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RESEARCH ARTICLE

Vitamin D Combined with Resveratrol Prevents Cognitive Decline in SAMP8 Mice

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Abstract: ***Background:*** Vitamin D (VD) and resveratrol (RSV) are two nutritional molecules that have reported neuroprotective effects, and findings from cellular models suggest that resveratrol could potentiate vitamin D's effects. The senescence-accelerated mouse-prone 8 (SAMP8) is a useful model of Alzheimer's disease (AD)-related memory impairment.

Objective: We aimed to explore how the combination of vitamin D with resveratrol would affect memory impairments shown by SAMP8 mice, as well as the potential mechanisms.

Method: SAMP8 mice and their control senescence-accelerated mouse resistant 1 (SAMR1) mice (10 weeks old) were divided into 5 groups, i.e. SAMR1 group, SAMP8 group, SAMP8 mice supplemented with VD group, SAMP8 mice supplemented with RSV group and SAMP8 mice supplemented with both VD and RSV group. At the end of the intervention, Morris water maze (MWM) test was used to assess cognitive function. Hippocampus and parietal cortex were dissected for further analysis.

Results: The combination of VD and RSV significantly increased time spent in target quadrant and the number of crossing *via* MWM test. In hippocampus, the combined intervention significantly reduced soluble $A\beta_{42}$ level and BACE1 protein expression. In cortex, the combined treatment significantly reduced phosphorylation of tau at serine404 and p-p53, as well as enhanced p-CREB protein expression. The combination also significantly reduced GFAP and p-NFκB p65 in both hippocampus and cortex.

Conclusion: The combined intervention might exert greater neuroprotective effects in SAMP8 mice, this might be associated with the fact that the combined intervention could positively affect amyloidogenic pathways, neuroinflammation, tau phosphorylation and probably apoptosis markers.

Keywords: Vitamin D, resveratrol, Alzheimer's disease, amyloid β, tau phosphorylation, neuroinflammation, cognitive function, endoplasmic reticulum stress.

INTRODUCTION

Alzheimer's disease (AD) is a progressive neurodegenerative disease and the most prevalent cause of progressive dementia in the elderly [1]. Over the past two decades, scientists have studied both pharmacological and lifestyle interventions in the prevention and treatment of AD, however the overall findings have been disappointing [2]. Therefore, it is urgent to explore effective strategies for the prevention and treatment of AD.

Vitamin D (VD) has been recognized as a neurosteroid hormone which exerts anti-neurodegenerative properties *via* the regulation of neurotrophic factor production, oxidative stress and calcium homeostasis [3-4]. Resveratrol (RSV) is a naturally occurring polyphenolic compound mainly found in grape skins, berries and peanuts [5]. RSV is capable of crossing the blood-brain barrier and is water soluble, consequently eliciting neuroprotective effects in the brain [6]. It has been reported that RSV could affect the pathogenesis of AD *via* multiple mechanisms such as Aβ plaque disruption, tauopathies, inflammation and oxidative stress [7]. Especially, recently Witte *et al*. [8] reported that supplementary resveratrol could improve memory performance in healthy older subjects. While both VD and RSV's individual biological mechanisms in the prevention and/or treatment of AD are being intensively investigated, neither studies have explored the combined effects of vitamin D and resveratrol on the pathogenesis of AD. Hayes proposed that some of the biological processes and mechanisms are common to both res-

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veratrol and vitamin D; meanwhile they also have important mutual processes and interactions [9], suggesting that neuroprotective actions of VD and RSV might be affected if they were intervened together. Thus, it is of importance to explore how combination of VD with RSV would affect AD pathology.

The characteristic neuropathological alterations of AD include senile plaques, neurofibrillary tangles (NFTs), neuroinflammation, synaptic dysfunction and neuronal loss in the brain [10-13]. Senile plaques are extracellular aggregates consisting of Aβ peptides [10]. Aβ is produced by sequential proteolytic cleavage of amyloid precursor protein (APP) by β- and γ-secretases [14]. β- secretases are the rate-limiting enzyme in Aβ genesis, which include, among others, the β-site APP cleaving enzyme 1 (BACE1) and cathepsin B [15], thus they both are emerged as potential drug targets for the treatment of AD [16-17]. NFTs are composed of hyperphosphorylated tau protein [11]. Various proline-directed serine-threonine protein kinases including glycogen synthase kinase-3β (GSK-3β) and cyclin-dependent kinases 5 (CDK5) are implicated in hyperphosphorylating tau [18]. Neuroinflammation plays a critical step in the progression of AD [12]. Synaptophysin, brain derived neurotrophic factor (BDNF) and post-synaptic density protein 95 (PSD-95) are synaptic plasticity-related proteins and thus play important roles for maintaining synaptic function, as well as cognitive function [13]. Consequently, strategies that could positively affect tau pathology, neuroinflammation and synaptic plasticity have also been the focus of AD therapy.

Recent evidence suggests that endoplasmic reticulum (ER) stress is also involved in the pathogenesis of AD. For example, there is elevated protein expression of ER stress markers such as glucose-regulated protein 78 (GRP78/BiP), protein disulfide isomerase (PDI), protein kinase-like ER kinase (PERK) and phosphorylation of eukaryotic initiation factor 2α (p-eIF2α) in AD postmortem brain [19-20]. Mechanistically, evidence suggests that ER stress is crucial in both intraneuronal accumulation of Aβ [21], and intracellular NFTs of hyperphosphorylated tau [20]. Consequently, the modulation of ER stress could be another suitable therapeutic strategy to delay or stop neuronal degeneration.

The senescence-accelerated mouse-prone 8 (SAMP8) has been recognized as a suitable model of age-related AD [22]. The mice exhibit not only age-related cognitive impairment, but also AD-related pathology such as abnormal APP processing by secretases and altered Aβ proteins [23], elevated phosphorylation of tau [24], as well as synaptic and dendritic alterations [25] with aging.

With the above points in mind, the aim of the present study was to explore how the combination of vitamin D with resveratrol will affect the memory impairments shown by SAMP8 mice. In particular, in the present study, it has been checked the effects of vitamin D and RSV on SAMP8 mice regarding cognitive status *via* Morris water maze test; Aβ genesis (APP, BACE1 and cathepsin B); hyperphosphorylation of tau, ER stress and apoptosis markers, synaptic markers (PSD95, synaptophysin and BDNF) in both hippocampus and cortex from SAMP8 mice.

MATERIALS AND METHODS

Materials

Reagents for SDS-PAGE were from Beyotime Institute of Technology (Jiangsu, China). Molecular weight marker and nitrocellulose membranes for SDS-PAGE were from Bio-Rad (CA, USA). Immobilon western chemiluminescent HRP substrate (cat#WBKLS0100) was purchased from Millipore (MA, USA). Antibodies against APP(cat#2450), BACE1 (cat# 5606), tau (cat#4019), p-CREB (cat#9198), p-STAT3 at tyrosine 705 (cat#9145), synaptophysin (cat# 5461), p-NF-κBp65 at serine536(cat#3033), glial fibrillary acidic protein (GFAP) (cat#3670), p-glycogen synthase kinase 3β (GSK3β) at serine 9 (cat#5558), p-AMPK(cat#2535), p-p53(cat#9284), PP2A (cat#2041), eIF2α (cat#9722) and beta-Actin (cat#4970) were purchased from Cell Signaling (MA, USA). PSD95 (cat# AJ1661a) was from Abgent (SD, USA). An antibody against BDNF (cat#ab108383) was from alomone labs (Jerusalem, Israel). Antibodies against p-Tau serine396 (cat#YP0263), p-Tau serine404 (cat#YP0264), GRP78(cat#YT2245), p-eIF-2α(cat#YP0093), p-PERK(T981) (cat#YP1055), PERK (cat#YM0517) were from ImmunoWay Biotechnology Company (DE, USA). Horseradish peroxidase-conjugated donkey anti-rabbit and goat anti-mouse IgG secondary antibodies were purchased from Jackson Immuno-Research Laboratories (PA, USA). *Trans*-resveratrol (cat#70675) was obtained from Cayman Chemicals with a purity of ≥ 98% (KS, USA). Vitamin D_3 injection was obtained from Shanghai general pharmaceutical Company (Shanghai, China).

Treatment of Animals

Ten week old male senescence-accelerated mouse resistant 1 (SAMR1) (N=10) and SAMP8 mice (N=40) were purchased from Animal Service of Health Science Center, Peking University (Beijing, China). All animals were treated in accordance with the Guidelines in the Care and Use of Animals and with the approval of the Animal Studies Committee of Soochow University, Suzhou, China. Mice were housed together in groups of two animals in standard plastic mouse cages ($30\times20\times13$ cm^3) and were acclimated to the animal housing facility for 1 week prior to the start of intervention. The mice were allowed an AIN-93G diet and drinking water *ad libitum* during the whole period of experimentation (i.e. 24 wks). The mice were randomly divided into 5 groups of 10 animals each, i.e. SAMR1 group (R1), SAMP8 group (P8), SAMP8 mice supplemented with Vitamin D group (VD P8), SAMP8 mice supplemented with RSV group (RSV P8) and SAMP8 mice supplemented with both vitamin D and RSV group (VDRSV P8). For the vitamin D intervention, the mice were administered a intramuscular injection of 500 IU vitamin D_3 once *per* week; for the RSV intervention, the mice were allowed a AIN-93G diet supplemented with 0.03% RSV (i.e. 30mg RSV dissolved in 100g food), which is roughly equal to 43mg/kg/d if assuming a mice consumed 40g food per week with a body weight of 40g. We chose these dosages of vitamin D and resveratrol are based on published findings from other research groups [26-29] and preliminary data from our laboratory. Body weight and food intake were determined every other week for the duration of the study.

Morris Water Maze (MWM) Test

Two days post-the last bout of vitamin D and RSV intervention, hippocampal dependent, long term spatial learning and memory retention were assessed in mice using a Morris water maze modified from Morris [30]. Behavioral tests were performed between 9:00 a.m. and 1:00 p.m.. In brief, the maze was a tank (120cm in diameter and 30 cm in height) filled with water at approximately 24±1°C. The tank was divided into 4 quadrants, one of which contained a circular escape platform (10 cm in diameter, 24 cm in height) placed at a fixed position, 1.0 cm below the surface of the water. There were visual cues around the water maze. Oriented navigation trials were performed 4 times per day for a total of 4 days. In each trial, the animal was placed into the water in a different quadrant and given 60 sec to search for the platform. If the mouse escaped successfully onto the platform within the given time, it was allowed to stay on the platform for 10 sec. If the mouse failed to find the platform within the given time, it was guided to the platform and allowed to stay for 20 sec. After this, a new trial would begin. Behavior was recorded by a computerized video tracking system, and the time that a mouse took to reach the submerged platform (escape latency) was recorded to assess spatial learning ability. On the fifth day, the platform was removed from the tank, and a spatial probe trial was performed. Mice were placed in the tank in the quadrant opposite to the quadrant that previously held the platform, and were allowed to swim freely for 60 sec. Two indexes were calculated to assess spatial memory ability: (1) the time spent by a mouse in the target quadrant in which the platform was hidden; (2) the number of mice exactly crossing over the previous position of the platform. The target quadrant was defined as the quadrant that previously held the platform, whose radius was limited to 70 cm in this assessment. Ethovision XT tracking software (VA, USA) was used for data collection

Brain tissue Collection and Preservation

Two days post-behavioral test, mice were sacrificed, and the brains were immediately removed and washed with ice-cold PBS; the hippocampus and parietal cortex from the hemi-brains were dissected, snap frozen in liquid nitrogen and then stored at -80°C for further Western blot and ELISA analysis. The other hemispheres were fixed in 4% paraformaldehyde, and embedded in paraffin for immunohistochemistry and TUNEL assay.

Immunohistochemistry

Paraffin sections were cut into 5 μm thickness and stored at room temperature until staining. In brief, endogenous peroxidase was first quenched with 0.3% H_2O_2 for 10min, and non-specific binding were blocked with normal goat serum for 1h. The sections were induced with mouse antibody p-tau at ser404 overnight at 4°C. Next, the sections were incubated with biotinalyted anti-mouse IgG for 1h, and followed by avidin-biotin-HRP complex for 1h. Immunohistochemical reaction was performed using 0.05% 3,3' diaminobenzidine (DAB) and 0.03% H_2O_2 as chromogen. Light microscopic images were taken.

TUNEL Assay

In situ detection of apoptotic cells was performed with commercially available in situ cell death detection kit POD (Roche, 11684817910). Paraffin embedded sections were cut into 5 μm thickness, and the slides were incubated with 20μg/ml proteinase K in PBS for 15min followed by washing in distilled water. Subsequently, the slides were incubated with the TUNEL reaction mixture containing TdT and fluorescein-dUTP for 2 hr at 37°C, and put in stop/wash buffer for 10 min. After washing to remove unbound enzyme conjugate, we visualized the POD retained in the immune complex using a DAB substrate kit (Dako company, Glostrup, Denmark).

Western Blot Analysis

Protein was extracted from the hippocampus and parietal cortex of the mice, and the content of APP, BACE1, cathepsin B, p-Tau ser396 and ser404, p-GSK3β ser9, CDK5, p25/35, PP2A, GFAP, p-NF-κB p65, p-CREB, p-STAT3, synaptophysin, PSD95, BDNF, p-eIF2α/ eIF2α, GRP78 and p-PERK/PERK were determined by Western blotting according to Wan *et al*. [31-33]. Signals were visualized using Immobilon western chemiluminescent HRP substrate and captured using a Syngene chemi-imaging system (MD, USA). Subsequently bands were quantified by densitometry *via* Gene Tool according to the manufacturer's instructions (SynGene, ChemiGenius2, PerkinElmer). Protein contents of phosphorylated protein were quantified and normalized to the total levels of these proteins. Beta actin was used as internal controls.

Quantification of $A\beta_{42}$ by ELISA

For analysis of total soluble $A\beta_{42}$ burden, the hippocampus was homogenized in 70% formic acid (i.e.10mg of tissue in 70μL of 70% formic acid). Homogenates were then centrifuged at 100,000g for 20 min at 4°C. The supernatant was neutralized with a 20-fold dilution in 1M Tris base, and $A\beta_{42}$ levels were measured *via* a high-sensitive enzyme linked immunosorbent assay kit from Wako (cat#292-64502, Osaka, Japan) following the manufacturer's instructions.

Serum 25 Hydroxyvitamin D [25(OH) D] Measurement

Serum 25(OH)D was analyzed using 25 OH Vitamin D reagent based on the chemiluminescent immunoassay (CLIA).

Statistical Analysis

Data are expressed as means ±SEM. For the MWM test, escape latency times in the hidden platform trial were analyzed *via* two-way ANOVA of repeated measures, while one-way ANOVA was conducted for the probe trial followed with Tukey's post hoc test, as well as for all biochemical data. Statistical significance was established at a $p < 0.05$.

RESULTS

Serum 25OHD Level

To determine if interventions affected vitamin D status, serum levels of 25OHD were determined using CLIA

method. 25OHD is the most widely used index of vitamin D status because it has a long half-life of 2-3 weeks [34]. As shown in Table **1**, there is no difference for serum 25OHD levels between SAMP8 group and SAMR1 group. VD injection significantly elevated serum 25OHD levels in both VD and VDRSV group compared to both SAMR1 and SAMP8 group; while the serum 25OHD level was significantly reduced in RSV group compared to SAMR1 group.

Cognitive Function *via* MWM Test

As shown in (Fig. **1A**), compared to SAMR1 group, SAMP8 group demonstrated longer latency to platform during navigation trials; and VD, RSV as well as VDRSV intervention reversed the prolonged escape latency because there is no difference for escape latency in VD, RSV and VDRSV groups in the testing 4 days compared to SAMR1 group. SAMP8 mice also demonstrated reduced time spent in target quadrant and reduction in the number of crossings during the spatial memory testing compared to SAMR1 group (Fig. **1B&C**). VD and RSV intervention alone increased the time spent in target quadrant with no effect on the number of crossing; meanwhile the combination of VD and RSV significantly increased the time spent in target quadrant and the number of crossing. Collectively, our results suggested that VD and RSV supplementation could reverse the cognitive impairment in 34 weeks old of SAMP8 mice, with the combined supplementation of VD with RSV being more effective than intervention alone.

APP, BACE1, Cathepsin B Protein Expression in Both Hippocampus and Cortex and Hippocampal $A\beta_{42}$ Level

As shown in (Fig. **2A**), in hippocampus, SAMP8 mice has elevated APP and BACE1 protein expression compared to SAMR1 mice; the combination of VD with RSV significantly reduced the protein expression of BACE1 compared to SAMP8 group; while there is no difference for the cathepsin B expression among groups. In cortex, SAMP8 group has elevated APP, BACE1 and cathepsin B protein expression compared to SAMR1 group, VDRSV group has significant reduced expression of both APP and cathepsin B expression compared to SAMP8 group; meanwhile RSV group also has significant reduced expression of APP protein expression compared to SAMP8 group (Fig. **2B**). We also quantified hippocampal $A\beta_{42}$ levels to confirm whether altered APP processing would lead to altered $A\beta_{42}$ burden. As shown in (Fig. **2D**), $A\beta_{42}$ levels were significantly elevated in SAMP8 mice compared to R1 mice, and the combined intervention significantly reduced $A\beta_{42}$ levels. It should be noted that we only measured $A\beta_{42}$ burden in hippocampus but not in cortex because the detection of $A\beta_{42}$ in cortex appeared at a much later timepoint than in hippocampus [35].

Tau Phosphorylation and Tau Kinases Protein Expression in Cortex

As shown in (Fig. **3A**), there is elevated phosphorylation of tau at serine396 and 404 in SAMP8 mice compared to SAMR1 mice, VD, RSV and their combination significantly reduced p-Tauser396; while only the combination of VD and RSV reduced elevated phosphorylation of tau at ser404. We also measured key kinases involved in tau phosphorylation and dephosphoryation. As shown in (Fig. **3B**), compared to SAMR1 mice, the phosphorylation of GSK3β at serine9 was significantly reduced in SAMP8 mice; while there is no difference for the protein expression of CDK5, p25/35 and PP2A among groups. We also performed immunohistochemistry using p-Tau ser404 antibody in the parietal cortex to confirm the above results. As shown in (Fig. **3D**), notable positive immunoreactivity of p-tau was observed in the parietal cortex of SAMP8 mice compared to SAMR1 mice, vitamin D, resveratrol independently and their combination partially restored elevated p-Tau ser404.

GFAP and p-NFκB p65 Protein Expression in Both Hippocampus and Cortex

As shown in (Fig. **4A&B**), there is elevated protein expression of GFAP and p-NF-κB p65 in hippocampus and cortex of SAMP8 mice compared to SAMR1 mice; the combination of vitamin D and RSV significantly reversed elevated GFAP and p-NF-κB p65 in both hippocampus and cortex. Meanwhile, in hippocampus, RSV also restored elevated p-NF-κB protein expression; in cortex, VD and RSV independently also significantly reduced elevated GFAP protein expression compared to SAMP8 mice.

p-CREB, p-STAT3, Synaptophysin, PSD95 and BDNF Protein Expression in Both Hippocampus and Cortex

Although there is no difference for p-CREB in both hippocampus and cortex from SAMP8 mice compared to SAMR1 mice, in cortex the combination of vitamin D and RSV significantly enhanced p-CREB (Fig. **5A&B**). There was a significant reduction in p-STAT3 in hippocampus from SAMP8 mice compared to SAMR1 mice, the combination of vitamin D with RSV significantly enhanced p-STAT3. However, there is no difference for pre-synaptic (PSD95) and synaptic markers (synaptophysin and BDNF) in both hippocampus and cortex among groups (Fig. **5C&D**).

ER Stress Markers in Both Hippocampus and Cortex

We measured key markers involved in ER stress including p-eIF2α, GRP78 and p-PERK. As shown in (Fig. **6**), there was no difference for the above markers in hippocampus. There was elevated markers of p-eIF2α and p-PERK in cortex of SAMP8 mice compared to SAMR1 mice.

Table 1. Serum 25OHD level from different groups.

group	SAMR1	SAMP8	VDP8	RSVP8	VDRSVP8
25OHD (ng/mL)	26.99±1.27	24.63±0.56	84.24±3.43 *,#	21.80±1.32 *	83.50±2.62 *,#

*, $p<0.05$, vs. SAMR1; #, $p<0.05$, vs. SAMP8.

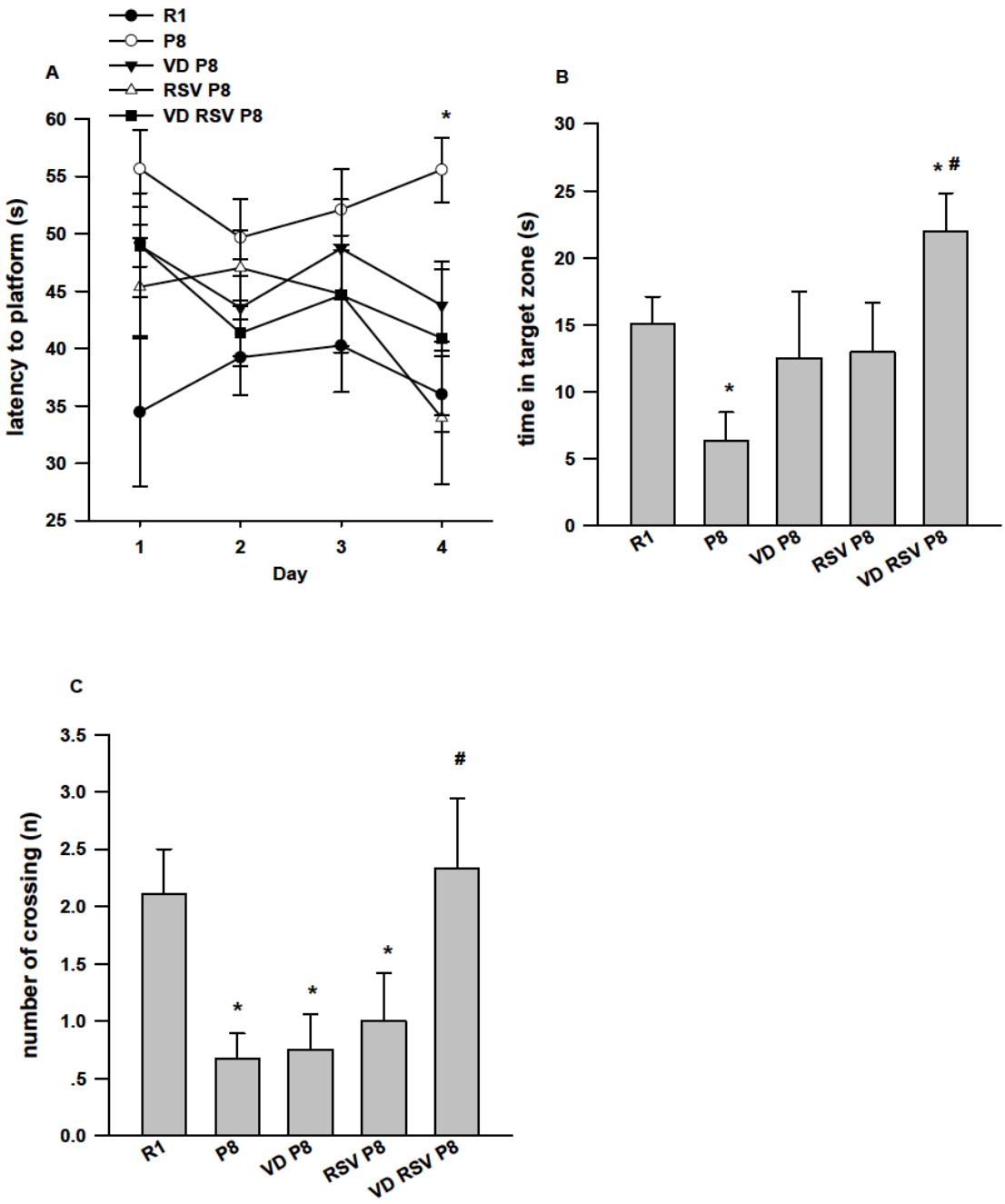

Fig. (1). Vitamin D and resveratrol improved cognitive impairment shown by SAMP8 mice *via* MWM test. (A) Mean latency in the hidden platform test; (B) Comparison of the time spent in the target quadrant and (C) the number of crossings over the exact, former location of the platform during the probe test. Data are presented as means +SEM for 10 mice per group. * $p<0.05$ versus SAMR1 group. # $p<0.05$ versus SAMP8 group.

Apoptosis Markers in Cortex

As shown in (Fig. **7A**), p-p53 was significantly elevated in SAMP8 mice compared to SAMR1 mice, VD, RSV and their combination significantly reduced elevated p-p53 from SAMP8 mice. We also measured neuronal apoptotic cell death in cortex *via* TUNEL methods. As shown in (Fig. **7B**), SAMP8 mice had a significant elevation in apoptotic neuronal cell death compared to SAMR1mice, vitamin D, resveratrol and their combination partially restored apoptotic neuronal cell death.

DISCUSSION

The main findings of the present study is that the combination of VD and RSV is more efficacious for improving age-associated memory dysfunction *via* MWM test. This might be associated with that the combined intervention could positively affect key markers involved in APP processing, neuroinflammation, tau phosphorylation, p-STAT3 and p-CREB; while it seems that synaptic plasticity related proteins, ER stress were not involved in the combined effects of VD and RSV against cognitive impairment.

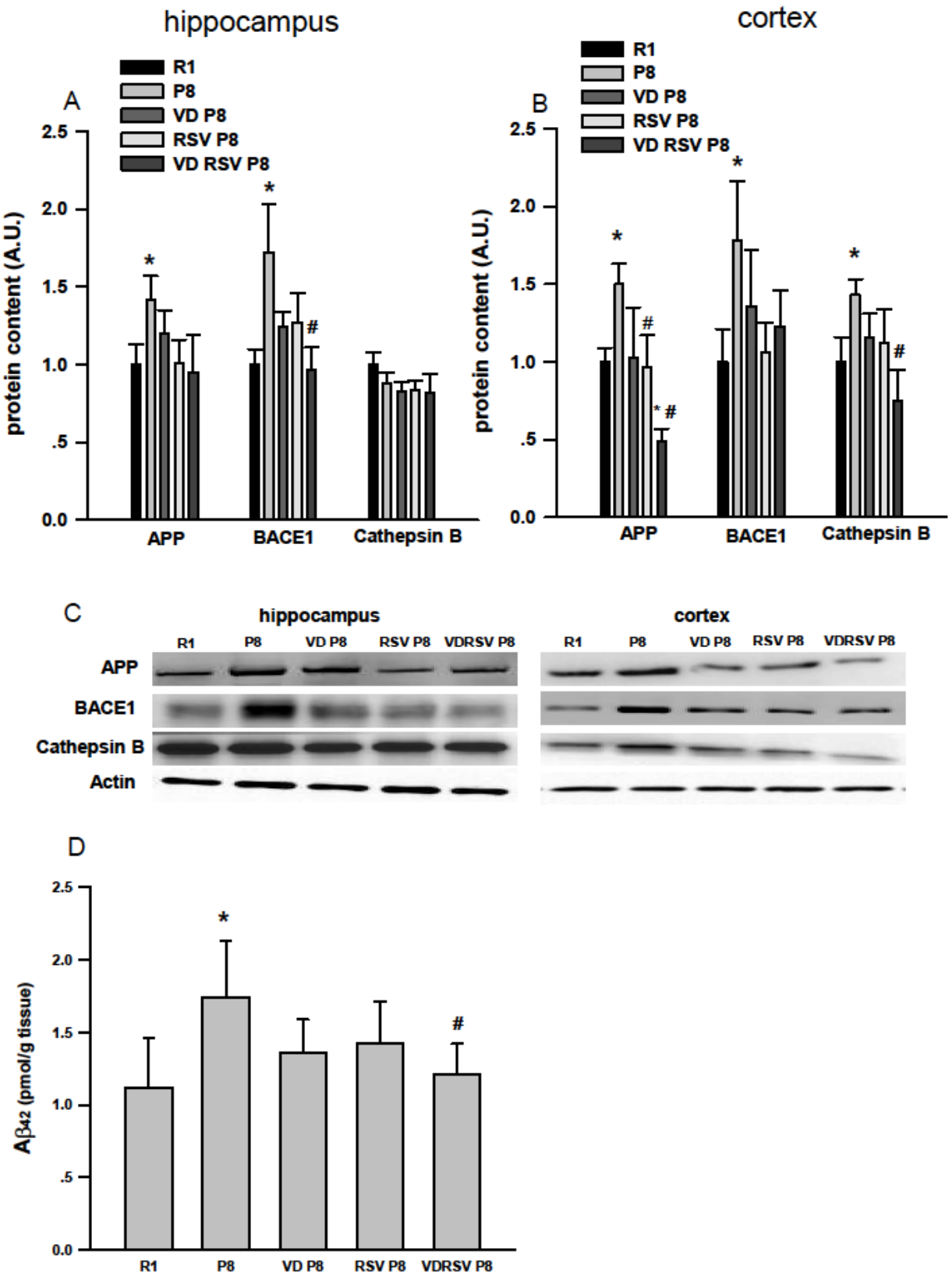

Fig. (2). Vitamin D and resveratrol affected protein expression of APP, BACE1, cathepsin B in hippocampus and cortex, as well as hippocampal soluble $A\beta_{42}$ levels. Protein expression of APP, BACE1, cathepsin B in hippocampus (A) and cortex (B) from all groups measured *via* western blotting; (D) hippocampal soluble $A\beta_{42}$ levels *via* ELISA assay. Representative blots are shown in panel C. A.U. means arbitrary units. Data are presented as means +SEM for 10 mice per group. * $p<0.05$ versus SAMR1 group. # $p<0.05$ versus SAMP8 group.

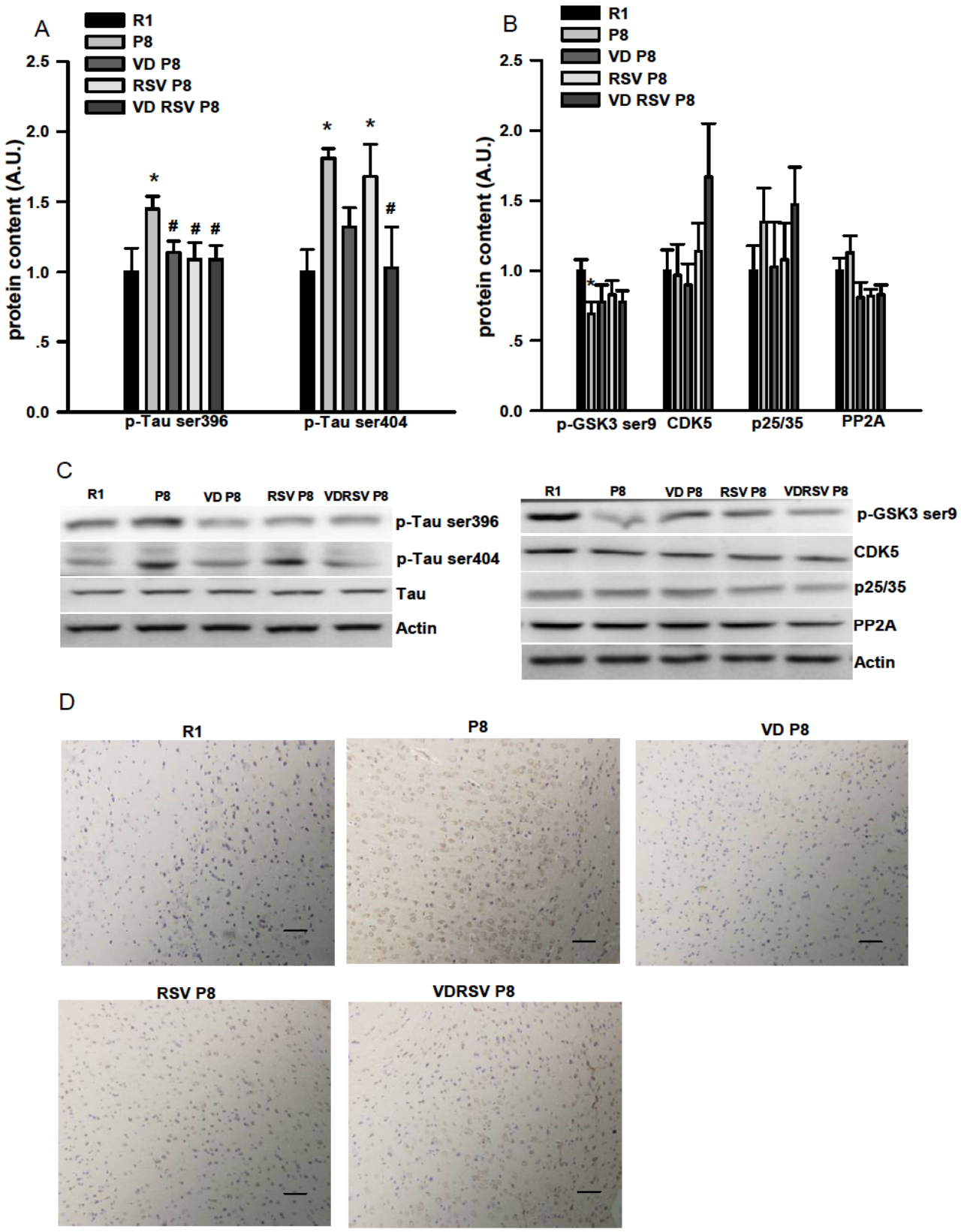

Fig. (3). Vitamin D and resveratrol affected phosphorylation of tau at serine396 and 404 in cortex of SAMP8 mice. Protein expression of (A) p-tau ser396 and serine404 and (B) tau kinases including p-GSK3β ser9, CDK5, p25/35 and PP2A in cortex from all groups. (D) Representative immunostaining of p-tau ser404 in cortex. Scale bar 20μm, magnification ×200. Representative blots are shown in panel C. A.U. means arbitrary units. Data are presented as means +SEM for 10 mice per group for A&B, and 4 mice per group for D. * $p<0.05$ versus SAMR1 group. # $p<0.05$ versus SAMP8 group.

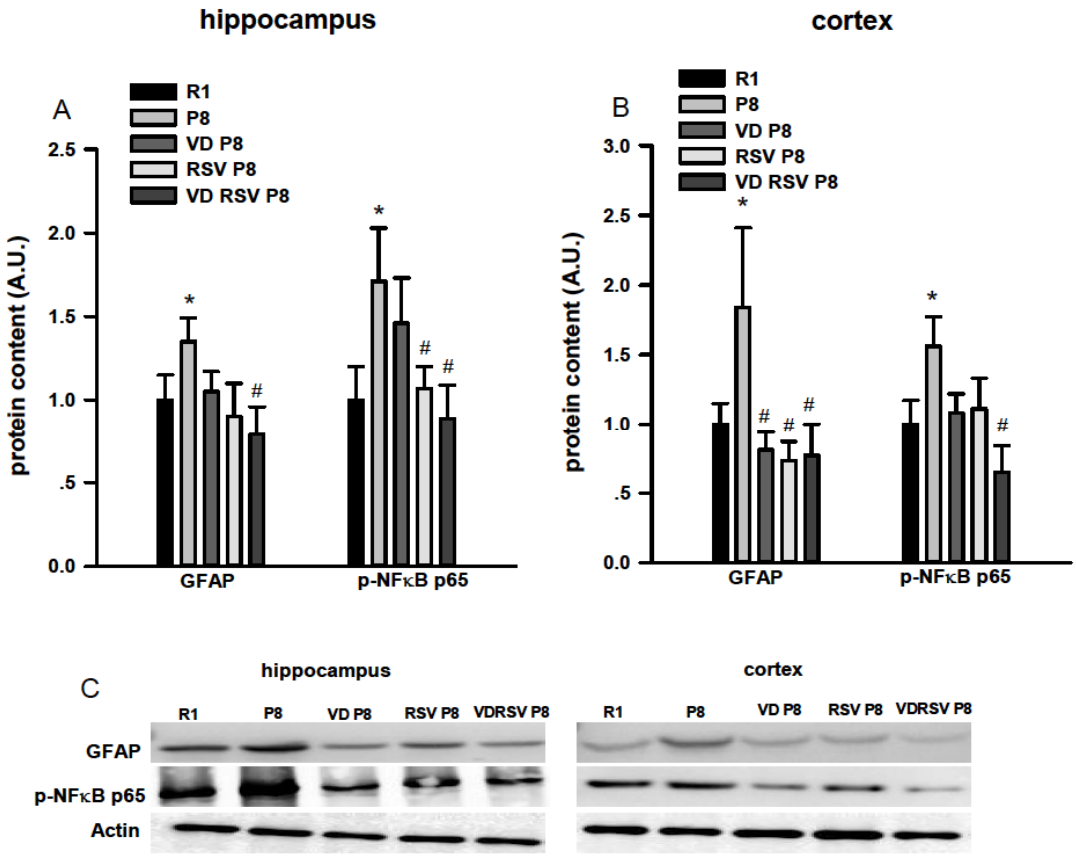

Fig. (4). Vitamin D and resveratrol affected GFAP and p-NF-κB p65 in both hippocampus and cortex of SAMP8 mice. Protein expression of GFAP and p-NF-κB p65 in (A) hippocampus and (B) cortex of all mice. Representative blots are shown in panel C. A.U. means arbitrary units. Data are presented as means +SEM for 10 mice per group. * $p<0.05$ versus SAMR1 group. # $p<0.05$ versus SAMP8 group.

Although not a universal finding [36], VD and resveratrol have been reported to improve cognitive function in animal models of AD [26, 37-39]. Our study is the very first to demonstrate that the combination of VD with RSV might be more effective for improving cognitive impairment in SAMP8 mice than intervention independently. At the cellular level, RSV has been reported to potentiate 1α,25dihydroxyvitaminD$_3$ binding to vitamin D receptor (VDR) in different cell lines [40], suggesting that the bioactions of vitamin D may be further potentiated by RSV. Hayes also proposed that some of the biological processes and mechanisms are common to both resveratrol and vitamin D [9]. Taken together, it is suggested that the combined intervention of VD with RSV might be an promising strategy for fighting against AD, whereas the underlying mechanisms need to be explored.

Although the role of Aβ in AD is being questioned [41], Aβ aggregation has been considered as a key event in the pathogenesis of AD [42]. Especially, the role of Aβ in soluble forms has been emphasized in the pathogenesis of AD. It has been suggested that at early stages of AD, soluble Aβ species tend to be more toxic to neuronal cells than fibrils [43]. Findings from human studies also confirmed that soluble Aβ species levels correlates with cognitive impairment and synaptic dysfunction in AD subjects [44]. Our present study demonstrate that the combination of VD with RSV could significantly reduce hippocampal soluble Aβ_{42} level, this may explain, at least partially, the improved cognitive function in VDRSV group. To probe the mechanisms by which VD and RSV attenuates the accumulation and effects of Aβ peptides, we first measured APP, as well as BACE1 and cathepsin B, key enzymes that initiates the production of Aβ peptide from APP [14-15]. We demonstrated that BACE1 was significantly reduced in hippocampus, APP and cathepsin B were significantly reduced in cortex. Therefore inhibition of APP, BACE1 and cathepsin B may represent the mechanism by which VDRSV attenuated Aβ_{42} accumulation in SAMP8 mice brain tissue. To the best of our knowledge, our study is the very first to demonstrate that the combination of VD and RSV prevents the progression of AD by reducing Aβ accumulation through the repression of amyloidogenic pathway.

Neurofibrillary tangles composed of hyperphosphorylated tau protein is another hallmark in AD [11]. Especially, GSK-3β induced tau hyperphosphoryation is pivotal in the events leading to NFTs formation [45]. It is also important to note that early tau phosphorylation, and not necessarily NFTs, may critically contribute to the development of synaptic dysfunction and neurodegeneration [46]. The levels of phosphorylated tau at ser396/ ser404 have been considered as reliable markers of the severity of AD [47]. Previously, Parque *et al.* [37] demonstrated that resveratrol supplementation could reduce p-Tau ser396 in cortex of SAMP8 mice *via* affecting CDK5 activity. Our present study is the very first

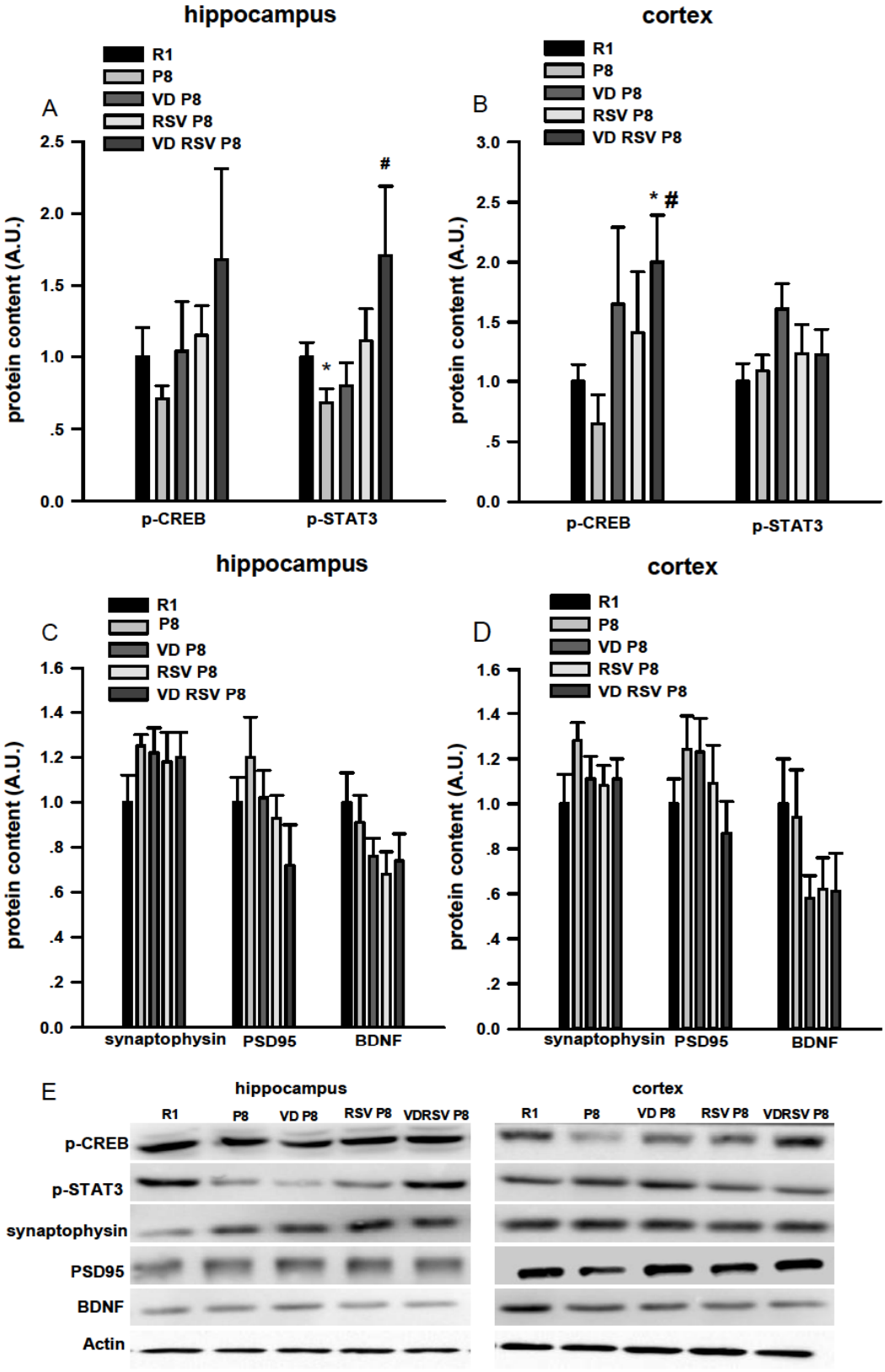

Fig. (5). Vitamin D and resveratrol affected p-CREB and p-STAT3 protein expression in both hippocampus and cortex of SAMP8 mice, while had no effects on synaptophysin, PSD95 and BDNF protein expression. Protein expression of p-CREB, p-STAT3, synaptophysin, PSD95 and BDNF in (A&C) hippocampus and (B&D) cortex of all mice. Representative blots are shown in panel E. A.U. means arbitrary units. Data are presented as means +SEM for 10 mice per group. * $p<0.05$ versus SAMR1 group. # $p<0.05$ versus SAMP8 group.

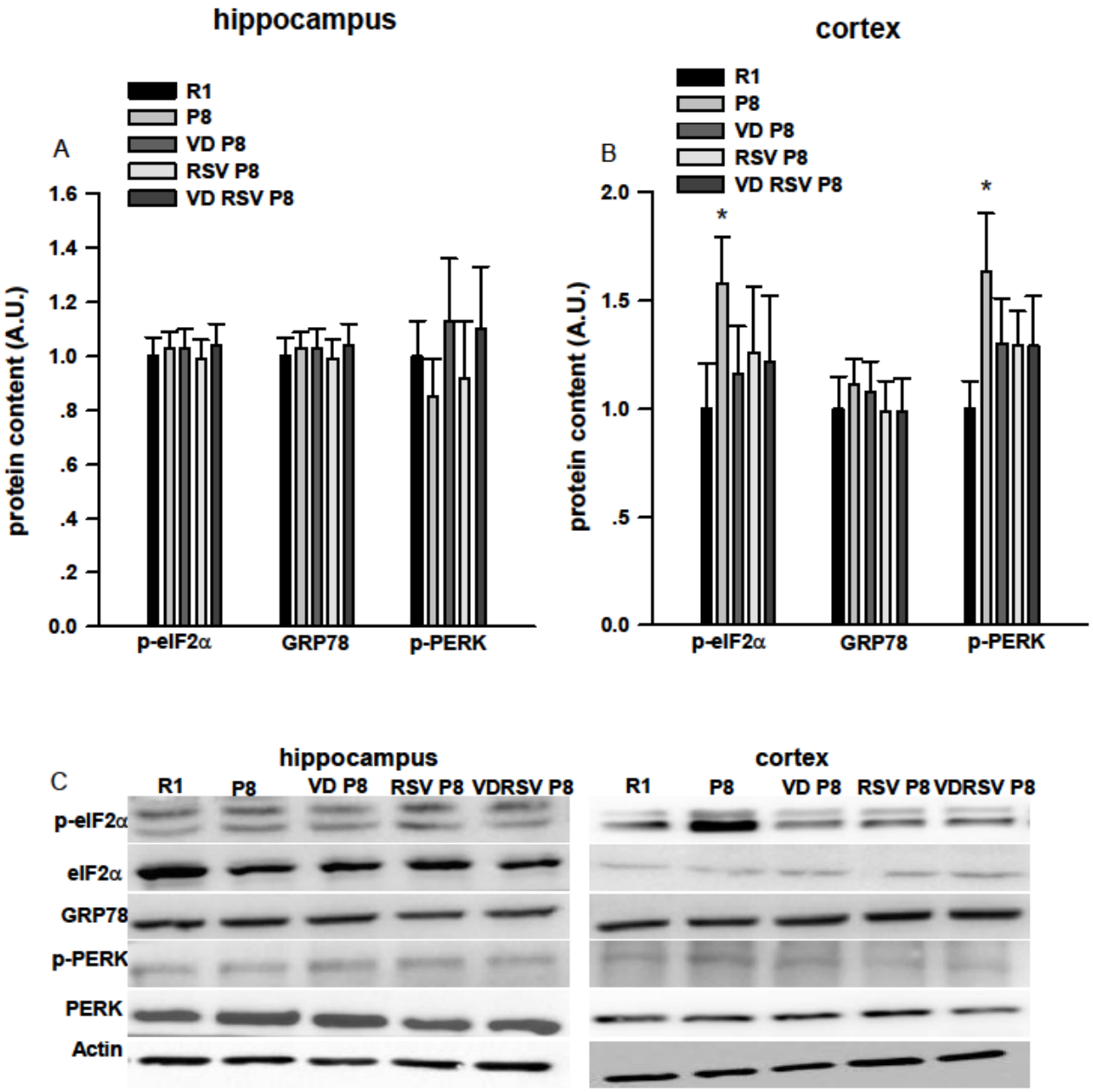

Fig. (6). Vitamin D and resveratrol had no effects on the protein expression of ER stress markers including p-eIF2α, GRP78 and p-PERK. Protein expression of p-eIF2α, GRP78 and p-PERK in (A) hippocampus and (B) cortex of all mice. Representative blots are shown in panel C. A.U. means arbitrary units. Data are presented as means +SEM for 10 mice per group. * $p<0.05$ versus SAMR1 group.

to demonstrate that vitamin D independently could reduce elevated p-Tau ser396, and that the combination of VD with RSV could significantly restore elevated p-Tau ser404 in cortex of SAMP8 mice. However, our results did not suggest that changes in the kinase activity of GSK-3β nor CDK5 nor the phosphatase activity of PP2A played a causal role in the reduction in phosphorylated tau from VDRSV group. Further studies are required to explore which kinases are involved in tau phosphorylation with CaMKII as a promising candidate [48].

Neuroinflammation, which is primarily mediated by activated glial cells, appears to play a critical role in AD progression [49]. An increased GFAP expression has been widely used as a marker for astrogliosis [50]. Meanwhile, the inhibition of NF-κB in astrocytes may also be regarded as a potential therapy for AD because the blockage of NF-κB transcriptional activity in astrocytes can extensively reduce inflammation and improve recovery [51]. Our present study demonstrated that the combination of VD with RSV could significantly reduce GFAP in hippocampus, as well as p-NF-κB p65 in cortex. Thus, inhibition of neuroinflammation post VD and RSV intervention might also be one of the reasons for explaining improved cognitive function in VDRSV group as observed in our present study.

Synaptic dysfunction is another major contributor to cognitive disturbance in AD [52]. STAT3 and CREB are two

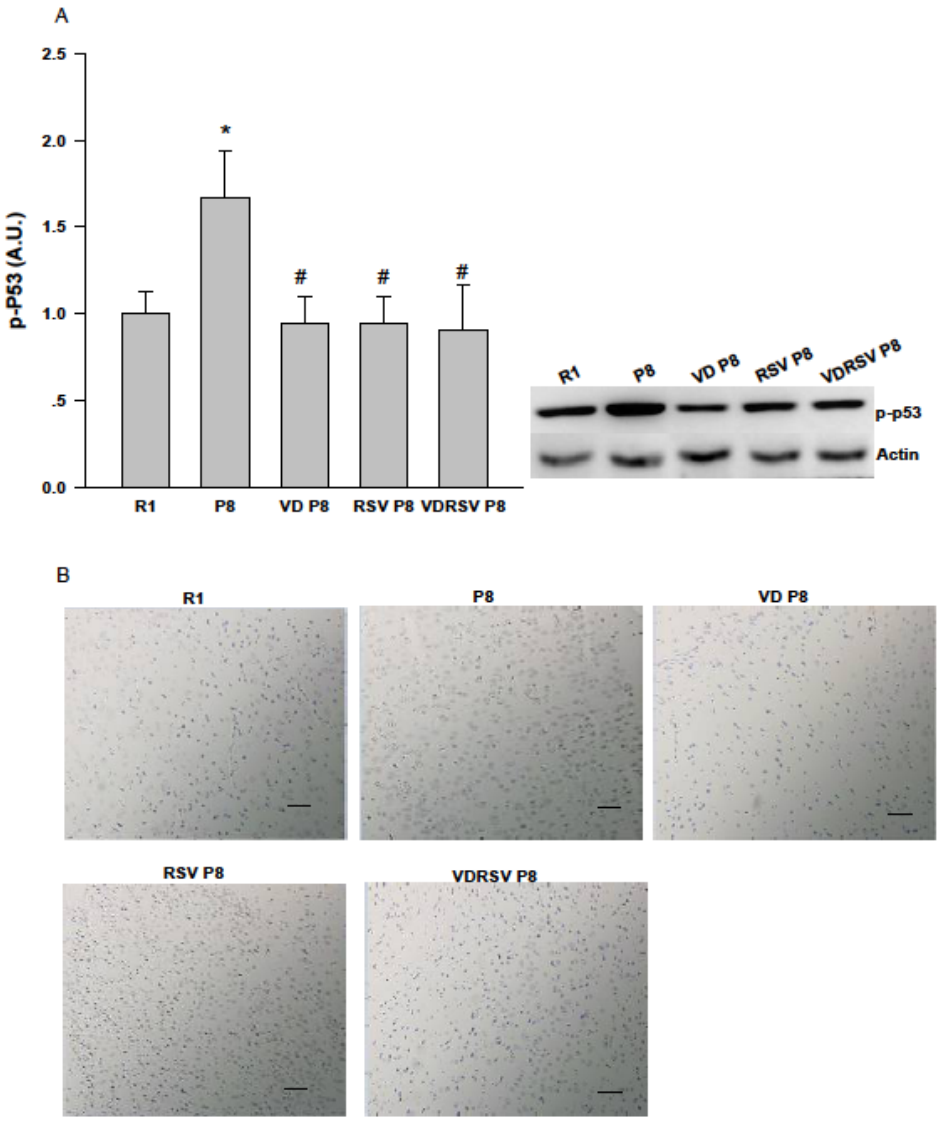

Fig. (7). Effects of Vitamin D and resveratrol on p-p53 protein expression and apoptosis *via* TUNEL assay in cortex of SAMP8 mice. (A) Protein expression of p-p53 in cortex from all groups. (B) Representative immunostaining of apoptotic cells *via* TUNEL assay. Scale bar 20μm, magnification ×200. Representative blots are shown in the right of quantified data in A. A.U. means arbitrary units. Data are presented as means +SEM for 10 mice per group for A, and 4 mice per group for B. * $p<0.05$ versus SAMR1 group. $^{\#}$ $p<0.05$ versus SAMP8 group.

critical factors involved in regulating transcription of synaptic plasticity related proteins including BDNF, synaptophysin and PSD95 [53-54]. We found that the combination of VD with RSV could significantly increase p-STAT3 in hippocampus and p-CREB in cortex although no difference were observed for BDNF, synaptophysin and PSD95 protein expression. Previously, Latimer *et al*. [26] reported that in middle-aged F344 rats, approximately 6 months vitamin D intervention (serum 25OHD: ~37ng/mL) could improve hippocampal-dependent learning and memory *via* MWM test, this might be associated with improved synaptic markers. Thomas *et al*. [55] demonstrated that RSV supplementation in streptozotocin induced diabetic C57Bl/6 mice could improve makers involved in hippocampal synaptic plasticity and neurogenesis. It is likely that VDRSV intervention could also affect synaptic plasticity related protein except BDNF, synaptophysin and PSD95 in our present study.

ER stress has also been proposed as a critical factor involved in the pathogenesis of AD, which is involved not only in intraneuronal accumulation of Aβ [21], but also NFTs of hyperphosphorylated tau [20]. Elevated p53 protein expression has also been observed in the brains of sporadic AD patients and transgenic mouse models carrying mutant familial AD genes [56]. In our present study, elevated ER stress markers, p-p53 and apoptotic markers (as evidenced by TUNEL assays) were observed in cortex from SAMP8 mice, meanwhile, VD, RSV independently and their combination could significantly reduce elevated neuronal apoptosis. It is suggested that the improved cognitive function in VDRSV group might not be owing to the improvement in ER stress markers, but may be partially associated with improved apoptosis.

In our present study, serum 25(OH)D level from SAMR1 and SAMP8 group were around 25ng/mL, which is within

the borderline of Vitamin D deficiency, while VD intervention successfully elevated serum 25(OH)D level, reaching the optimal vitamin D status. However, RSV intervention slightly but significantly reduced serum 25(OH)D level, suggesting the absorption of vitamin D might be affected by resveratrol. Because resveratrol is a naturally occurring polyphenolic compound mainly found in grape skins, berries and peanuts [5], it is important to consider this interaction between vitamin D and resveratrol for dietary recommendation. To be specific, the grape skin has a high concentration of resveratrol ranging from 50 to 100μg/g [57]. Accordingly, red wine is by far the major human dietary source of resveratrol [58], and *trans*-resveratrol levels in wine ranged from 0 to 14.3 mg/L [59]. In raw peanut kernels, peanut butters and peanut oil, the concentration of resveratrol is in the range from 0.02 to 1.92μg/g, 0.1 to 0.8μg/g [60], and 10.9 to 16.9 ng/g [61], respectively. In our present study, the level of vitamin D post-vitamin D intervention is easily to be obtained *via* commercially available vitamin D supplements. The RSV dosage utilized in our present study is roughly 43mg/kg/d, thus, for a 60kg man, it seems that he or she needs to consume 2.58g resveratrol *per* day to meet this requirement, which is unrealistic to obtain. It should also be realized that the oral bioavailability of resveratrol was <1% [62]. However, it is likely that there might be species difference for the physiological responses to the same amount of resveratrol, and that synergistic effects might existed between vitamin D and resveratrol *in vivo*. Therefore, further studies in this area are required to explore the appropriate intake of vitamin D and resveratrol for the development of a practical and powerful non-pharmacological therapeutic strategy that can be applied at various stages of AD, to supplement current pharmaceutical clinical trials that prove unsuccessful. Especially, whether the bioavailability of resveratrol might be affected by vitamin D supplementation is of importance to answer.

CONCLUSION

In conclusion, we demonstrated that the combined intervention of VD with RSV might exert greater neuroprotective effects in SAMP8 mice, this might be associated with the fact that the combined intervention could positively affect amyloidogenic pathways, neuroinflammation, tau phosphorylation and probably apoptosis markers.

ABBREVIATIONS

Aβ	=	amyloid β
AD	=	Alzheimer's disease
APP	=	amyloid precursor protein;
BACE1	=	β-site APP cleaving enzyme 1
BDNF	=	brain derived neurotrophic factor
cdk5	=	cyclin-dependent kinases 5
CLIA	=	chemiluminescent immunoassay
DAB	=	diaminobenzidine
ER	=	endoplasmic reticulum
GRP78/BiP	=	glucose-regulated protein 78
GSK-3β	=	glycogen synthase kinase-3β
MWM	=	Morris water maze
NFTs	=	neurofibrillary tangles
p-eIF2α	=	phosphorylation of eukaryotic initiation factor 2α
PSD-95	=	post-synaptic density protein 95
PDI	=	protein disulfide isomerase
PERK	=	protein kinase-like ER kinase
RSV	=	resveratrol
SAMP8	=	senescence-accelerated mouse-prone 8
VD	=	vitamin D

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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