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Research Article

Gastrodiae Rhizoma Improve Cognitive Functions and Regulate Keap1/Nrf2-ARE Signal Pathway in APP/PS1 Transgenic Mice

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Abstract

Background and Objective: *Gastrodiae Rhizoma* (GR, Chinese name "Tian Ma") is known as a precious traditional Chinese medicine with a wide range of clinical applications. This study aimed to investigate the neuroprotective effects and the potential therapeutic mechanisms of GR in APPswe/PSEN1dE9 double-transgenic (APP/PS1_DT) mice. **Materials and Methods:** Mice in the prevention group were intragastrically administrated with 1.5 g kg^{-1} GR daily from the age of 2 months to 6- and 10 months, respectively, mice in the treatment group were treated at the age of 6- and 10 months and continued for 14 days. The learning and memory abilities, synapse morphology, the mRNA and protein levels of synaptic and Keap1/Nrf2-ARE signalling pathway proteins in the hippocampus and the level of oxidative stress were detected. **Results:** Compared to the APP/PS1_DT mice, GR effectively improved the learning and memory abilities, decreased the Aβ deposition, attenuated the Aβ-induced synaptic loss and oxidative stress, increased synaptic proteins levels, maintained the synapse morphology and activated the Keap1/Nrf2-ARE signalling pathway. Compared with the treatment group, the above changes in the prevention group were more obvious. **Conclusion:** The GR could ameliorate the learning and memory ability of APP/PS1_DT mice, which may be related to its attenuation of the neurotoxic effects of Aβ *in vivo*.

Key words: *Gastrodiae Rhizoma*, Keap1/Nrf2-ARE signalling pathway, oxidative stress, synapses, Alzheimer's disease, APPswe/PSEN1dE9 double-transgenic mice, Aβ-induced synaptic loss

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Alzheimer's Disease (AD) is the leading cause of dementia. Its main clinical manifestations are progressively cognitive dysfunction, abnormal mentality and personality, dwindling social interaction and adaptability[1,2]. Senile plaques (SP), intraneuronal neurofibrillary tangles (NFTs) and neurons loss are the major pathophysiological hallmarks of AD[3].

β-amyloid peptide (Aβ) is the major component of SP (38-43 amino acids peptide). Of note, excessive Aβ deposition is the core mechanism of AD. The Aβ deposition is associated with synaptic network dysfunction, longitudinal cognitive decline and neuronal calcium homeostasis. Therefore, reducing the deposition of Aβ or attenuating its neurotoxicity are promising potential strategies for AD. The precursor protein of Aβ is amyloid precursor protein (APP), mutations in the APP gene cause abnormalities in the APP metabolism and lead to excessive Aβ deposition in the brain[4]. The APPswe/PSEN1dE9 double-transgenic (APP/PS1_DT) mice express human APP (APPswe) and presenilin (DeltaE9) mutated fusions, thereby elevating the production and deposition of Aβ in the mouse brain (6-7 months old) and showing spatial memory and learning deficit and mimic the neuropathological changes of AD.

Synaptic impairment is highly correlated to the neuropathological characteristics and cognitive impairment of AD. Synapses are intercellular junctions that transmit information between neurons[5]. It is well known that synapse loss and the impairment of synaptic function are closely related to the cognitive dysfunction of AD patients[6]. The previous data also indicated that the numbers of the synapse, synaptic vesicles and synaptic proteins were decreased significantly in the hippocampus of APP/PS1 mice, suggesting that was connected to the decline of cognitive function[7].

Oxidative stress (OS) is also an essential and early mechanism in AD pathogenesis[8]. The Aβ is an inducer of OS and OS will increase the Aβ production and deposition, thereby forming a vicious cycle that accelerates AD progression. The reactive oxygen species (ROS) overproduction and the antioxidant defenses reduction directly damage synaptic activity and neurotransmission, leading to cognitive dysfunction[9]. As a transcription factor, nuclear factor erythroid 2 related factors 2 (Nrf2) can regulate the antioxidant proteins and redox balance in eukaryotic cells. Nrf2 plays a key role in the oxidative status of cells and robust protection against oxidative challenge by regulating the genes that possess the antioxidant/electrophile response elements (ARE/EpRE)[10]. In the absence of external stimuli, Nrf2 and Kelch-like ECH-associated protein 1 (Keap1) form a complex in the cytoplasm and is cleared through the ubiquitination degradation pathway. When the cell is in oxidative stress disturbance, the Keap1/Nrf2 complex will dissociate, Nrf2 is released into the cytoplasm and then enters the nucleus to activate the transcription of protective genes, including NAD(P)H-quinone oxidoreductase 1 (NQO1), thioredoxin reductase 1 (Txnrd1), Glutathione peroxidase 1 (GPX1), ferritin and heme oxygenase-1 (HO-1), glutathione-S-transferase (GST)[11]. Insufficient Nfr2 activation is also related to AD pathogenesis and the neuroprotective effects of the Nrf2/HO-1 redox signal pathway under normal and pathological conditions have recently become the major subject in research[12]. Therefore, stimulating Nrf2 and regulating the Keap1/Nrf2-ARE pathway will be provided promising potential therapeutic ideas and targets for AD treatment.

Gastrodiae Rhizoma (Tian Ma, GR), the dried rhizome of *Gastrodia elata* Blume, is a precious traditional Chinese herb listed in the Chinese Pharmacopoeia. Accumulating evidence has been conducted on its pharmacological properties and suggested that GR have wide therapeutic effects and was extensively applied to prevent and ameliorate central nervous system diseases in Traditional Chinese Medicine. It is widely applied to treat convulsions (also in children), tetanus, headache, vertigo, epilepsy, numbness, rheumatic arthralgia, etc.[13]. It worked by attenuating amyloid and tau levels, inhibiting the autophagy and apoptosis of hippocampus neurons and alleviating inflammation[14]. Recent growth data have found that GR induces the expression and nuclear translocation of Nrf2 and increases the expression of antioxidant genes (such as HO-1 and GCLM) in astrocytes, indicating that it may have potential value in the treatment of AD[15].

In the present study, we used APP/PS1_DT mice models to investigate the effects of GR on OS, synapse morphology, synaptic proteins expression levels, cognitive functions and the Keap1/Nrf2-ARE signalling pathway. The results may provide a certain theoretical basis for the prevention, relief and treatment of AD by GR.

MATERIALS AND METHODS

Study area: The study was carried out at the Key Laboratory of Endemic and Minority Disease, Ministry of Education and School of Pharmacy of Guizhou Medical University, Guiyang, China from 1 September, 2017 to 7 November, 2019.

Table 1: Primers for analysis of the mRNA levels of Keap1, Nrf2, HO-1 and NQO1 and β-actin by RT-qPCR

Genes	Primer sequences (5′→3′)	Amplicon size (bp)
β-actin	GTGCTATGTTGCTCTAGACTTCG	174
	ATGCCACAGGATTCCATACC	
Keap1	CGGGGACGCAGTGATGTATG	85
	TGTGTAGCTGAAGGTTCGGTTA	
Nrf2	ACCAAGGGGCACCATATAAAAG	114
	CTTCGCCGAGTTGCACTCA	
HO-1	AGGTACACATCCAAGCCGAGA	86
	AGGATGGGAGGTACTCGAATC	
NQO1	AGGATGGGAGGTACTCGAATC	127
	TGCTAGAGATGACTCGGAAGG	

Reagents and antibodies: Antibodies used in the study were listed as follows: Rabbit anti-SYN antibody (ab32594) was purchased from Abcam, USA. Rabbit anti-PSD95 antibody (GTX133091), rabbit anti-DYN1 antibody (GTX110379), rabbit anti-β-actin antibody (GTX109639), rabbit anti-Keap1 antibody (GTX54329), rabbit anti-Nrf2 antibody (GTX103322), rabbit anti-HO-1 antibody (GTX101147), rabbit anti-NQO1 antibody (GTX100235) were purchased from Gene Tex, USA. The HRP-labelled anti-mouse secondary antibody (#7076) and HRP-labelled anti-rabbit secondary antibody (#7074) were purchased from Cell Signaling Technology, USA. Mouse anti-Aβ monoclonal antibody 6E10 (SIG39320) was purchased from Biolegend company, USA.

Other reagents were as follows: GR powder from Guizhou Yikang Pharmaceutical Co., Ltd. China. The primers for amplification of Keap1, Nrf2, HO-1, NQO1 and β-actin were synthesised by Shanghai Genecore Biotechnologies, Shanghai, China, the primer sequences were listed in Table 1. The ABC immunohistochemistry kit was from Vectastain, USA. The other chemicals used were purchased from Sigma. The MDA content test kit, SOD, GOT, GPT enzyme activity kits were from Nanjing Jiancheng Bioengineering Research Institute, Nanjing, China.

Reproduction and treatment of APP/PS1_DT mice: About 20-30 g male APP/PS1_DT mice (N = 60) (B6. Cg-Tg) were purchased from Shanghai Southern Model Biology Co., Ltd. (animal license No. SCXK 2014-0002). The PCR was performed to confirm the genotypes of these mice with the presence of the APPswe/PSldE9 gene mutations. The identification of APP/PS1_DT mice was carried out according to the previous method and the expected length of PCR products was 400 and 600 bp, respectively[7].

The APP/PS1_DT mice were treated with GR for grouping (15 mice for each group):

- **Control group:** Wild-type C57 mice were intragastrically administrated with normal saline daily from 2-6 and 10-months of age
- **APP/PS1 group:** APP/PS1_DT mice were intragastrically administrated daily with normal saline from 2-6 and 10 months of age
- **Prevention group:** APP/PS1_DT mice were intragastrically administrated with 1.5 g kg^{-1} GR daily from 2-6 and 10 months of age
- **Treatment group:** APP/PS1_DT mice were intragastrically administrated with 1.5 g kg^{-1} GR daily at the 6th and 10th months for 14 days

Morris water maze test (MWM): The MWM was established for testing hippocampal-dependent learning, including acquisition and long-term spatial memory[16]. Every mouse was subjected to 4 pieces of training a day for 4 days consecutively, with an inter-trial interval for 5-7 min. Video tracking software (ViewPoint, DNS-2) was used to record the movement of each mouse simultaneously. In each training, the escape latency (the duration of locating the escape platform, the shorter the escape latency), the quadrant search time (the time spent in the platform quadrant, the length of the time, the stronger the learning and memory ability of mice) were determined. On the 5th day, the number of passing through the platform within 60 sec after removing the platform of each mouse was recorded. The more times the mouse passes through the platform within 60 sec, the stronger the learning and memory ability of the mouse was.

Observing the synapse morphology by TEM: The hippocampus tissue was rinsed 3 times with PBS after fixing with 4% glutaraldehyde solution for more than 2 hrs. Samples were then fixed by 1% osmium tetroxide for 2 hrs, then dehydrated with gradient ethanol and acetone. Finally, specimens were impregnated, embedded and polymerized by epoxy resin. Ultratome was used to prepare 0.5 μm and 60 nm slices and then the slices were double-stained with lead citrate and uranyl acetate. Using H-7500 electron microscope (HITACHI) to observe and record the morphology changes of synapse in the hippocampal. Using Image-pro plus 6.0 software to analyze the synapses number, the synaptic cleft width, the post-synaptic thickness and the synaptic interface curvature.

Analyzing the expression of $A\beta_{1-42}$ and PSD95 by immunohistochemistry: Immunohistochemistry was performed following our previous report[7]. Briefly, embedded the brain tissue with paraffin after 24 hrs fixing by 4% paraformaldehyde in PBS. Then, the embedded hippocampal tissue was cut into 4 μm slices and then deparaffinized with xylene, rehydrated with ethanol and then incubated in 3%

H_2O_2 for 30 min. Afterwards, the slices were subjected to antigen retrieval with citrate buffer and then blocked with 5% goat serum for 30 min, incubated with PSD95 (1:100) and $A\beta_{1-42}$ (1:100) primary antibodies overnight at 4°C. After overnight incubation with primary antibody, a peroxidase-conjugated anti-mouse ABC kit (Vector, vectastain ABC Kit) was used to detect the monoclonal antibodies. For negative control, primary antibodies were replaced with nonimmune serum. The pictures were obtained by using a Nikon microscope (Nikon, Model Eclipse Ci-E) and analyzed with NIS-Elements AR software.

Analyzing the mRNA levels of Keap1/Nrf2/ARE pathway proteins by RT-qPCR: The mRNA levels of Nrf2/ARE/HO-1 signalling pathway proteins (Keap1, Nrf2, HO-1, NQO1) and β-actin were determined by RT-qPCR as described previously[17]. Briefly, 2 μg of total RNA from the hippocampus of each mouse were reverse transcribed to cDNA by using the oligo-dT strategy. The RT-qPCR was conducted on ABI 7300 Real-time system and analyzed with the Applied Biosystems SDS2.1 software. Amplifications were performed with the universal TaqMan 2×PCR SYBR Green I Master mix according to the manufacturer's instructions. Reactions were carried out in a 25-μL volume with 2.5 μL cDNA and 1.25 μL each primer for analyzing the relative level of each protein, the primer sequences were listed in Table 1. The SDS2.1 software was used for analyzing the $^{\Delta\Delta}$Ct and relative quantity (RQ) value ($RQ = 2^{-\Delta\Delta}Ct$) of each gene. With β-actin as an internal control, the relative levels of Keap1, Nrf2, NQO1 and HO-1 mRNA were calculated.

Analyzing the protein levels of synaptic proteins and Keap1/Nrf2/ARE pathway by western blot: The levels of synaptic proteins and Keap1/Nrf2/ARE signal pathway proteins in brain tissue lysates were quantified by western blot as described previously[17]. In brief, proteins extracted from the hippocampus were quantitated by BCA Assay. The proteins were separated with 10% SDS-PAGE electrophoresis, transferred to polyvinylidene difluoride membranes and blocked with 5% skimmed milk for 2 hrs at room temperature (RT). Then, the membranes were incubated with primary antibodies (against SYN, DYN1, Keap1, Nrf2, HO-1 or NQO1) at 4 overnight. After rinsing, a secondary antibody conjugated with HRP was added to the membranes and incubated for 1 hrs at RT. Finally, the protein blots were detected with chemiluminescence substrate (ECL) and exposure to film.

After striping the antibodies of the membranes, they were incubated again with a primary mouse antibody against β-actin (1:5000) for 120 min and then incubated with HRP-conjugated anti-mouse IgG for 60 min at RT. Using ImageJ software to analyze the intensity of each protein band and the β-actin band as an internal control to calculate the relative expression levels of proteins.

Analyzing the MDA content and SOD activity: The malondialdehyde (MDA) level and the activities of superoxide dismutase (SOD) in the homogenate of mice hippocampus of each group were determined by commercial kits according to the protocol (Jiancheng Institute of Biotechnology, Nanjing, China).

Statistical analysis: All data were presented as Means±SD. The values were examined using two-tail Student's t-test and one-way ANOVA with SPSS 22 software (USA), differences between means were considered statistically significant when $p<0.05$.

RESULTS

GR enhanced spatial learning and memory abilities of APP/PS1_DT mice: The MWM assay was measured the neuroprotective function of GR on alleviating the decline of spatial memory and learning ability of APP/PS1_DT mice. The escape latency was much longer in APP/PS1 groups at 6 and 10 months old (18.20±2.15 sec, $p<0.01$ and 17.64±3.71 sec, $p<0.05$) than the control group (9.59±0.97 and 11.08±1.56 sec). However, administration of GR could alleviate the elongated escape latency both in prevention at 6 and 10 months old (6.73±1.76 sec, $p<0.01$ and 8.84±1.64 sec, $p<0.01$) and treatment groups (7.51±1.13 sec, $p<0.01$ and 10.25±1.8 5 sec, $p<0.05$) (Fig. 1a). On the 5th day, the number of crossing the platform within 60 sec after removing the platform of each mouse was recorded. The numbers of crossing platform of APP/PS1 groups at 6 and 10 months old were reduced (1.63±0.38, $p<0.01$ and 2.17±0.32, $p<0.01$) compared with controls at 6 and 10 months old (5.00±0.72 and 4.05±0.66), while the time staying on the platform of APP/PS1 groups at 6 and 10 months old were decreased (10.78±0.89 sec, $p<0.01$ and 15.27±1.60 sec, $p<0.05$) compared with the control groups (22.53±0.88 and 20.50±1.20 sec). In the GR-treated mice, the numbers of crossing and the time staying on the platform were dramatically increased at 6 months old (4.40±0.87, $p<0.01$, 19.33±1.30 sec, $p<0.01$) and 10 months old (3.50±0.45, $p<0.05$, 24.16±1.72, $p<0.01$) (Fig. 1b, c). In the GR-prevention group, the numbers of crossing and the time staying on the platform were dramatically increased at 6 months old (4.40±0.87, $p<0.01$, 19.33±1.30 sec, $p<0.01$)

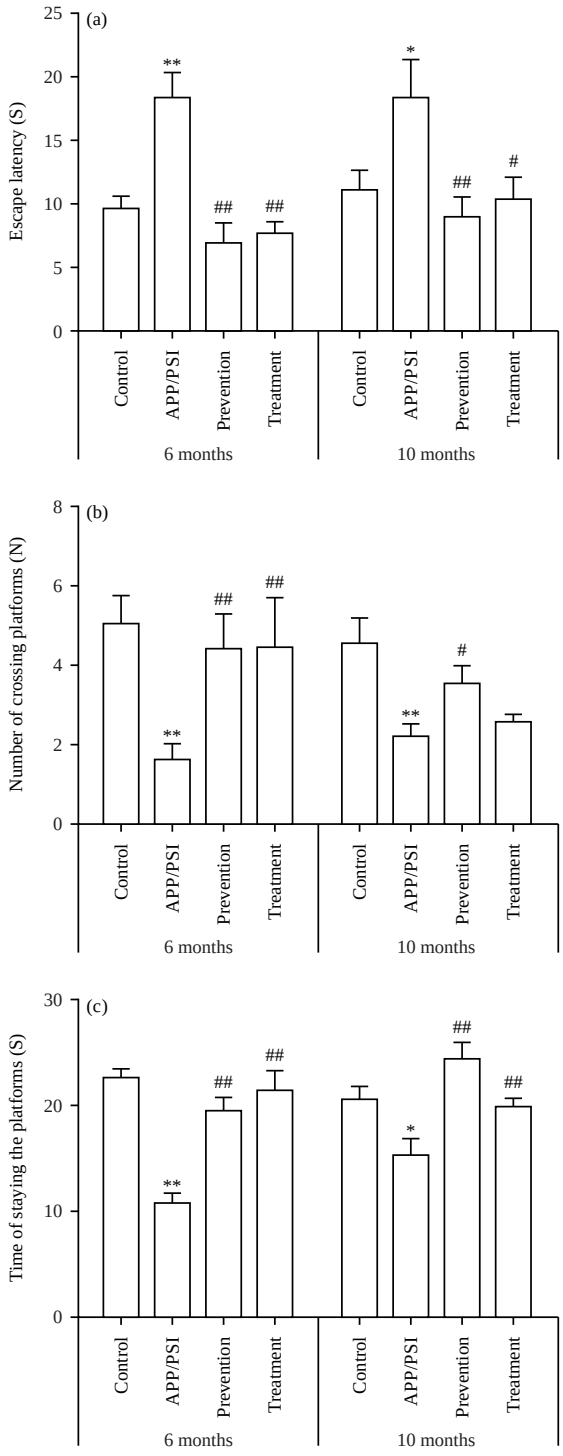

Fig. 1(a-c): GR enhanced learning and memory abilities in APP/PS1_DT mice, (a) Escape latency (second, s), (b) Numbers of crossing (times, N) and (c) Time of staying on the platform (sec)

Values were shown as the Means±SD. *p<0.05, **p<0.01 in comparison to control, #p<0.05, ##p<0.01 in comparison to APP/PS1

and 10 months old (3.50±0.45, p<0.05, 24.16±1.72, p<0.01). In the GR-treatment group, the numbers of crossing platforms were dramatically elevated at 6 months old (4.40±1.27, p<0.01), whereas, there are not altered at 10 months old (2.50±0.24). The time staying on the platform were dramatically raised at 6 and 10 months old (21.30±1.90 sec, p<0.01, 19.75±0.85, p<0.01). Taken together, these results indicate that GR rescues the spatial learning and memory decline in APP/PS1_DT mice.

GR attenuated the deposition of Aβ in the hippocampus of APP/PS1_DT mice: The Aβ deposition and Aβ integrated optical density (IOD) were alleviated significantly both in the 6 and 10 months old (69131.50±5386.79, p<0.01in Fig. 2a, b, and 97698.43±6880.97 p<0.01 in Fig. 2c, d) APP/PS1_DT mice groups. Figure 2a-c shows that, there was no obvious Aβ deposition in the brain of the 6 and 10 months mice in the control group. Whereas, in the GR prevention mice group brains, the Aβ deposition and the Aβ IOD level were significantly alleviated at 6-months old and 10-months (5936.67±586.13, p<0.01 in Fig. 2a, b and 6497.00±1030.36, p<0.01 in Fig. 2c, d) and the GR treatment mice group brains, the Aβ deposition and the Aβ IOD level were also significantly reduced at 6 and 10 months (9246.00±1388.90, p<0.01 in Fig. 2a, b and 10294.15±895.82, p<0.01 in Fig. 2c, d). There was no difference in Aβ1-42 deposition in the hippocampus of 6 months old mice between the prevention and treatment group (p>0.05), but the Aβ deposition in the brain tissue of 10 months old mice in the prevention group was alleviated more pronounced than the treatment group. The results showed that GR could decrease the Aβ deposition in the APP/PS1_DT mice brain, thereby alleviating the neurotoxic effects of Aβ.

GR treatment increased the level of synaptic proteins in the hippocampus of APP/PS1_DT mice: The level of synaptic proteins (SYN, DYN1) at protein levels in the hippocampus was determined. A decreased DYN1 protein levels were found in hippocampal of APP/PS1_DT mice group at 6 and 10 months (74.90±2.43, p<0.01, 71.95±3.93, p<0.01) compared with that in the control mice group (100.00±6.10, 100.00±9.81, Fig. 3a). After GR treatment, the expressions of DYN1 in the prevention group were significantly increased at 6 and 10 months (96.00±7.06, p<0.05 and 94.95±4.29, p<0.05), while in the treatment group, the level of DYN1 increased only in the 10 months group treated by GR (92.83±5.50, p<0.05, Fig. 3a). Decreased SYN protein levels were also detected in

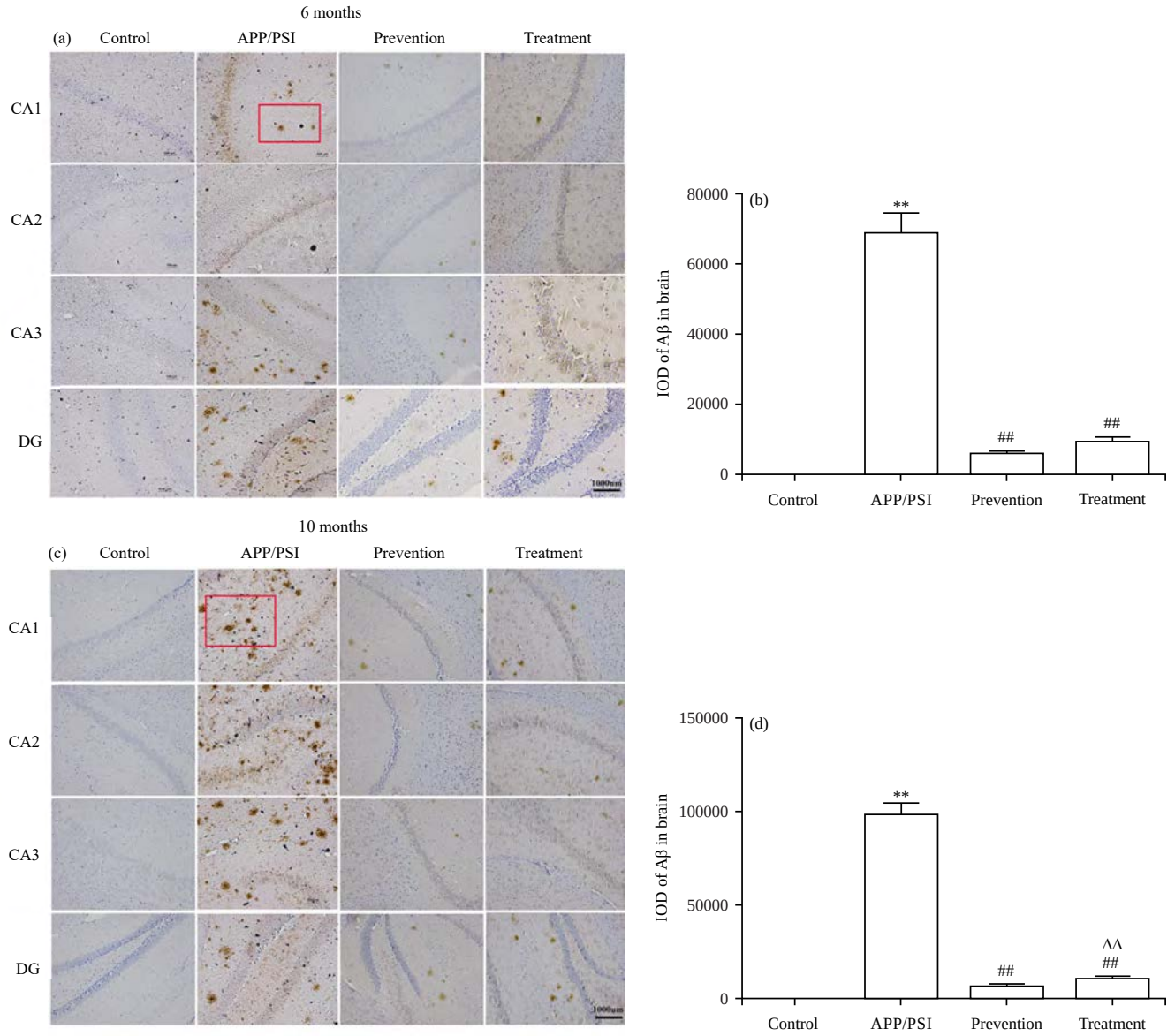

Fig. 2(a-b): GR reduced the Aβ deposition in the hippocampus of APP/PS1_DT mice, (a-b) Histological observation and optical density analysis of $A\beta_{1-42}$ in brain tissue of mice in 6 months old groups and (c-d) Histological observation and optical density analysis of $A\beta_{1-42}$ in brain tissue of mice in 10 months old groups. B and D represented the IOD statistical value of Aβ in the brain of mice at the age of 6 and 10 months, respectively

Values are shown as the Means±SD, $^{**}p<0.01$ in comparison to the control group, $^{\#\#}p<0.01$ in comparison to the APP/PS1 group and $^{\Delta\Delta}p<0.01$ in comparison to the prevention group

hippocampal of APP/PS1_DT mice group at 6-months old and 10 months (84.42±5.30, $p<0.05$, 80.63±4.01, $p<0.05$) compared with that in the control mice group (100.00±9.08, 100.00±6.11, Fig. 3b). There was no difference in the level of SYN protein level in the hippocampus compared with mice in the prevention and treatment groups (87.08±4.18 and 87.63±5.23, Fig. 3b). Taken together, these data indicated that GR partially reversed the decreased synaptic protein levels.

GR treatment increased the expression of PSD95 in the mice model: The PSD95 is a key synaptic protein that participates in synaptic connections and postsynaptic signal transduction. Its decreased expression indicates abnormal synaptic function. The results of immunohistochemical staining (Fig. 4a-c) showed the integrated optical density (IOD) of PSD95 were decreased significantly in the 6 (14452.73±2811.64, $p<0.05$ in Fig. 4a, b) and 10 months old (10767.96±984.67, $p<0.01$ in

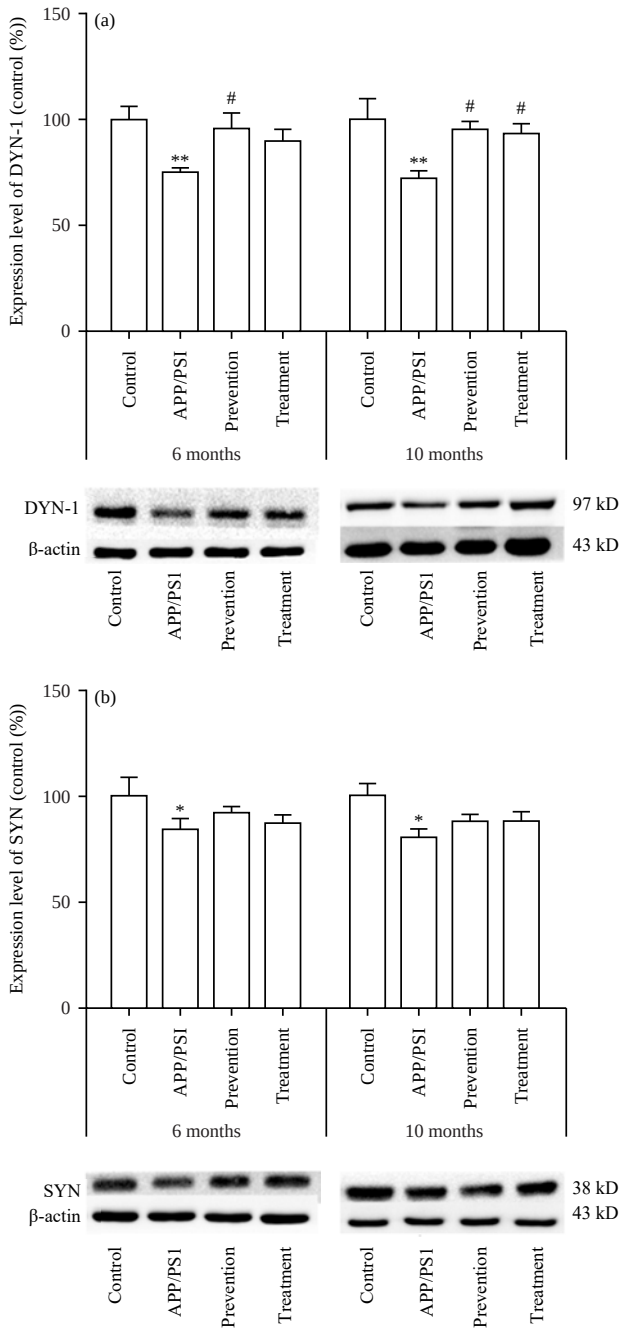

Fig. 3(a-b): GR treatment increased the level of synaptic proteins in the hippocampus of APP/PS1_DT mice, Levels of (a) DYN1 and (b) SYN proteins in the hippocampus of wild-type normal mice (Control), APP/PS1_DT mice (APP/PS1), APP/PS1_DT mice treated with long-term GR (Prevention group) and APP/PS1_DT mice treated with short-term GR (Treatment group) were determined by Western blot

Values shown as a percentage of the control by relative quantification were Mean±SD, *p<0.05, **p<0.01 compared with control, #p<0.05 compared with APP/PS1_DT

Fig. 4a-c) APP/PS1_DT mice group and it decreased more obviously in the 10 months old mice. Interestingly, the PSD95 expression was significantly alleviated by GR treatment both in the brain of the prevention (31035.23±2971.60, p<0.01) and treatment groups of 10 months (16517.59±1170.17, p<0.01) (Fig. 4a-c), while there were no changes at the 6 months old group in GR prevention and treatment groups (5936.67±586.13 and 6497.00±1030.36) (Fig. 4a, b). Compared with the prevention group of the same age, there was no change of IOD value of PSD95 protein in 6 months old mice between GR prevention and treatment group (14436.54±3189.73), while it was increased significantly in the hippocampus of the GR prevention group compared with GR treatment group at 10 months old mice. Taken together, these data indicated that GR reversed the decreased postsynaptic protein levels.

Observation synapse morphology by TEM: The TEM image in Fig. 5 showed that the synapse number was decreased, the synaptic cleft became wider and the post-synaptic dense substance became thinner in the hippocampus of 6 and 10 months old APP/PS1 mice as compared to the control group (Fig. 5a, b). After GR treatment, the post-synaptic dense substance in the prevention groups (6 or10 months old) were thicker than APP/PS1 mice. The number and characteristics of synapses in the treatment group also ameliorated compared to APP/PS1 group, the synaptic interface curvature became smaller in 6 months old mice (Fig. 5a-b). These results indicated that GR could maintain the synaptic morphology and increase the synapse number, thus enhancing the synaptic functions (Fig. 5).

Table 2 and 3 show that, the number of synapses decreased, the synaptic cleft widened, the post-synaptic dense substance became thinner and the synaptic interface curvature became smaller in hippocampal neurons of both 6 and 10 months old APP/PS1 mice. Compared with APP/PS1 group, the synapse number was increased significantly, the synaptic cleft narrowed, the thickness of postsynaptic membrane density and the curvature of the synaptic interface increased in the prevention group, while there was no chance of these results in the 10 months old treatment group compared with APP/PS1 group. Above all, GR could maintain the synaptic structure integrity and increase the synapses number, thereby improving synaptic function, which was key in AD prevention.

GR activated the Keap/Nrf2/ARE pathway in the hippocampus of APP/PS1_DT mice: This study investigated the effect of GR on Keap/Nrf2/ARE signaling pathway in APP/PS1_DT mice. As presented in Fig. 6a, the mRNA level of

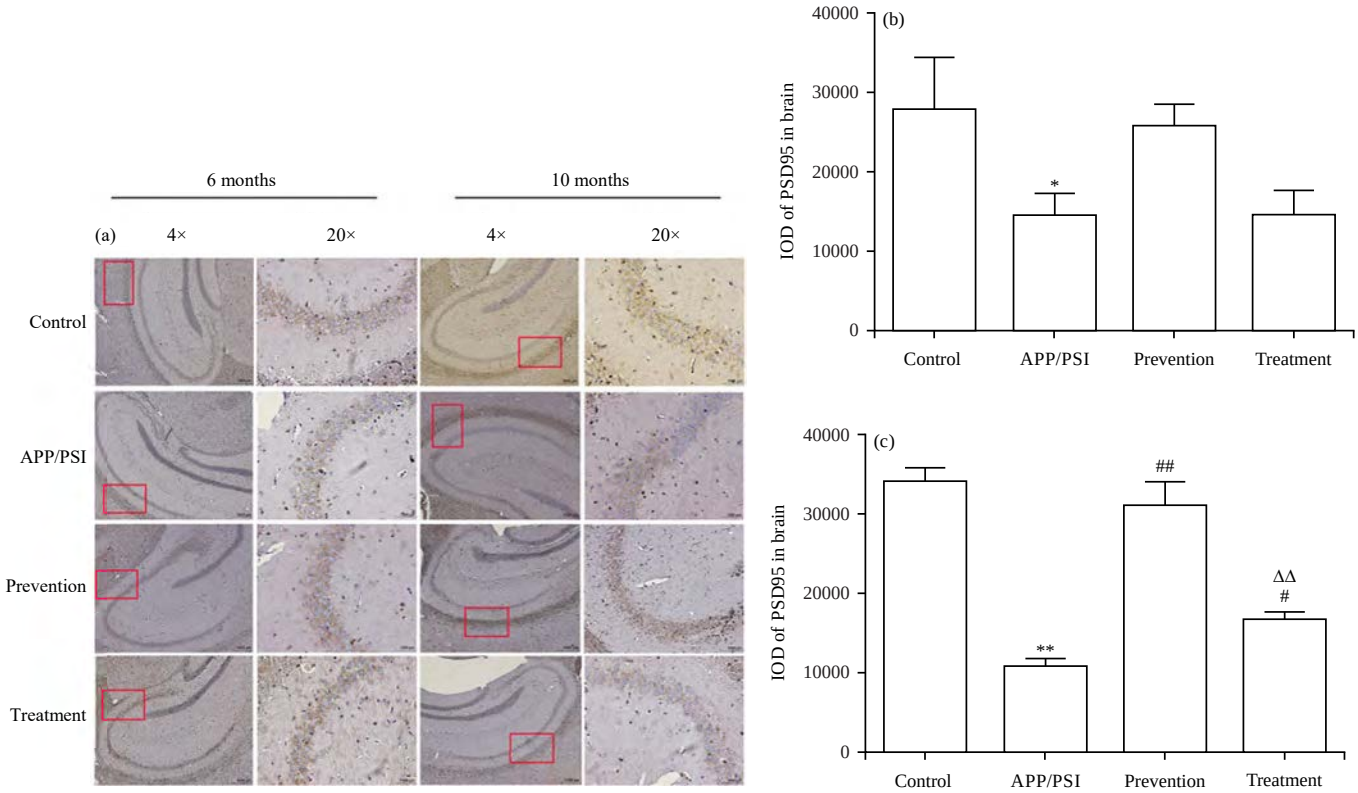

Fig. 4(a-c): GR treatment increased the PSD95 expression in the hippocampus of APP/PS1_DT mice, (a) Histological observation and optical density analysis of PSD95 in brain tissue of mice in 6 and 10 months groups, (b-c) IOD statistical value of PSD95 in the brain of mice at the age of 6 and 10 months, respectively

Values were shown as the Means±SD, $^{*}p<0.05$, $^{**}p<0.01$ compared with control, $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with APP/PS1 group, $^{\Delta\Delta}p<0.01$ compared with prevention group

Table 2: Hippocampal synaptic number and structural parameters of 6-months old mice $\bar{x}\pm$SD

Group	Number of synapses	Synaptic cleft width (nm)	Thickness of postsynaptic membrane density (nm)	Curvature of synaptic interface
Control	12.71±1.30	23.33±1.85	41.55±7.45	1.10±0.01
APP/PS1	8.10±0.80**	26.38±1.75**	33.15±4.98**	1.04±0.01**
Prevention	15.00±1.18##	23.39±2.88##	40.15±5.20##	1.08±0.01#
Treatment	10.27±0.95	24.15±2.33#	39.74±5.99##	1.07±0.01

$^{*}p<0.05$, $^{**}p<0.01$ compared with control, $^{\#}p<0.05$ and $^{\#\#}p<0.01$ compared with APP/PS1 group

Table 3: Hippocampal synaptic number and structural parameters of 10-months old mice $\bar{x}\pm$SD

Group	Number of synapses	Synaptic cleft width (nm)	Thickness of postsynaptic membrane density (nm)	Curvature of synaptic interface
Control	12.10±2.07	22.03±2.76	51.35±5.20	1.06±0.03
APP/PS1	7.75±0.50**	25.68±1.84*	44.40±4.43*	1.03±0.03**
Prevention	12.33±1.74##	22.17±3.05#	49.43±6.16#	1.06±0.02##
Treatment	10.80±1.46	23.38±4.43	43.04±6.51ΔΔ	1.05±0.02

$^{**}p<0.01$, $^{*}p<0.05$ in comparison to the control group, $^{\#\#}p<0.01$, $^{\#}p<0.05$ in comparison to the APP/PS1 group and $^{\Delta\Delta}p<0.01$ in comparison to the prevention group

Keap1 was no significant difference at 6 and 10 months old (1.04±0.18 and 1.03±0.19) compared to control group, no changes were found between both groups at 6 and 10 months old (GR prevention group: 0.96±0.18 and 0.98±0.08, GR treatment group: 0.95±0.05 and 1.01±0.13), the decreased mRNA level of Nrf2 were detected in the hippocampus of APP/PS1_DT mice at 6 and 10 months old (0.60±0.06, $p<0.01$ and 0.69±0.08, $p<0.01$, Fig. 6b) compared to control group, at the same time, the elevated mRNA level of Nrf2 were found in both GR prevention and treatment group at 6 months old (1.30±0.09, $p<0.01$ and 1.03±0.06, $p<0.01$), whereas, the mRNA level of Nrf2 was increased in GR

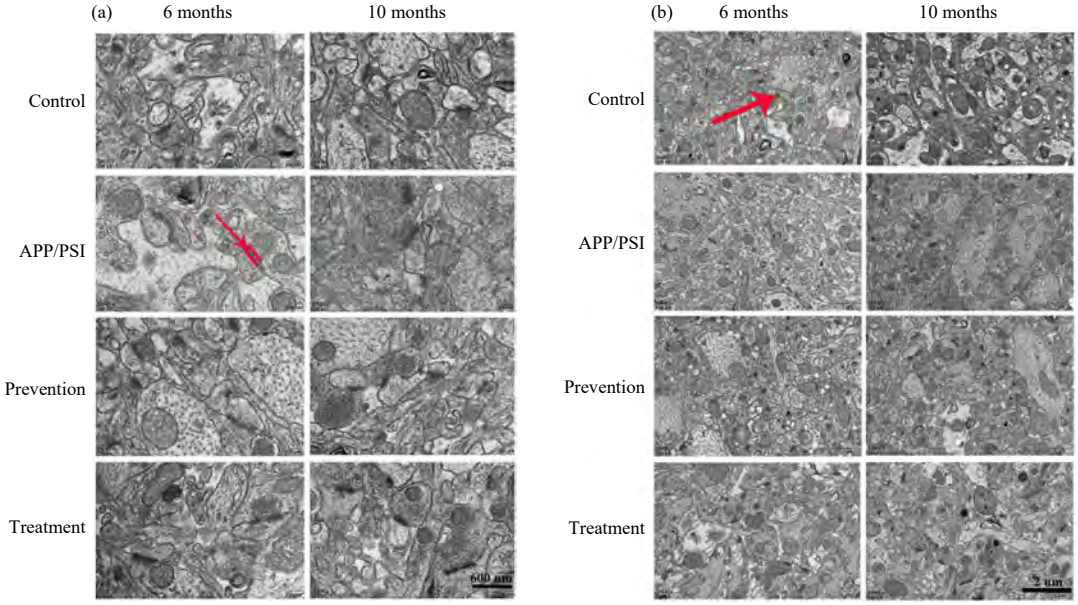

Fig. 5(a-b): GR treatment increased the synapse number and maintained synaptic morphology in the hippocampus of APP/PS1_DT mice. The synapses were visualized by using TEM. The magnification of TEM is (a) 20K times and (b) 50K times, respectively. The number and synaptic characteristics in 6 and 10 months old mice were shown above
The arrows indicated the synapse in A and the synaptic cleft in B

treatment group at 6 months old (1.14±0.14, $p<0.05$) while no obvious change was found in GR treatment group at 10 months old (0.92±0.10, Fig. 6b), the mRNA level of HO-1 was significantly decreased in the hippocampus of APP/PS1_DT mice at 6 and 10 months old (0.69±0.05, $p<0.01$ and 0.83±0.03, $p<0.01$, Fig. 6c) compared to control group, the increased HO-1 mRNA level was found in both groups at 6 and 10 months old (GR prevention group: 1.05±0.08, $p<0.01$ and 1.43±0.11, $p<0.01$, GR treatment group: 1.11±0.04, $p<0.01$ and 0.94±0.13, $p<0.01$, Fig. 6c), the mRNA level of NQO1 was decreased in APP/PS1_DT mice at 6 and 10 months old (0.75±0.02, $p<0.05$ and 0.68±0.08, $p<0.01$, Fig. 6d) compared to control group, the elevated NQO1 mRNA level was detected in GR prevention group at 6 and 10 months old (1.45±0.16, $p<0.01$ and 1.34±0.07, $p<0.01$, Fig. 6d), while no alteration was found in GR treatment group at 6 and 10 months old (0.68±0.01 and 0.90±0.10), the Keap1 protein level was no significant alteration at 6 and 10 months old (106.58±2.85 and 99.50±10.68) compared to control group (100.00±12.16 and 100.00±10.88), no changes were found between both groups at 6 and 10 months old (GR prevention group: 103.26±4.09 and 93.60±8.17, GR treatment group: 95.72±6.15 and 103.20±7.96, Fig. 7a), the Nrf2 protein level was reduced in APP/PS1_DT mice at 6 and 10 months old (67.10±6.30, $p<0.05$ and 63.63±5.06, $p<0.05$, Fig. 7b) compared to control group (100.00±9.10 and 100.00±17.51), the elevated Nrf2 protein level found in GR prevention group at 6 and 10 months old (100.40±5.90, $p<0.05$ and 98.38±6.75, $p<0.05$) while no obvious change was found in GR treatment group at 6 and 10 months old (82.00±10.40 and 82.16±8.41, Fig. 7b), the protein level of HO-1 was reduced in the hippocampus of APP/PS1_DT mice at 6 and 10 months old (60.45±5.67, $p<0.05$ and 72.34±9.97, $p<0.01$, Fig. 7c) compared to control group (100.00±6.67 and 100.00±6.18), the HO-1 protein level was raised in GR prevention group at 6 and 10 months old (85.25±4.62, $p<0.05$ and 97.94±5.82, $p<0.01$, Fig. 7c), no change was found in GR treatment group at 6 months old (72.03±3.60) while the increased HO-1 protein level was presented at 10 months old (93.17±6.00, $p<0.05$), the decreased protein level of NQO1 was found in the hippocampus of APP/PS1_DT mice at 6 and 10 months old (46.49±2.31, $p<0.05$ and 50.52±10.76, $p<0.05$, Fig. 7d) compared to control group (100.00±14.36 and 100.00±15.31), the NQO1 protein level was increased in GR prevention and treatment group at 6 and 10 months old (GR prevention group: 106.63±21.32, $p<0.05$ and 112.28±24.47, $p<0.01$, GR treatment group: 102.10±24.04, $p<0.01$ and 69.09±18.67, $p<0.05$, Fig. 7d). Taken together, in this current study, GR treatment could obviously reversed the decreased mRNA and protein levels of Nrf2, HO-1 and NQO1 in the hippocampus of APP/PS1_DT mice , especially in the prevention group of 10 months old mice. These results indicated that GR activated the Keap/Nrf2/ARE signaling pathway.

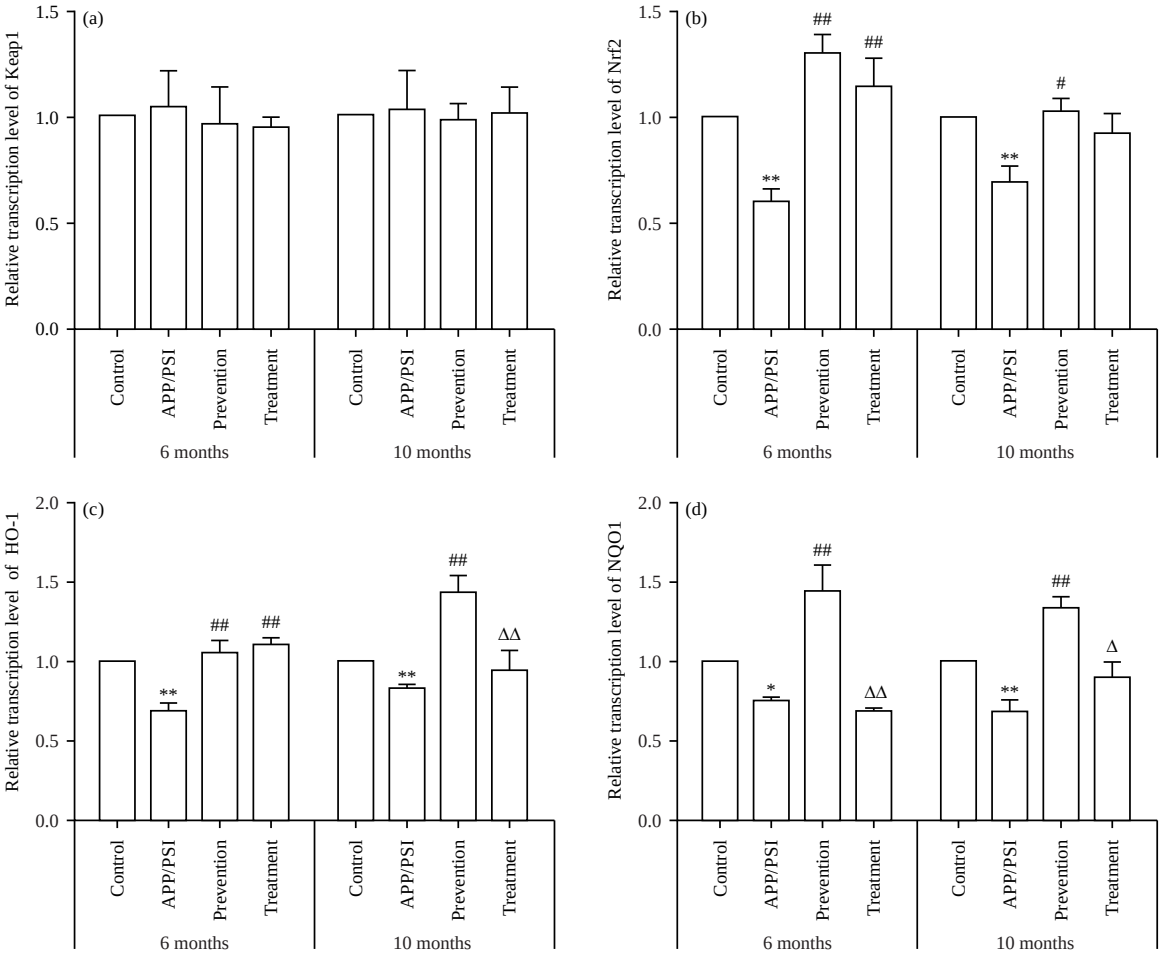

Fig. 6(a-d): GR treatment increased the mRNAs level of Keap/Nrf2/ARE pathway in the hippocampus of APP/PS1_DT mice. The levels of (a) Keap-1, (b) Nrf2, (c) HO-1 and (d) NQO1 mRNAs in the hippocampus of wild-type normal mice (Control), APP/PS1_DT mice, APP/PS1-DT mice treated with long-term GR (Prevention group) and APP/PS1_DT mice treated with short-term GR (Treatment group) as determined by RT-qPCR

Data presented were Mean±SD, $^{*}p<0.05$, $^{**}p<0.01$ in comparison to control group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ in comparison to APP/PS1_DT, $^{\Delta}p<0.05$ and $^{\Delta\Delta}p<0.01$ in comparison to prevention

Table 4: MDA content and SOD activity in the hippocampus of 6-months old APP/PS1_DT mice and GR treated APP/PS1_DT mice

Group	MDA (nmol mg^{-1})	SOD (U mg^{-1})
Control	1.6898±0.1229	2.3867±0.1231
APP/PS1	2.6328±0.24678**	1.3229±0.4060**
Prevention	1.5733±0.16153$^{\#\#}$	1.9900±0.1819$^{\#}$
Treatment	1.5008±0.1799$^{\#\#}$	2.0675±0.1933$^{\#}$

$^{**}p<0.01$, $^{*}p<0.05$ compared with control, $^{\#\#}p<0.01$ and $^{\#}p<0.05$ compared with the APP/PS1 group

Table 5: MDA content and SOD activity in the hippocampus of 10-months old APP/PS1_DT mice and GR treated APP/PS1_DT mice

Group	MDA (nmol mg^{-1})	SOD (U mg^{-1})
Control	3.0323±0.2705	2.2178±0.1573
APP/PS1	4.6172±0.2892**	1.4797±0.1943**
Prevention	3.1243±0.2206$^{\#\#}$	1.9522±0.1391$^{\#\#}$
Treatment	4.0954±0.1340$^{\Delta}$	1.6323±0.0791$^{\Delta}$

$^{**}p<0.01$, $^{*}p<0.05$ compared with control, $^{\#\#}p<0.01$, $^{\#}p<0.05$ compared with the APP/PS1 group, $^{\Delta}p<0.05$ compared with the prevention group

GR reduced the level of OS and increased the activity of SOD in the hippocampus of APP/PS1_DT mice: The content of MDA was widely received as a biomarker reflecting the lipid peroxidation level. The antioxidant enzymes SOD plays important role in maintaining the appropriate cellular redox status and the level is significantly lower in AD patients. The present study showed that the MDA content increased and the SOD activity decreased in the brain tissue of the 6 and 10 months APP/PS1 group, which was the same as previous studies (Table 4 and 5). As compared to the APP/PS1 group, the content of MDA in brain tissue of 6 and 10 months prevention groups were significantly reduced and the SOD activity was significantly elevated, MDA content in brain tissue of 6 months treatment group

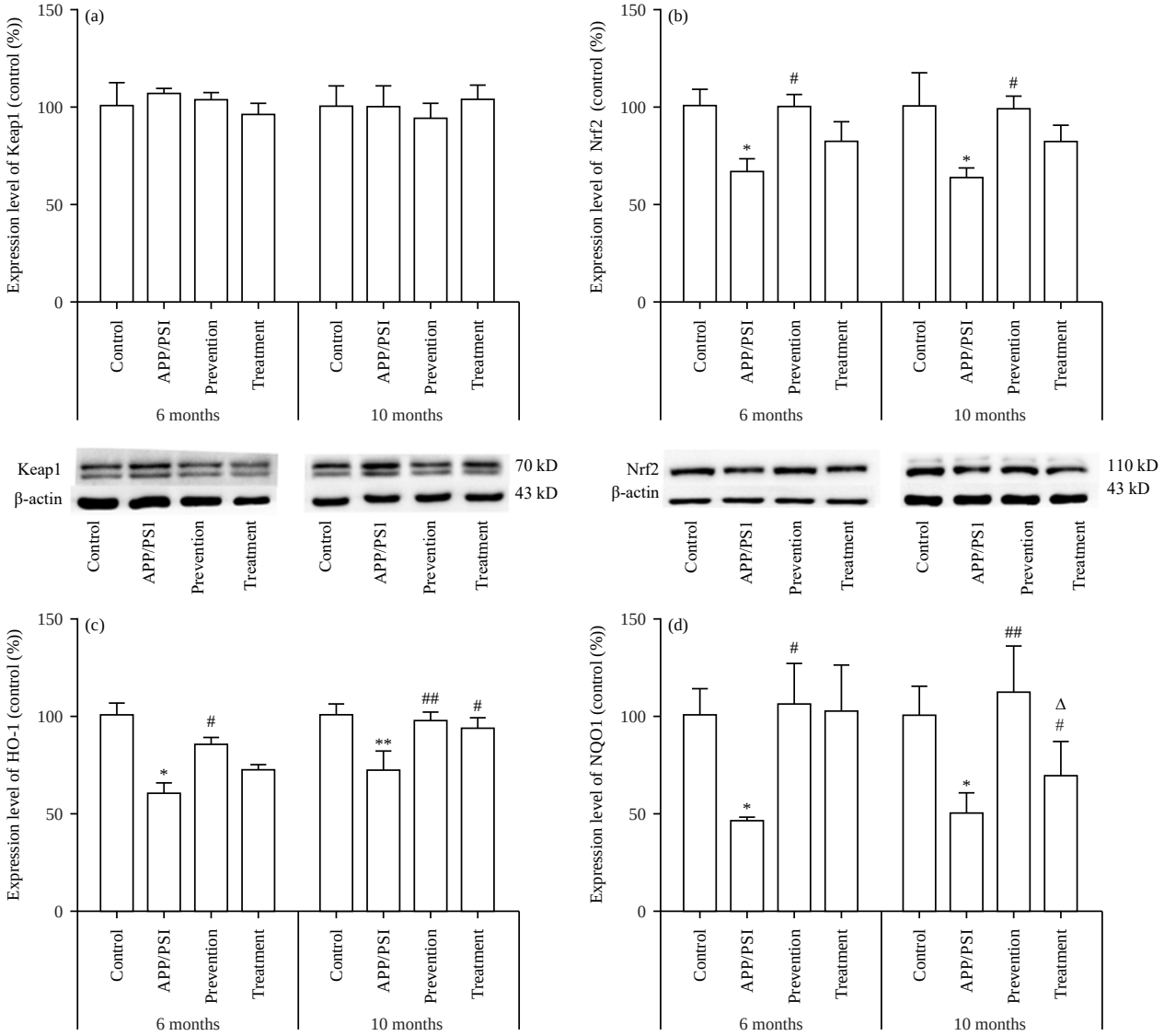

Fig. 7(a-d): GR treatment increased the protein expression of Keap/Nrf2/ARE pathway in the hippocampus of APP/PS1_DT mice. The levels of (a) Keap-1, (b) Nrf2, (c) HO-1 and (d) NQO1 proteins in the hippocampus of wild-type normal mice (Control), APP/PS1_DT mice (APP/PS1), APP/PS1-DT mice treated with long-term GR (Prevention group) and APP/PS1_DT mice treated with short-term GR (Treatment group) were determined by Western blot

Values shown as a percentage of the control by relative quantification were Mean±SD, $^{*}p<0.05$, $^{**}p<0.01$ compared with control group, $^{\#}p<0.05$, $^{\#\#}p<0.01$ compared with APP/PS1 group $^{\Delta}p<0.05$ and $^{\Delta\Delta}p<0.01$ compared with prevention group

was significantly reduced and SOD activity increased, while there were no statistically significant differences between the 10 months treatment group mice.

DISCUSSION

The AD is the leading type of dementia in the world. The Alzheimer's disease international survey suggested that there were more than 50 million AD patients worldwide and it was still growing. With the ageing of the world's population, the number of AD patients would exceed 130 million by 2050[18]. One of the key pathological features of AD is the formation of SP, which is mainly deposited of Aβ. Reducing Aβ deposition in the brain and countering the toxic effects might be a potential therapeutic method. The GR, traditional Chinese medicine in ameliorating cerebral-vascular diseases and neurological diseases, can be a potential promising medicine to alleviate cognitive impairment[19]. Gastrodin, one of the

main bioactive components of GR, was reported to have protective effects on the cytotoxicity induced by 1-methyl-4-phenylpyridinium in human dopaminergic neuroblastoma cells and other studies also showed that GR has neuroprotective effects[20,21]. However, the neuroprotective mechanism of GR on cognitive function, Aβ deposition, synaptic function, oxidative stress and its related signal pathway shall be further elucidated. Therefore, we aimed to evaluate the possible mechanism and therapeutic effects of GR on the neuropathogenesis of AD by using APP/PS1_DT mice models.

The results showed that Aβ was deposited in the hippocampus of the APP/PS1_DT mice model. The MDA content increased, SOD activity decreased and the expression level of synapse-related protein decreased. Furthermore, declining learning and memory abilities were recorded in APP/PS1_DT mice model. With aged growth, more Aβ was deposited in the hippocampus region and synaptic morphology changes were observed. The number of synapses decreased, the synaptic cleft became widened, the post-synaptic dense substance became thinner and the curvature of the synaptic interface was smaller in the hippocampal neurons of 6 and 10 months old APP/PS1_DT mice than in the control group. As reported before, the Aβ deposition might be the primary cause of these changes and then resulted in damaging of hippocampus neuron, impairing synaptic plasticity and decreasing learning and memory abilities in APP/PS1_DT mice models.

After being treated with GR, the deposition of Aβ was significantly decreased in the brain tissue of the mice at the 6 and 10 months old treatment group. Interestingly, Aβ deposition was alleviated more obviously in that prevention group. The result indicated that GR could reduce the Aβ deposition in the brain of APP/PS1 mice, therefore, alleviating the pathological process of AD. It also showed that the early preventive intervention of APP/PS1_DT mice model with GR was better than GR treatment after pathological features appeared. Meanwhile, the learning and memory ability was significantly improved both in the prevention and treatment of APP/PS1 mice at 6 and 10 months old. As Aβ plays a major role in the cognitive dysfunction in AD, GR-improved learning and memory ability of APP/PS1 mice could be connected to the reduction of deposition of amyloid peptide in the brain by GR. Further, the mechanism of GR against the neurotoxicity of Aβ was explored.

The studies on the postmortem brains of AD patients and animal models indicate that synapses are affected at the earliest stages of the neurodegenerative processes, which is closely related to cognitive function[22]. One of the pathophysiological characteristics of AD is several synapses and neuron loss resulting in a gradual decline in cognitive function[23]. Growth pieces of evidence have shown that the Aβ deposition impairing synaptic integrity and function, which is considered to be the vital pathological factor of cognitive decline in AD[4]. Synaptic function is greatly correlated with synaptic proteins, including pre-and post-synaptic membrane proteins and vesicle-associated proteins. SYN is a specific marker of vesicles and plays a key role in neural plasticity, which reflects the density and distribution of synapses. Down-regulation of SYN could form abnormal synapses morphology, such as swollen and clumping of synaptic vesicles[24]. The postsynaptic density of 95 kDa (PSD-95) is a neuronal PSD-95/Dlg/ZO-1 (PDZ) protein, which can associate with receptors and cytoskeletal elements, orchestrate synaptic development and regulate the stabilization and plasticity of synapse[25]. The previous and present data all indicated that Aβ accumulation down-regulated the synaptic-associated proteins levels, including SYN, PSD95 and DYN1 in APP/PS1_DT mice model with age-related manna and then exacerbated the synaptic function[7]. Synapses are the basic unit of communication and information transmission between neurons. Synaptic transmission efficiency is highly correlated with synaptic cleft width, postsynaptic density and curvature. With the progress of age, the number of synapses decreases, the synaptic cleft widens, the post-synaptic dense substance becomes thinner and the synaptic interface curvature becomes smaller in the hippocampal neurons in APP/PS1 mice. Interestingly, these results suggested that GR could largely restore the expression level of SYN, DYN1 and PSD95, which have been down-regulated with the increasing Aβ deposition in hippocampus of APP/PS1 mice model in both prevention and treatment groups. Results also indicated that GR could improve the synaptic function significantly which may be connected to its effects on maintaining the synaptic structure integrity and increasing synapse number. The increase of SYN, DYN1, PSD95 proteins, synapse number and changes of synaptic morphology after GR treatment could improve the synaptic function against Aβ toxicity, which leads to the improvement of cognitive functions. Similarly, this part of the research also suggested that the long-term use of GR to prevent AD was better than treatment.

Many studies and our previous studies have confirmed that OS is an important pathological feature in AD pathogenesis[17,26]. The deposition of Aβ can induce oxidative stress damage to nerve cells and OS promotes the production and aggregation of Aβ, forming a vicious circle between them[27]. In addition, the occurrence of OS also damages neurons and accelerates their ageing, leading to learning and

memory disorders. Nrf2 is a major transcription factor and can regulate genes related to OS and its promoter contains antioxidant response elements[28]. The Nrf2 structural and functional changes were found in most neurodegenerative diseases, such as AD[29], Parkinson's disease (PD)[30] and amyotrophic lateral sclerosis[31]. The Nrf2 protects the body from oxidative stress damage by up-regulating antioxidant defence pathway activation, inhibiting inflammation and maintaining protein homeostasis. The expression of Nrf2 is reduced in the brains of AD patients and AD animal models. The Nrf2 expression in the brain of APP/PS1 transgenic mice was reduced with the deposition of Aβ. The Nrf2 has been proved to be a protective factor in AD and has become a novel therapeutic target for AD[32]. Recent studies have shown that Nrf2 activators have therapeutic effects in AD animal models and cultured human AD-like cells[32]. More specifically, there is an inevitable connection between Aβ, synaptic damage and oxidative stress. Therefore, Nrf2, which is one of the regulators of oxidative stress, has become a hot spot in the current research on treating AD.

The GR also has a significant role in antioxidants. It was found that gastrodin induced Nrf2 up-regulation and nuclear translocation and subsequently increased the level of antioxidant genes GCLM and HO-1 in astrocytes[33]. In addition, gastrodin combination or pretreatment significantly inhibited cell death caused by excessive ROS production and the PAPR-1 increase[33] caused by Zn^{2+}. In the present study, the mRNA and protein levels of Keap1 and Nrf2 in the hippocampus of each group of mice and the downstream antioxidant genes HO-1 and NQO1 were detected. The level of Keap1 mRNA in the hippocampus of all mice at the age of 6 and 10 months did not change. It is speculated that GR may not affect the level of Keap1, but affect its binding ability with Nrf2 by changing the protein structure to regulate the expression of downstream genes. The mRNA and protein levels of Nrf2, HO-1, NQO1 in the hippocampus of APP/PS1 mice decreased significantly, while the levels increased in brain of mice in 6 and 10 months old GR prevention group. The 6 and 10 months old GR treatment group mice were not as effective as the prevention group. These findings indicated that GR might enhance the antioxidant capacity by activating the Keap1-Nrf2/ARE pathway and its downstream gene expression.

After clarifying that GR may regulate the oxidative stress state of cells by activating the Keap/Nrf2 pathway, the study further tested the activities of the oxidative stress product malondialdehyde and the antioxidant enzyme SOD in the hippocampus of each group. The present study showed that the MDA content increased and the activity of SOD decreased in the brain tissue of the 6 and 10 months APP/PS1 group mice, which was the same as previous studies. Compared with the APP/PS1 group, the MDA content in the brain tissue of 6 and 10 months old prevention groups were significantly reduced and the SOD activity was significantly elevated, MDA content in brain tissue of 6 months treatment group was significantly reduced and SOD activity increased, while there were no statistically significant differences between the 10 months treatment group mice. These results further proved that GR could enhance the antioxidant capacity in the brain tissue of APP/PS1 transgenic mice, which might be related to the reduction of Aβ deposition and the improvement of the learning and memory ability of APP/PS1_DT mice.

CONCLUSION

Above all, we investigated the possible therapeutic role and underlying mechanism of GR and the neuropathological process of AD by using APP/PS1_DT mice models. The GR could improve the learning and memory abilities of APP/PS1 double transgenic mice, reduce the deposition of Aβ in the mouse brain and increase synaptic function via regulating Keap1/Nrf2/ARE.

SIGNIFICANCE STATEMENT

This study discovered GR could improve the learning and memory abilities of APP/PS1 double transgenic mice, reduce the deposition of Aβ and increase synaptic function via regulating Keap1/Nrf2/ARE, which can be beneficial for treatment in AD, this study provided the possible therapeutic role and underlying mechanism of GR attenuated neuropathological process of AD.

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