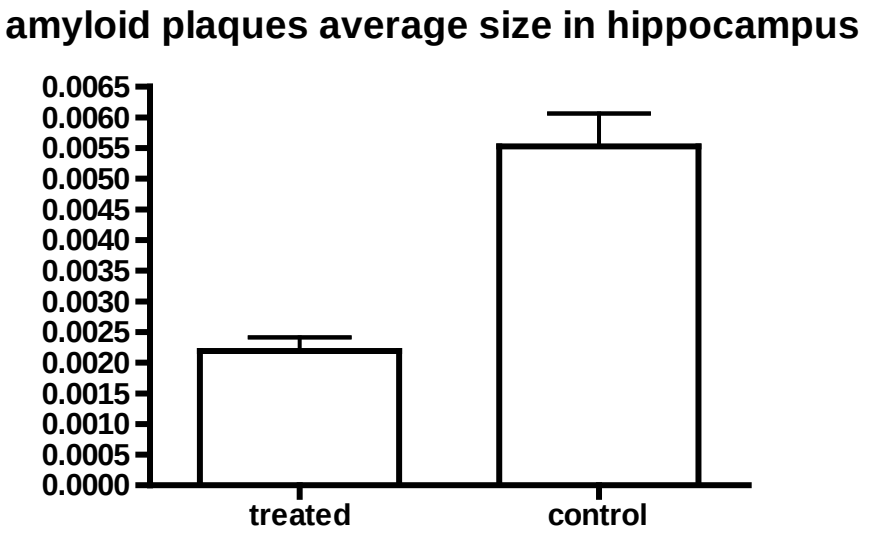
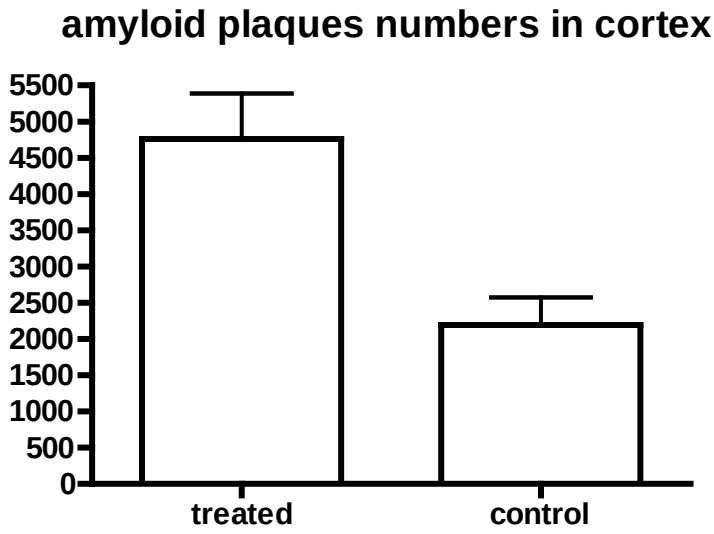


Figure 5-8. Analysis of dense-core amyloid deposits in APP/PS1 female mice hippocampus area. Deposits visualized by thiazine red staining. a) Amyloid plaques total numbers. b) Average size of amyloid plaques. Bars represent group means ± the standard error.

a.



b.

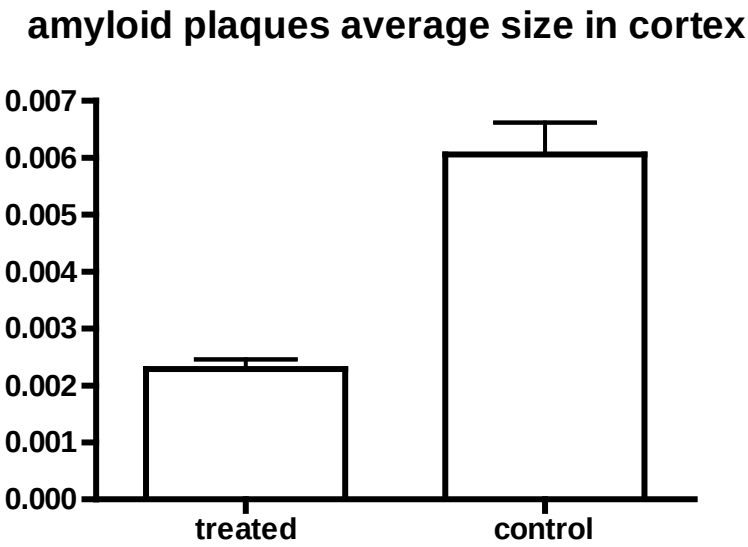


Figure 5-9. Analysis of dense-core amyloid deposits in APP/PS1 female mice cortex area. Deposits visualized by thiazine red staining. a) Amyloid plaques total numbers. b) Average size of amyloid plaques. Bars represent group means ± the standard error.

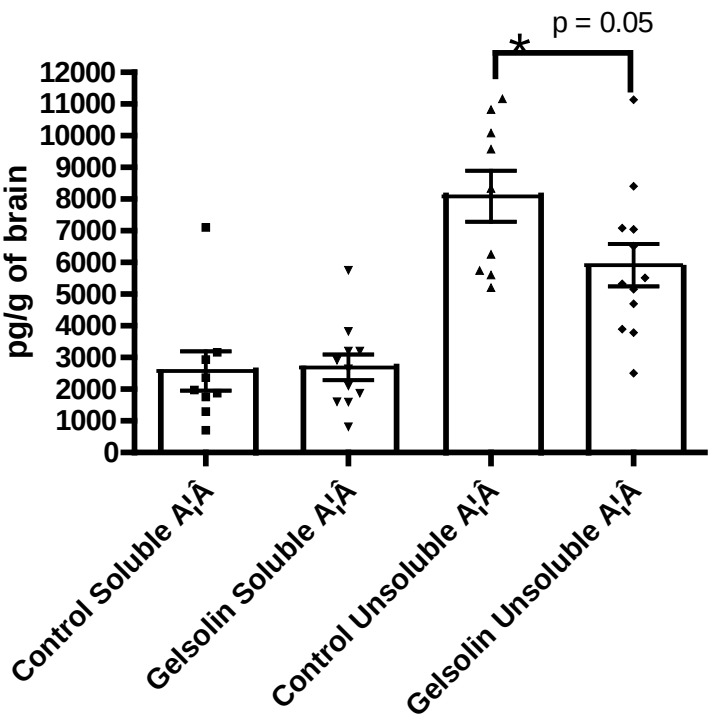


Figure 5-10. ELISA analysis of the Aβ42 concentration in the female mice hippocampus section. There is no difference of soluble Aβ42 concentration between treated group and control group, but the illustration shows that there are significant differences of unsoluble Aβ42 concentration between treated group and control group.

CHAPTER 6
NEW ELISA METHOD FOR HUMAN PLASMA GELSOLIN QUANTIFICATION STYELS

Methods of Protein Concentration Quantification

We have shown that peripheral human plasma gelsolin gene delivery via AAV-8 can retard the memory loss observed in APP/PS1 male mice and this effect may be due to the reduced Aβ burden in the brain, especially in the hippocampus area. These results encourage us to try to develop the PK/PD model of this treatment in order to help us understand how this treatment works. Low level of plasma gelsolin correlated with severe trauma, sepsis, myonecrosis, conditioning regimens for stem cell transplantation, acute respiratory distress syndrome (ARDS), and liver necrosis, [122, 123, 124, 125, 126, 127]. Increase plasma gelsolin level could improve pulmonary injury in rodents which was experienced to hyperoxia or burns, [124, 128]. Due to these observations and the fact that a biotechnology company has begun to produce gelsolin for future clinical use, knowledge of human plasma gelsolin metabolism appears worthy of consideration, and development of PK model is necessary. Currently there is no published data for the PK/PD parameter of the plasma gelsolin. Human plasma gelsolin has high homology with mouse plasma gelsolin. In order to quantify human plasma gelsolin concentration in the mice we had to develop a method which can be used to differentiate the signal of the human and mouse plasma gelsolin.

We tried several methods to quantify plasma gelsolin concentration. First, we tried a gelsolin function assay to quantify gelsolin concentration. Since enzyme-like properties of gelsolin could polymerize actin monomer and cleave actin filaments under different pH, ion strength. The time of reactions is dependent on the gelsolin concentration, so we can measure the reaction signal to quantify the gelsolin

concentration. There are two standard gelsolin functional assays. One assay is to cleave rhodamine phalloidin labeled actin filaments; the other assay is to polymerize pyrine labeled actin monomer. Fluorescence intensity was measured to quantify gelsolin concentration. [129, 130] We used rhodamine phalloidin method to quantify gelsolin concentration. Rabbit muscle actin was dissolved in a solution (TRIS 10 mM, CaCl2 0.2 mM, ATP 0.5 mM and dithiothreitol 0.2 mM, pH 7.4). Then it was diluted to 10 μM with a polymerising buffer (KCl 150 mM, MgCl2 2 mM, TRIS 10 mM, CaCl2 0.2 mM, ATP 0.5 mM and dithiothreitol 0.2 mM), incubated at room temperature for 2 hours and stored at – 20°C. Rhodamine phalloidin was dissolved in DMSO at 500 μM and stored at - 20°C. Human plasma gelsolin was dissolved in a solution (KCl 75 mM, EGTA 0.2 mM, and HEPES 10 mM) at 400 nM, and stored at -80°C. The solution used throughout the experiments. The assay is based on the fluorescence increase of rhodamine-labelled phalloidin after binding to polymerized actin. [131, 132] Fluorescence intensity was measured by a fluorescence microplate reader, using 96-well black clear-bottom plates. We used transmittance at 540 and 590 nm for excitation and emission. Actin was incubated with phalloidin for at least 8 minutes before test. Adding of gelsolin would reduce the fluorescence. The gelsolin concentration was normalised by using phalloidin plus actin and monomer actin alone as 100% and 0%. (Fig 6-1) These methods cannot be used because the physiological concentration of gelsolin in the plasma is 179μg/ml and the background signal of gelsoin is too strong to eliminate.

Then we attempted to use the monoclonal antibodies and develop western blot methods to quantify human plasma gelsolin concentration in the mouse plasma. Antibodies were developed by us with help from the CDBR lab. The western blot is used

to detect gelsolin concentration. It uses electrophoresis to separate denatured proteins. Then the proteins are transferred to a PVDF membrane, where they are tested by antibodies. [133, 134] But this method still cannot be used because this method requires denaturation of the protein resulting in the lose their three dimensional structure and turn to linear structure exposing all the antibody binding sites making it difficult to differentiate the signal of human and mouse plasma gelsolin.

Since western blot cannot differentiate signals between mouse and human plasma gelsolin, we attempted to develop an ELISA method using commercial antibodies. First we tried to develop Sandwich ELISA. The his-tag polyclonal antibody in the Sandwich ELISA was used to selectively bind to the commercial his-tagged human plasma gelsolin, which should to eliminate the influence of mouse plasma gelsolin and help to quantify the amount of human plasma gelsolin more precisely.

Figure 6-3 shows that there were not enough differences by comparing with the control groups. So the Sandwich ELISA cannot being used to quantify human plasma gelsolin, either.

New ELISA Method for Gelsolin

We concluded that the his-tag antibody might not bind the human plasma gelsolin as a reason why this method did not worked. The new ELISA method was developed by coating plasma gelsolin sample on plates directly. Figure 6-4 showed the standard curve looks very well on linear relationship between O.D. value and the gelsolin concentration from 0 ng to 20 ng range. As gelsolin concentration increased, antibodies became more and more saturated so that curve turned to flat.

LOD & LOQ

The limit of detection (LOD) and the limit of quantitation (LOQ) were used to verify the effecacy of our experiments.

LOD was around 0.18ng and LOQ was around 2.5ng, both of them were lower than 5ng.This result means that the standard curve range which covers from 5ng to 20ng might be effective to quantify the amount of human plasma gelsolin.

The Results of Gelsolin Spiked with Mouse Plasma Compared with Those in PBS

The further experiment was done to compare the results between gelsolin spiked with mouse plasma and in PBS (Figure 6-5). We noticed that both of curves show same trend and almost parallel. But the O.D. value in spike sample is lower than PBS sample. We hypothesized that this may due to proteases in the mouse plasma that degrade human plasma gelsolin protein which gives us a warning that for further experiments we might have to use protease inhibitors.

ELISA Results Analysis

In this study, we developed a new method to quantify the human plasma gelsolin, the beauty of this method is that it can differentiate human and mouse plasma gelsolin signal which provide a powerful tool for the future research.

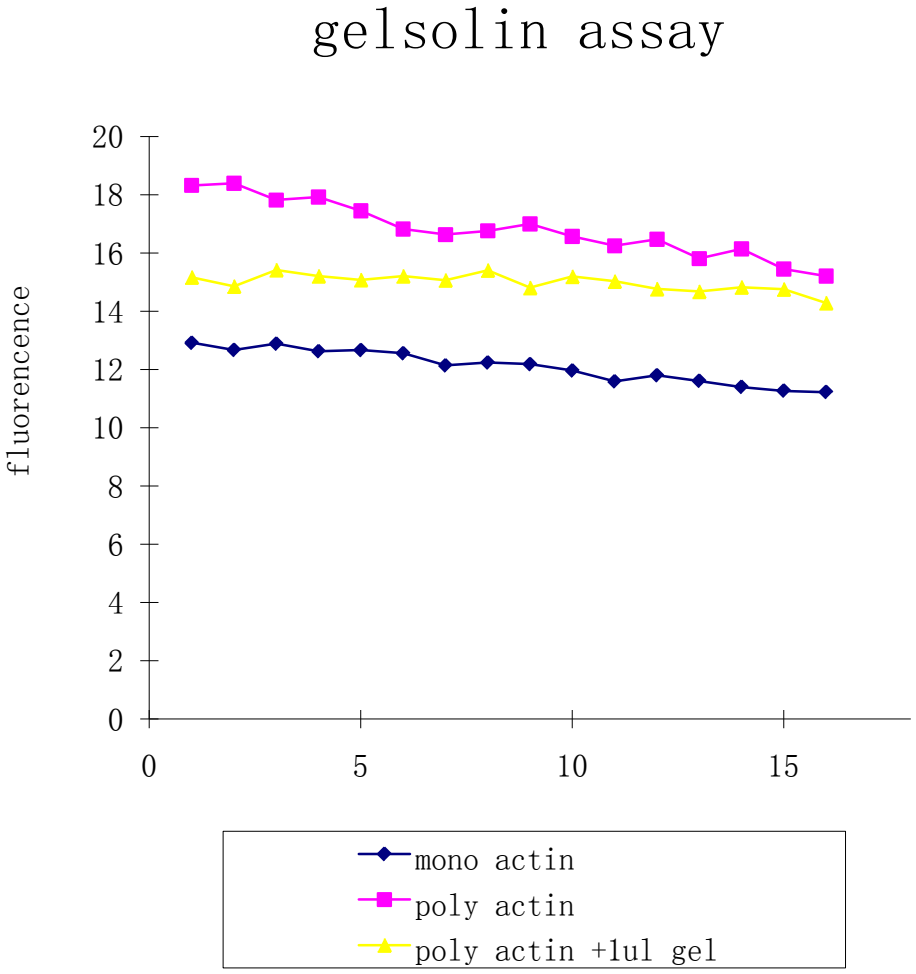


Figure 6-1. Gelsolin function assay. Assay shows that it works for quantify gelsolin concentration because monomer actin solution show lowest signal strength, polymerized actin binding by rhodanmine phalloidin show strongest signal strength and add-in gelsolin polymerized actin solution's signal strength between monomer and polymerized actin.

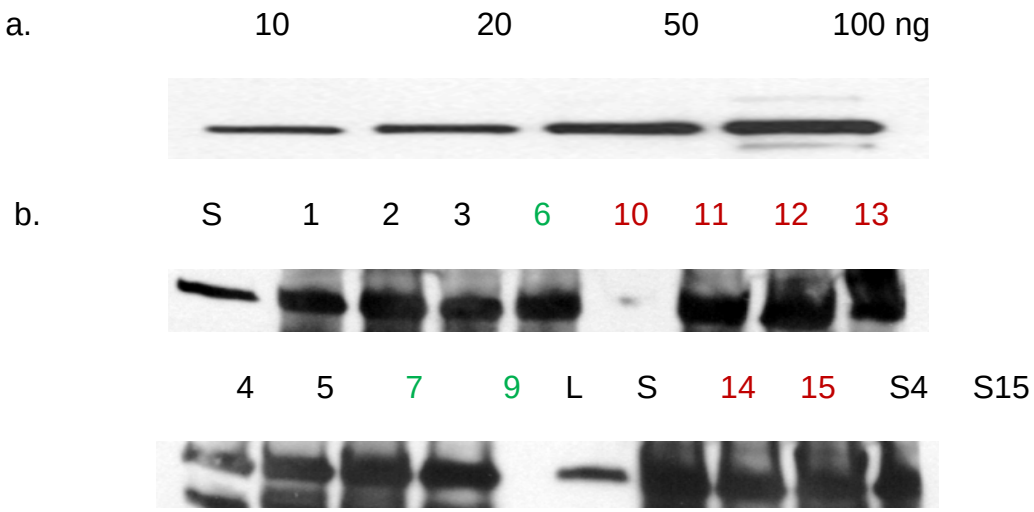


Figure 6-2. Western blot of gelsolin. Western blot results illustrated it could be use to quantify human plasma gelsolin concentration alone, but it failed when it is used to try to quantify human plasma gelsolin in mouse plasma. a) western blot signal is stronger when gelsolin amount increase. b) western blot signal show no difference among different groups of mice. S, standard gelsolin; L, ladder; black numbers: 1, 2, 3, 4, 5 are mice number in control group; green numbers: 6, 7, 9 are mice number in 6 month old treated group; red numbers: 10, 11, 12, 13, 14, 15 are mice number in control group; S4, standard gelsolin spike with NO. 4 mouse; S15, standard gelsolin spike with NO. 15 mouse.

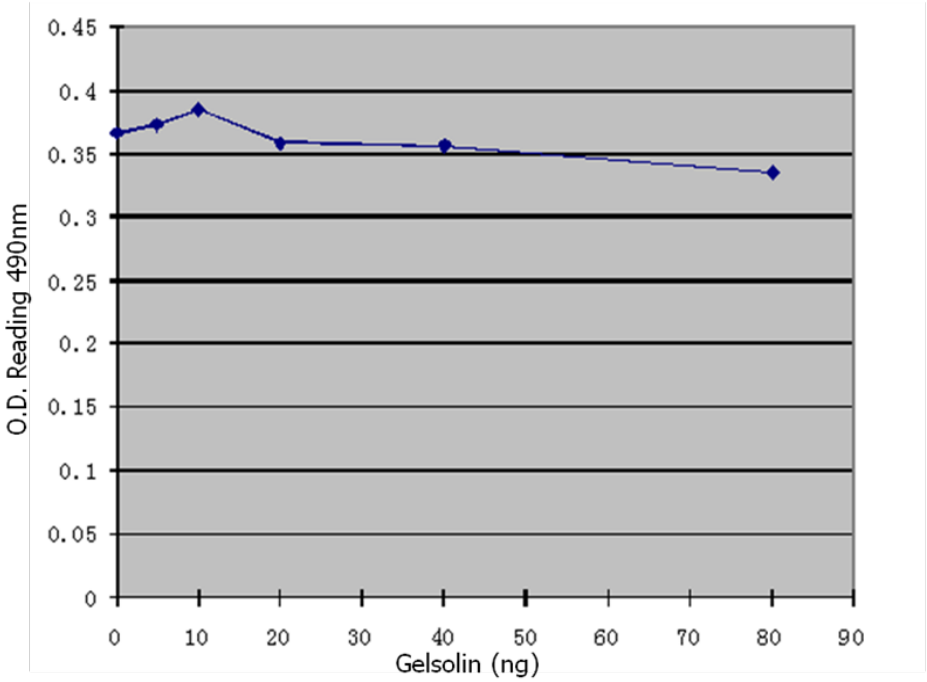


Figure 6-3. Showed that O.D. value not changed when gelsolin amount increased.

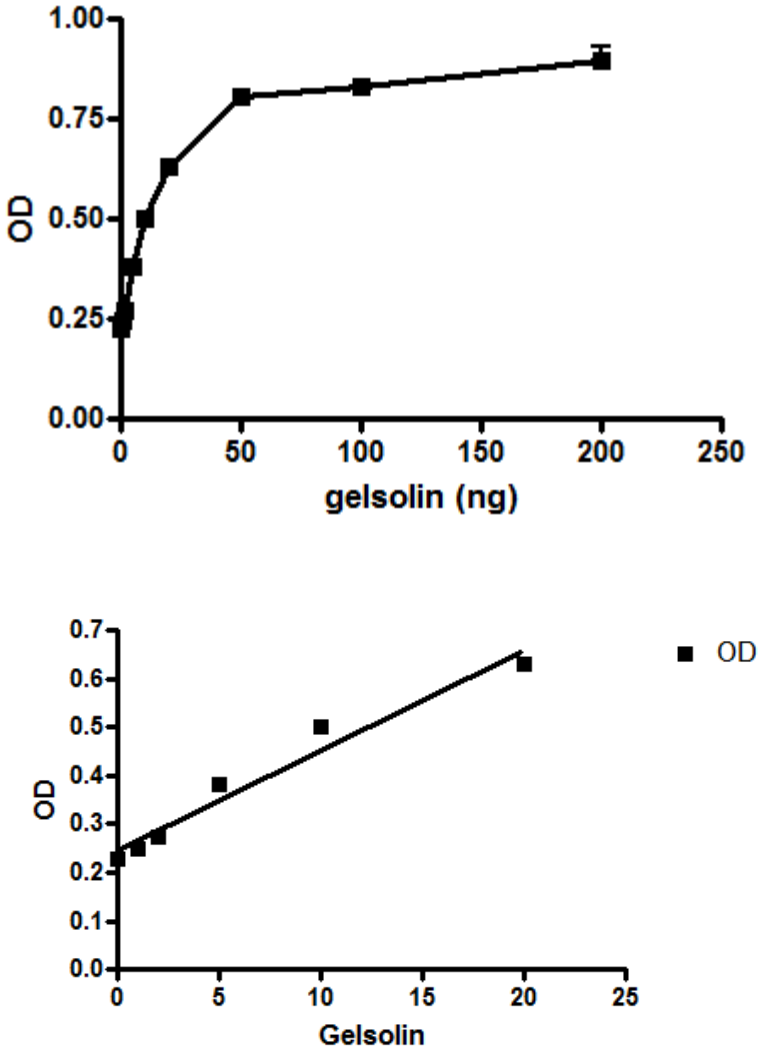


Figure 6-4. Gelsolin standard curve.

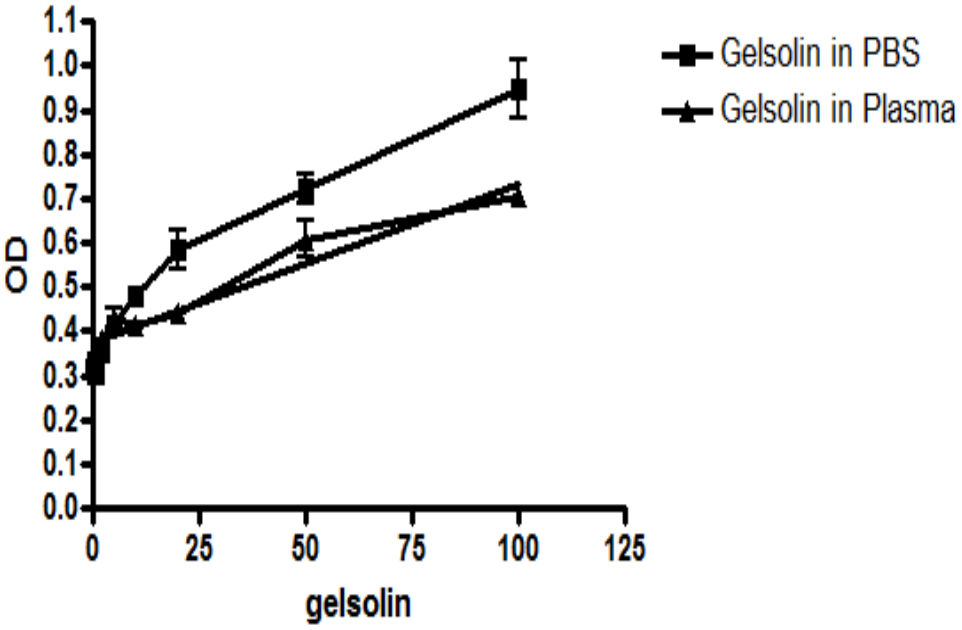


Figure 6-5. Standard curve of gelsolin in PBS and spike with mouse plasma.

CHAPTER 7
DISCUSSION AND FUTURE DIRECTIONS

Previous studies have demonstrated that human plasma gelsolin can bind to amyloid β and disassemble, even prevent the assembly of amyloid fibrils. [107, 108] Matsouka showed that administration of bovine plasma gelsolin could reduce amyloid levels in huAPP/PS1M146L mice. [110] Hirko et al. showed that peripheral delivery of human plasma gelsolin plasmid DNA could reduce amyloid levels in APP/PS1 mice. [112] Based on these results, we hypothesized that with human plasma gelsolin gene delivery via AAV-8 peripherally would be a single dose, long term ideal treatment for AD.

To test this hypothesis, first we prepared large amount of human plasma gelsolin plasmid for AAV-8 packaging. Then we injected AAV-8 coding for human plasma gelsolin into the AD mouse model – APP/PS1 mice through the tail vein. To test the effect of the treatment, we performed behavior training and finally sacrificed the mice and examined their brains for amyloid pathology.

Through comparing the results of behavior training from the Morris Water Maze test and radial arm water maze test, we determined that memory loss associated with the APP/PS1 line of mice used had been retarded in the male mice that received gelsolin gene delivery compared to the control males. We did not oberve any effect on memory in female mice through behavior training. The learning ability showed no difference for all mice. Arendash reported similar radial arm water maze behavior training results comparing APP/PS1 and wild type male mice. [117] Their results showed that wild type male mice slow down memory loss by comparing with APP/PS1 male mice and learning ability showed no difference between those two groups of male mice. That means our gelsolin gene delivery via AAV-8 treatment in male mice behavior

training results is matching the wild type mice. The reason that the female mice did not retard memory loss may due to gender behavior differences. Although memory loss had been retarded after treatment, we did not find any significant difference of brain slice staining results between treated male mice and control mice. A larger sample group size to show the statistical significant difference may be needed.

From the ELISA analysis we find that after treatment, unsoluble Aβ42 concentration decreased and soluble Aβ42 concentration increased in the brain by comparing the treated mice and control mice. At the same time, thioflavin-S and thiazine red staining of brain slices show that amyloid deposits in the brain decreased in the treated mice compared to the control mice, especially in the hippocampus area which is a critical structure for learning and memory. These results may support the sink theory. Gelsolin gene expression in the body after gene delivery via AAV-8, results in the production of gelsolin protein. Gelsolin can bind soluble Aβ42 peripherally in the body and keep it soluble such that Aβ42 can be cleared by other enzymes quickly, and that would shift the equilibrium of soluble Aβ42 from the CNS to the periphery and resulting in reduced Aβ42 in the brain, reducing amyloid deposition in the brain.

Our treatment reduced Aβ42 burden in the brain and retarded memory loss in APP/PS1 male mice. Therefore, we believe that the treatment strongly showed promising evidence of Aβ42 as a promising target for the Alzheimer's disease treatment and sink theory may be used to explain how peripherally expressed gelsolin acted as a chemical which can reduce brain Aβ42.

Nearly all clinical trials for Alzheimer's disease have failed the past several years. We suspect that this may be due to poor volunteer selection. First, we recognized that

one chemical for the treatment may focus on one biomarker. We understand there are many hypotheses to explain which one is the critical reason for Alzheimer's disease progression, such as amyloid beta, tau, neuron cell signal receptors, etc. It may be true that there is more than one factor for Alzheimer's disease progression that may require the need to differentiate Alzheimer's disease into several subtypes. This may help to make more clear how to explain that Alzheimer's disease can be induced and explore more chemicals to focus on different biomarkers due to different hypothesis. Before we do clinical trials, we need to screen the volunteers by using different biomarkers in order to target therapies via the specific chemicals, then we have a higher chance to find a fitting treatment chemical for a specific subtype of Alzheimer's disease patient. It may be necessary to go back and check all chemicals we have to find useful chemicals. Second, we always choose aged volunteers for the clinical trials. It may be another reason for the trial failure because we know if a volunteer shows symptoms, that means neurons have already been injured and lost, and no method can be used recovery them, then memory will be lost forever due to neurons dying. This may be the reason clinical trials fail on not show significant results at halting the progression of memory loss by comparing the treatment groups with control groups. In the future, we can choose volunteers through biomarker screens in order to find high risk group base on the specific biomarkers for the clinical trials and perform clinical trials earlier before symptoms have already been shown.

For the methods to quantify human plasma gelsolin concentration, by comparing the three methods of the western blot, sandwich ELISA, the new ELISA method, the new ELISA method showed to be a better method. The standard curve showed a good

linear relationship in a measurable range. This advantage made it possible to quantify human plasma gelsolin. Although the data in vitro appeared positive, it did not work very well *in vivo*. There were no significant differences between administration groups and the background. We hypothesis that i.v. injection of human plasma gelsolin may bind to blood cells or tissue, so when we harvest plasma samples there was no human plasma gelsolin signal showing in the plasma fraction. We need to perform more experiments to confirm our hypothesis.

The vast majority of gene therapy approaches for AD involve delivery of vectors coding for amyloid degrading enzymes (neprilysin or insulin degrading enzyme) (Eckman and Eckman, 2005; Marr et al., 2003). the rationale behind using amyloid degrading enzymes is to increase the clearance of amyloid.

Future directions for study should include the development of a PK/PD model of AAV-8 with human plasma gelsolin gene delivered peripherally, determination which plasma Aβ is a good biomarker for the treatment, and test a cocktail treatment by combine three gene therapies: siRNA for γ-secretase, human plasma gelsolin and amyloid degrading enzymes. The first two studies will be important for understanding how the treatment works, and the cocktail treatment will provide an ideal treatment because it combines treatments to decrease Aβ production, to keep it soluble, and finally increase clearance.

In conclusion, our study demonstrates that peripheral delivery of AAV-8 coding for human plasma gelsolin may be a potential treatment for AD because it results in retarding memory loss and reduces Aβ burden in the brain. This treatment has the

advantage of being a one dose, long term treatment compared with the traditional therapeutic approaches for AD.

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BIOGRAPHICAL SKETCH

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