rAAV/ABAD-DP-6His attenuates oxidative stress-induced injury of PC12 cells

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Abstract
Our previous studies have revealed that amyloid β (Aβ)-binding alcohol dehydrogenase (ABAD) decoy peptide antagonizes Aβ25-35-induced neurotoxicity. However, whether it improves oxidative stress injury remains unclear. In this study, a recombinant adenovirus constitutively secreting and expressing Aβ-ABAD decoy peptide (rAAV/ABAD-DP-6His) was successfully constructed. Our results showed that rAAV/ABAD-DP-6His increased superoxide dismutase activity in hydrogen peroxide-induced oxidative stress-mediated injury of PC12 cells. Moreover, rAAV/ABAD-DP-6His decreased malondialdehyde content, intracellular Ca2+ concentration, and the level of reactive oxygen species. rAAV/ABAD-DP-6His maintained the stability of the mitochondrial membrane potential. In addition, the ATP level remained constant, and apoptosis was reduced. Overall, the results indicate that rAAV/ABAD-DP-6His generates the fusion peptide, Aβ-ABAD decoy peptide, which effectively protects PC12 cells from oxidative stress injury induced by hydrogen peroxide, thus exerting neuroprotective effects.

Key Words: nerve regeneration; neurodegenerative disease; gene therapy; Alzheimer’s disease; amyloid beta peptide; amyloid beta binding alcohol dehydrogenase; adeno-associated virus; hydrogen peroxide; oxidative stress; mitochondrial dysfunction; NSFC grant; neural regeneration

Introduction
Alzheimer’s disease is a predominantly late-onset neurodegenerative disease that is age-dependent, and characterized by the progressive decline of memory, cognitive functions, and changes in behavior, and personality (Selkoe, 2001; Mattson, 2004; Reddy and Beal, 2008; Wang et al., 2013). Amyloid beta (Aβ) is considered to be an important initiating molecule in the pathogenesis of Alzheimer’s disease (Selkoe, 2002). Recent findings have confirmed that intraneuronal accumulation of Aβ precedes the deposition of amyloid plaques and the appearance of neurofibrillary tangles, which are consistent with the first pathological manifestation of deficits in synaptic plasticity and learning and memory (Billings et al., 2005; Wirths and Bayer, 2012). Using the yeast two-hybrid system, Yan et al. (1997) have shown that Aβ-binding alcohol dehydrogenase (ABAD) in the mitochondrial matrix is involved in multiple aspects of metabolic homeostasis as a short-chain dehydrogenase. Aβ-ABAD distorts the structure of the enzyme and modifies its functions in metabolic homeostasis related to energy metabolism and catabolism of isoleucine branched-chain fatty acids. This binding also leads to accumulation of multiple metabolic intermediates and decreases activities of the Krebs cycle and cellular respiration. These findings indicate that the effects of Aβ-ABAD may exacerbate Alzheimer’s disease pathology. Therefore, blocking Aβ-ABAD-mediate effects with ABAD decoy peptide (ABAD-DP) may be a potential therapeutic strategy for Alzheimer’s disease (Xie et al., 2006; Yao et al., 2011).

Oxidative stress is thought to play a major role in the etiology of Alzheimer’s disease. Evidence suggests that reactive oxygen species (ROS) damage macromolecules, including lipids, proteins, and DNA (Jomova et al., 2010). Studies have also shown that the expression of oxidative stress markers is elevated in neurons surrounding Aβ deposits in transgenic mouse models of Alzheimer’s disease (Pappolla et al., 1998). Aβ accumulation also occurs in primary neurons with induced oxidative stress (Goldsbury et al., 2008).

Our group has previously established a lentiviral expression system in which thioredoxin-1-ABAD-DP (TA) allows the stable expression of small ABAD-DP by fusion with cytosolic thioredoxin-1. Overexpression of TA aptamer has been shown to protect PC12 cells from intracellular Aβ cytotoxicity (Yang et al., 2007). However, whether ABAD-DP inhibits oxidative stress is unclear. In the present study, we investigated the role of recombinant adeno-associated viral
vector (rAAV) with the fusion peptide fragment, ABAD-DP-6-His protein (6His), in oxidative stress and its effect on H2O2-induced oxidative stress in PC12 cells. To construct ABAD-DP-6His, we fused 6His to the 5′ end of ABAD-DP cDNA in the AAV.

Materials and Methods

Cell culture

Human embryonic kidney (HEK) 293T cells and PC12 cells (Chinese Academy of Sciences, Shanghai, China) were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The cells were digested with 0.02% ethylenediaminetetraacetic acid in PBS, and maintained at 37°C under 5% CO2 for 24–48 hours (Billings et al., 2005).

Construction of recombinant plasmid

ABAD-DP cDNA was synthesized in the plent/TRX-ABAD-DP-prepro plasmid by polymerase chain reaction (PCR) (Selkoe, 2001). The oligonucleotides (BioAsia Bioengineering Co, Shanghai, China) used were as follows: upstream primer, 5′-G CAC GTG GCA GGC ATC GCG GTG GCT AG-3′; downstream primer, 5′-G GGA TCC TCA TAC ATC AAG AAC TCG CTG G-3′. Amplifications were performed on a DNA Thermal Cycler (Sino-American, Zhengzhou, Henan Province, China) with standard PCR procedure temperatures (94°C pre-denaturation for 300 seconds, 94°C denaturation for 60 seconds, 57°C annealing for 60 seconds, 72°C extension for 70 seconds, for 30 cycles in total). ABAD-DP cDNA was assembled in a pGEM-T Easy plasmid (Promega, Madison, WI, USA), and the proper orientation was confirmed by restriction analysis using Pmacl and BamHI (Sino-American). The integrity of the coding sequence was purified and sequenced by Sanger dyeoxy sequencing (http://www.ncbi.nlm.nih.gov/Genbank) (Sanger et al., 1977).

A universal recombinant rAAV vector was created, according to Smith et al. (2000). The AAV Rep and Cap genes between inverted terminal repeats of pSSV9inte plasmid were replaced by the cassette from the pACCMVpLpA plasmid (Hua Guang Bioengineering Co, Beijing, China). The primer contained: an upstream primer, 5′-GAC GTG GCA GGC ATC GCG GTG GCT AG-3′; downstream primer, 5′-G GGA TCC TCA TAC ATC AAG AAC TCG CTG G-3′. Amplifications were performed on a DNA Thermal Cycler (Sino-American, Zhengzhou, Henan Province, China), and the proper orientation was confirmed by restriction analysis using Pmacl and BamHI (Sino-American). The integrity of the coding sequence was purified and sequenced by Sanger dyeoxy sequencing (http://www.ncbi.nlm.nih.gov/Genbank) (Sanger et al., 1977).

For the detection of ROS, fluorescent Real-Time PCR with SYBR Green I (Tiangen, Beijing, China). The primer contained: an upstream primer, 5′-C AAG TAC GCC CCC TAT TGA C-3′ and a downstream primer, 5′-AAG TCC GTG TGT TGA TTT TGG TG-3′. The recombinant virus was digested by Proteinase K, and the total DNA was extracted by the routing phenol-chloroform method (Reed et al., 2006). Amplifications were performed on the iQ5 Real-Time PCR Detection System (Tiangen) with the following temperature profile: 94°C for 180 seconds, 40 cycles at 94°C for 60 seconds, 54°C for 60 seconds, 68°C for 30 seconds, and extension at 72°C for 300 seconds.

Drug treatment of PC12 cells

PC12 cells were divided into four groups: (1) vehicle control, (2) 300 μmol/L H2O2, (3) AAV vector + 300 μmol/L H2O2, and (4) rAAV/ABAD-DP-6His + 300 μmol/L H2O2. Cells from all groups were cultured at 37°C with 5% CO2 for 24 hours. The AAV vector + H2O2 and rAAV/ABAD-DP-6His + H2O2 groups were infected by rAAV at a multiplicity of infection of 1:100 for 24 hours. Cells were then incubated at 37°C with 5% CO2 for 2 hours in fresh medium, and then incubated at 37°C with 5% CO2 for additional 24 hours. The morphological changes of PC12 cells were observed by an inverted phase-contrast microscope (Olympus, Tokyo, Japan).

3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay for PC12 cells

The MTT assay was used for the determination of cell viability (Billings et al., 2005). Briefly, cells were cultured at a concentration of 5,000 cells/well in 96-well plates, at 37°C with 5% CO2. After treatment, 20 μL MTT (5 mg/mL; Sigma-Aldrich, Los Angeles, CA, USA) was added to each well, and incubated for 4 hours at 37°C. The cell culture medium was then removed, and 100 μL of dimethyl sulfoxide was added to the wells. The plates were briefly shaken at 60 r/min for 5 minutes to dissolve the precipitates and remove the bubbles. Absorbance was read at 490 nm using the Model 550 microplate reader (Bio-Rad, Los Angeles, CA, USA).

Flow cytometry of PC12 cells for apoptosis, ROS, mitochondrial membrane potential (ΔΨm) and Ca2+ concentration

For the detection of apoptosis, 5 μL of Annexin V/fluorescein isothiocyanate and 10 μL of propidium iodide (20 μg/mL) (Beyotime, Beijing, China) were added to 100 μL of cell suspension. 2′,7′-Dichlorofluorescein diacetate (20 μmol/L), rhodamine123 (5 μmol/L), and acetoxymethyl ester (5 μmol/L) of Fluo-3 (Fluo-3/AM; Sigma-Aldrich) were used as fluorescent probes for the detection of ROS, ΔΨm, and Ca2+ concentration, respectively. Fluorescent agent (5 μL) was added to each test tube. PC12 cells were incubated for 30 minutes in the dark at 37°C, washed twice with 0.01 mol/L PBS, and centrifuged at 1,500 r/min for 5 minutes. The mean fluorescence intensity of 10,000 cells was measured for all groups using flow cytometry (BD, Los Angeles, CA, USA).
Biochemical assays of PC12 cells
PC12 cells were seeded into 75 mm² petri dishes at the density of 1 x 10⁵ cells/mm². After treatment with H₂O₂, cells were collected in 2-mL Eppendorf tubes, and washed three times with balanced salt solution; PC12 cells were incubated for 30 minutes in the dark at 37°C, washed twice with 0.01 mol/L PBS, centrifuged at 1,500 r/min for 10 minutes, and the supernatant was discarded. Balanced salt solution (1 mL) was added and the cells were lysed using ultrasound. The concentrations of malondialdehyde, ATP, and superoxide dismutase (SOD) were measured in the resulting homogenate using malondialdehyde test kit, ATP test kit, and SOD test kit, respectively (Nanjing Jiancheng Bioengineering Institute, Nanjing, Jiangsu Province, China). After the enzymatic reaction, the absorbance was detected using a spectrophotometer (Chengguang Corporation, Shanghai, China) at the excitation wavelengths of 532 nm, 636 nm, and 550 nm for malondialdehyde, ATP, and SOD, respectively.

Statistical analysis
All data are expressed as mean ± SD and were analyzed by one-way analysis of variance followed by the Student-Newman-Keuls test. SPSS 17.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis. Significance was reached at a values of 𝑃 < 0.05 or 𝑃 < 0.01.

Results
Identification of recombinant plasmid and packaging of the recombinant AAV system
The ABAD-DP fusion gene was assembled in the pGEM-T Easy plasmid, and its correct orientation was confirmed by restriction analysis with PmaI. A 93 bp fragment was observed by agarose gel electrophoresis (Figure 1B). The integrity of the coding sequence showed 100% consistency with the corresponding GenBank sequence (Figure 1C). The ABAD-DP cDNA was then inserted into the multiple cloning site of the pSSCMV/6His-prepro plasmid to generate the recombinant plasmid pSSHG/ABAD-DP-6His DNA sequencing and the comparison with our designed sequence using DNASIS software (MiraRio, Tokyo, Japan). File 1: Gene sequencing file of ABAD-DP-6His. File 2: Gene sequencing provided by GeneBank (http://www.ncbi.nlm.nih.gov/genbank). (D) Recombinant plasmid pSSHG/ABAD-DP-6His was digested with EcoRI and BamHI and confirmed that the ABAD-DO-6his fusion gene was 136 bp, which was consistent with the expected size. Lane 1: HBI 1.0 kb plus DNA ladder; lane 2: pSSHG/ABAD-DP-6His cut with EcoRI; lane 3: pSSHG/ABAD-DP-6His cut with XhoI; lane 4: HBI 1.0 kb plus DNA ladder, lane 5: λDNA/HindIII marker. (C) The results of ABAD-DP-6His DNA sequencing and the comparison with our designed sequence using DNASIS software (MiraRio, Tokyo, Japan). File 1: Gene sequencing file of ABAD-DP-6His. File 2: Gene sequencing provided by GeneBank (http://www.ncbi.nlm.nih.gov/genbank).

rAAV/ABAD-DP decreased H₂O₂-induced apoptosis in PC12 cells
PC12 cells in the control group exhibited normal morphology (Figure 2A1). Oxidant treatment caused damage to these cells, with many dead neurons and debris of disintegrated cells, and neurons with markedly swollen somas (Figure 2A2, 3). H₂O₂ co-treated with rAAV/ABAD-DP-6His induced similar damage, but with most neurons maintaining somewhat normal somas, intact axons, and exuberant synapse connections.

Flow cytometry results showed that the number of apoptotic cells was significantly (𝑃 < 0.05) higher in the H₂O₂ group and AAV vector + H₂O₂ group compared with the control group. Furthermore, number of apoptotic cells was significantly (𝑃 < 0.05) lower in the rAAV/ABAD-DP-6His + H₂O₂ group compared with the H₂O₂ group (Figure 2C). However, the H₂O₂ group and the AAV vector + H₂O₂ group
showed similar number of apoptotic cells (Figure 2B, C). MTT assay results indicated that cell viability in the H$_2$O$_2$ group and AAV vector + H$_2$O$_2$ group was significantly ($P < 0.05$) decreased compared with the control group. Furthermore, cell viability in the rAAV/ABAD-DP-6His + H$_2$O$_2$ group was significantly ($P < 0.05$) higher than the H$_2$O$_2$ group (Figure 2D). However, cell viability in the H$_2$O$_2$ group and AAV vector + H$_2$O$_2$ group was similar (Figure 2D).

rAAV/ABAD-DP increased the antioxidant capacity of H$_2$O$_2$-mediated damage to PC12 cells

Spectrophotometry results showed that SOD activity and ATP content were significantly ($P < 0.01$) decreased in the H$_2$O$_2$ group and rAAV/ABAD-DP-6His + H$_2$O$_2$ group compared with the control group (Figure 3A, C). Malondialdehyde content was significantly increased in the H$_2$O$_2$ group ($P < 0.05$) and rAAV/ABAD-DP-6His + H$_2$O$_2$ group ($P < 0.01$) compared with the control group (Figure 3B). Moreover, SOD activity and ATP content in the rAAV/ABAD-DP-6His + H$_2$O$_2$ group was markedly ($P < 0.01$) higher, but malondialdehyde content in this group was significantly ($P < 0.01$) lower than in the H$_2$O$_2$ group (Figure 3A–C). SOD activity, ATP content, and malondialdehyde content between the H$_2$O$_2$ group and the AAV vector + H$_2$O$_2$ group was similar (Figure 3A–C).

rAAV/ABAD-DP improved mitochondrial dysfunction in H$_2$O$_2$-mediated damage of PC12 cells

Flow cytometry results showed that the mitochondrial membrane potential was significantly reduced in the H$_2$O$_2$ group ($P < 0.05$) and rAAV/ABAD-DP-6His + H$_2$O$_2$ group.

Figure 2 Protective effect of rAAV/ABAD-DP on H$_2$O$_2$-mediated changes in morphology, viability, and apoptotic activity of PC12 cells.

(A) Effect of rAAV/ABAD-DP on the morphology of PC12 cells treated with H$_2$O$_2$ (× 200). (B, C) Flow cytometry showing the effect of rAAV/ABAD-DP on H$_2$O$_2$-induced apoptosis of PC12 cells. (D) 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay showing the effect of rAAV/ABAD-DP on H$_2$O$_2$-mediated proliferation of PC12 cells. (A1, B1, a) Normal control group; (A2, B2, b) H$_2$O$_2$ group; (A3, B3, c) AAV vector + H$_2$O$_2$ group; (A4, B4, d) rAAV/ABAD-DP-6His + H$_2$O$_2$ group. Each treatment was performed in triplicate. All data are expressed as mean ± SD and were analyzed by one-way analysis of variance followed by the Student-Newman-Keuls test. *$P < 0.05$, vs. normal control group; #$P < 0.05$, vs. H$_2$O$_2$ group. ABAD: Aβ-binding alcohol dehydrogenase; DP: decoy peptide; rAAV: recombinant adeno-associated viral vector; 6His: 6-His protein.
(P < 0.01) compared with the control group (Figure 3D). Ca\(^{2+}\) concentration and reactive oxygen species levels were significantly increased in the H\(_2\)O\(_2\) group (P < 0.05) and rAAV/ABAD-DP-6His + H\(_2\)O\(_2\) group (P < 0.01) compared with the control group (Figure 3E, F). Furthermore, ROS levels and Ca\(^{2+}\) concentration in the rAAV/ABAD-DP-6His + H\(_2\)O\(_2\) group was significantly (P < 0.01) lower than those of the H\(_2\)O\(_2\) group (Figure 3E, F); however, mitochondrial membrane potential was significantly (P < 0.01) higher than that of the H\(_2\)O\(_2\) group (Figure 3D). The mitochondrial membrane potential, Ca\(^{2+}\) concentration and reactive oxygen species levels between the H\(_2\)O\(_2\) group and the AAV vector + H\(_2\)O\(_2\) group were similar (Figure 3D–F).

Discussion

Vector construction

Yan et al. (1997) have shown that the L\(_\beta\) loop of ABAD contains a unique insertion, which is presumed to be a recognition site for Aβ. A peptide encompassing this region (residues 92–120) of human ABAD (termed ABAD-DP) was tested by surface plasmon resonance for its ability to inhibit the interaction of the intact ABAD with Aβ. ABAD-DP inhibits binding of Aβ40 and Aβ42 to immobilized intact ABAD with inhibitory constants of 4.9 and 1.7 mmol/L, respectively. In contrast, a scrambled peptide with the same amino acids but reversed sequence (ABAD [120–92] or ABAD reversed peptide [RP]) is inactive (Schwarze et al., 1999; Aarts et al., 2002). These data indicate that the L\(_\beta\) loop of ABAD is critical for Aβ binding to ABAD. Therefore, blocking Aβ-ABAD using ABAD-DP may be a potential therapeutic strategy for AD (Xie et al., 2006; Yao et al., 2011). However, because of its short half-life and high-synthesis costs, ABAD-DP cannot be clinically administered as a therapeutic agent.

In the present study, the ABAD-DP fusion gene was successfully inserted into the multiple cloning site of pSSGH/NT4-TAT-6His-prepro plasmid to generate a recombinant pSSHG/ABAD-DP-6His plasmid. The AAV vector was co-transfected with two helper plasmids into HEK293T cells by the calcium phosphate method and was harvested at a high titer. We sought to establish a safe and highly effective method of delivering the gene construct containing ABAD-DP cDNA into PC12 cells to constitutively produce ABAD-DP in vivo. AAV vectors are currently among the most frequently used viral vectors for gene therapy. AAV is a small (25-nm), non-enveloped virus that packages a linear single-stranded DNA genome (Liu et al., 2014). It belongs to the family of Parvoviridae, genus Dependovirus, thus enabling effective infection in the presence of a helper virus (either an adenovirus or herpes virus). The lack of pathogenicity, persistence, and many available serotypes of the virus have increased the potential of AAV as a delivery vehicle for gene therapy (Balazs et al., 2011; Rosenberg et al., 2012; J Dismuke et al., 2013; Ploquin et al., 2013). In the present study, the 6-His gene was inserted at the S\(_{\varepsilon}\) end of ABAD-DP cDNA as a molecular label to follow the expression of
the ABAD-DP gene. Because of a short half-life and high synthesis costs of ABAD-DP, the constructed rAAV/ABAD-DP-6His bound to intracellular Aβ peptide in the cytoplasm, in addition to its constitutive production and expression by AVV.

**Protective agent against oxidative stress**

Oxidative stress is recognized as an early event in neurodegenerative diseases such as Alzheimer's disease, and plays a key role in Aβ-induced cell death (Alhebshi et al., 2013). The complex nature and genesis of oxidative damage in Alzheimer's disease is partially due to mitochondrial abnormalities that can initiate oxidative stress. Several *in vitro* studies have shown that Aβ peptide exposure causes abnormalities of mitochondrial function, as characterized by excessive mitochondrial membrane potential depolarization, aggravated calcium buffering, uncoupling of the mitochondrial respiratory chain, reduced ATP, and increased production of ROS (Cha et al., 2012; Correia et al., 2012; Frangkio et al., 2012; Sciacca et al., 2012). These alterations are evident during Aβ oligomerization and also before the appearance of Aβ plaques and neurofibrillary tangles, thus supporting the view that oxidative stress occurs early in the development of the disease (Moreira et al., 2009). Therefore, decreasing oxidative damage and repairing antioxidant defenses are important strategies to target in early Alzheimer’s disease (Smith et al., 1997; Straface et al., 2005).

Lipids are the most targeted biomolecules of oxidative stress. SOD contributes to the reduction of oxidative stress and prevents lipid damage (Hosoki et al., 2012). Lipid oxidation also gives rise to a number of secondary products. These products are mainly aldehydes, which exacerbate oxidative damage (Uchida, 2000). SOD is a group of enzymes that plays a pivotal role in metabolizing superoxide radical (O$_2^-$), preempts oxidizing chain reactions that cause extensive damage, and prevents the formation of a cascade of deleterious ROS, including H$_2$O$_2$, hypochlorite, peroxynitrite, and hydroxyl radical (Miller, 2012). Hydroperoxyl radical (HO$_2^-$) is important for SOD binding to the membrane to intercept incoming superoxide. Defects in SOD that increase intracellular [O$_2^-$] cause damage to the cell envelope (Inlay and Fridovich, 1992). Because SOD catalyzes the dismutation of O$_2^-$ to H$_2$O$_2$, and is localized at distinct compartments (cytosol [for SOD1], mitochondria [for SOD2], and extracellular matrix [for SOD3]), they participate in compartmentalized redox signaling (Fukai and Ushio-Fukai, 2011). Therefore, in the present study, total SODs were tested to demonstrate the damage from oxidative stress. Malondialdehyde is the principal and most studied product of polyunsaturated fatty acid peroxidation (Marnett, 1998), and is similar to ROS forming DNA adducts, which are mutagenic. Malondialdehyde can be detected in relation with lipid peroxidation and oxidative stress (Nielsen et al., 1997). Several other pathologies involving oxidative stress have been recently studied in which malondialdehyde was used as a common oxidative stress biomarker. Malondialdehyde is the principal and most studied product of polyunsaturated fatty acid peroxidation (Del Rio et al., 2005). Our data showed that SOD activity was higher in the rAAV/ABAD-DP-6His + H$_2$O$_2$ group than in the H$_2$O$_2$ group, and malondialdehyde content was higher in the H$_2$O$_2$ group than in the rAAV/ABAD-DP-6His + H$_2$O$_2$ group. Therefore, our data indicate a protective effect of rAAV/ABAD-DP-6His against H$_2$O$_2$-induced oxidative damage.

Mitochondrial ATP production is the main energy source for intracellular metabolic pathways (Schapira, 2006). Mitochondria synthesize ATP from ADP in the mitochondrial matrix by using the energy provided by the proton electrochemical gradient (Capaldi et al., 1994; Nijtmans et al., 1995; Zeviani and Di Donato, 2004). The proton gradient establishes a proton-motive force, which has two components, a pH differential and an electrical membrane potential (Campanella et al., 2009). Ca$^{2+}$ plays an important role in the regulation of pH and electrical membrane potential. Studies have indicated that under basal conditions, total mitochondrial Ca$^{2+}$ content is low and that cytosolic free Ca$^{2+}$ increases in response to extrinsic agents. The latter is likely to provoke increases in intracellular Ca$^{2+}$ concentrations (Denton and McCormack, 1980; Hansford and Castro, 1981; Crompton, 1985). The consequence activation of oxidative metabolism would then provide an increased supply of reducing equivalents to drive respiratory chain activity and ATP synthesis (Tarasov et al., 2012). The mitochondrial matrix Ca$^{2+}$ overload can lead to enhanced generation of ROS, triggering mitochondrial permeability transition pore and cytochrome C release, and reducing equivalents to drive respiratory chain activity and ATP synthesis, which leads to apoptosis (Brookes et al., 2004). ROS, including superoxide, singlet oxygen, hydrogen peroxides, hydroxyl free radical and nitric oxide, which are mainly generated from mitochondria, play an important role in cell death (Griivennikova and Vi-nogradov, 2013). Accumulating evidence strongly suggests that ROS, specifically mitochondria-generated ROS, are involved in physiological signaling cascades regulating various cellular and organ functions (Afanas’ev, 2007; Valko et al., 2007; Leuner et al., 2012; Tang et al., 2013). The production of ROS by mitochondria is recognized as a crucial event in mitochondrial metabolism and oxidative stress (Akopova et al., 2014).

This study found that increasing intracellular Ca$^{2+}$ concentrations prevented the electric membrane potential caused by H$_2$O$_2$. The consequent activation of ROS release may increase the Ca$^{2+}$ overload thus preventing the electric membrane potential and inducing mitochondrial permeability transition pore. This response may lead to an increase in the level of ROS that can damage the respiratory chain and result in the decrease of ATP. This vicious circle results in oxidative stress and mitochondrial damage. Similarly, our results also showed enhanced ROS formation in mitochondria after oxidative damage, which was preventable by antioxidant rAAV/ABAD-DP-6His. The rAAV/ABAD-DP-6His inhibited the increase of Ca$^{2+}$ concentration, maintained the stability of electric membrane potential, decreased ROS levels, and ensured a constant content of ATP.

Studies addressing oxidative stress will enable researchers
to better understand the mechanisms underlying Alzheimer’s disease by identifying early markers of the disease. Our group has previously investigated the mechanisms and pathogenesis of Alzheimer’s disease to seek new therapeutic schedules to inhibit oxidative damage and Aβ injury of Alzheimer’s disease (Wang et al., 2012; Wang et al., 2013).

Conclusion
In this study, rAAV/ABAD-DP-6His induced neuroprotective effects by binding to Aβ and also protecting against oxidative stress in a non-specific manner. Therefore, these effects may be useful for developing therapeutic strategies with the aim that early treatment will slow or prevent the progression of Alzheimer’s disease by targeting oxidative stress.

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Author contributions: Jia MY conducted the experiments, performed statistical analysis, analyzed experimental data, and wrote the manuscript. Yang Y and Yang Y conceived and designed the study. Wang MY provided experimental data. Chen YX and Liu DJ integrated experimental data. Wang X and Song L assisted in conducting the experiments. Wu J was in charge of funds and revised the manuscript. All authors approved the final version of the paper.

Conflicts of interest: None declared.

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