AMELIORATIVE EFFECT OF NOVEL VITAMIN FORMULA WITH HERBAL EXTRACTS ON SCOPOLAMINE-INDUCED ALZHEIMER'S DISEASE

JEONG MIN LEE*, SANDESH SANCHETI, JAE WON PARK, ILL CHAN NOH, WOON DONG CHO, JUNG HO CHOI

Life Science R&D Center, Sinil Pharmaceutical Co., Ltd, Chungju, Republic of Korea.E-mail: bearlss@hanmail.net

ABSTRACT

Health supplements, vitamins and herbal extracts are gaining popularity as they have been found beneficial in preventing/treating various ailments. Also, combination of such supplements has been found to work synergistically. Therefore, in the present research, we designed and evaluated a novel formula designated as VDDS, consisting of water-soluble vitamins (B complex, vitamin C), coenzyme Q-10 (CoQ10) and herbal extracts (green tea and ginkgo leaf), in treating Alzheimer's Disease (AD). *In vitro,* we investigated neuroprotective effect using amyloid beta (Aβ)-induced neurotoxicity in human neuroblastoma SK-N-SH cells. Further, VDDS effect on learning and memory *In vivo* was scrutinized against scopolamine-induced mice model. Memory enhancing effects were evaluated using rectangular maze test and hot plate test. At the end of the study, brain homogenate was estimated for $A\beta_{1-40}$, $A\beta_{1-42}$, acetylcholinesterase (AChE), nitric oxide (NO), superoxide dismutase (SOD), and chemokine (IFN-γ, MCP-1 and RANTES) contents. *In vitro*, VDDS showed synergistic neuroprotective effect. In addition, it noticeably inhibited the development of AD *In vivo* (induced by scopolamine). The inhibitory effect of VDDS on AD might be associated with its Aβ and AChE inhibition, scavenging of free radicals and its ability to modulate pro-inflammatory chemokines. Thus, VDDS holds a significant therapeutic value in alleviating AD and its related complications.

Keywords: Alzheimer's Disease, Herbal extracts, scopolamine, Vitamins.

INTRODUCTION

Alzheimer's disease (AD) is characterized by progressive neurodegeneration leading to dementia, unusual behavior, personality changes and eventually death[1]. Accumulation of senile plaques (Aβ protein) and neurofibrillary tangles along with decline in cholinergic neurotransmission in brain might be the probable cause of dementia in AD patients[2,3]. Also, several hypotheses have been proposed suggesting key role of oxidative stress and inflammation in the progression of AD[4,5]. As part of the inflammatory response, altered levels of pro-inflammatory chemokine molecules (RANTES, MCP-1, etc.) were observed in AD patients[6]. Current AD treatments have several drawbacks including decreased efficacy over time, adverse/side effects, ineffective against some long-term complications and low-cost effectiveness[7].

CoQ10 is naturally occurring strong antioxidant and as per published preliminary clinical trials, it might offer promise in many brain disorders. Also, it is well tolerated in humans and thus may be a promising target for therapeutic trials in AD[8,9]. In addition, green tea is the most popular beverage consumed worldwide. Green tea (-) epigallocatechin-3-gallate was found to reverse oxidative stress and reduce acetylcholinesterase activity in a streptozotocin-induced model of dementia[10]. Another herbal extract gaining popularity is *Ginkgo biloba*. An extract of *Ginkgo biloba* leaves, EGb761, is a popular dietary supplement in the United States to enhance memory. The two potential mechanisms of action proposed for EGb761 are reducing oxidative damage and stimulating cell survival machinery[11].

In recent times, much effort has been put into the development of various dietary supplements/functional foods (vitamins, minerals, herbal extracts, etc.) for the prevention of various diseases, including AD. Among them, vitamins have been found beneficial to treat/prevent various ailments and thus widely consumed around the globe[12,13]. Combination of vitamins and herbal extracts has been imagined to work synergistically in the treatment of AD[14]. AD pathology has been related to multiple pathways such as Aβ deposition, plaques formation, cholinergic deficiency, oxidative stress, etc. Thus "single-molecule-single target" medicines may not be a potent strategy to delay or block the progress of AD[15]. Taking these facts into consideration, we developed a novel vitamin formula (VDDS) containing vitamins (B complex, vitamin C), CoQ10 and herbal extracts (green tea, ginkgo leaf) (Table 1) and evaluated its efficacy *In vitro*, *In vivo* and *ex vivo* for treating/preventing AD.

MATERIALS AND METHODS

VDDS components

Details of each constituent and its procuring source is as follows: Vitamin B1 (Hubei Huazhong pharma, China), Vitamin B2 (Hubei Guangji pharma, China), Vitamin B3 (Amsal chem., India), Vitamin B5 (Daiichi, Japan), Vitamin B6 (Zhejiang Tianxin pharma, China), Vitamin B7 (Merck, Switzherland), Vitamin B9 (DSM Nutritional Products, Switzerland), Vitamin B12 (Hebei Huarong pharma, China), Vitamin C (Northeast pharma, China), CoQ10 (Zhejiang Medicine, China), Green tea extract containing catechins 39.8%, caffeine 1.27% and polyphenols 53.7% (Monteloeder S.L., Spain) and Ginkgo extract with flavone glycosides 27.52% and ginkgolides 6.1% (Naturex, France). Composition of VDDS is detailed in Table 1.

In vitro neuroprotective effect of VDDS using MTT reduction assay

The experimental protocol followed in this study was according to previously described method, with slight modifications[16]. SK-N-SH cells (Korea cell line bank, Seoul) were cultured in modified Eagle's medium (10% fetal bovine serum and 1% penicillin (Gibco)), seeded at a density of $1x10^5$ cells/ml in 96 well plate, incubated overnight and treated with VDDS and its each component (100 μg/ml), exposed to $A\beta_{25-35}$ (BACHEM AG, Switzerland) (100 μM) and further incubated for 24 hours. ($A\beta_{25-35}$ was diluted in distilled water at a concentration of 1 mM and incubated for 3 days at 37 °C to aggregate and further stored at -20 °C). 0.5 mg/ml of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was added to each well and the plates were incubated for 2 hours at 37 °C in dark and then 100 μl of DMSO was added and the wavelength was measured at 540 nm using microplate reader.

Animals

Male ICR mice weighing 25-30 g were purchased from Central Lab. Animal, Inc (Seoul, Korea). All mice were maintained according to the Korea Food and Drug Administration guidelines for the care of animals. All animals were housed at a 12-hour light and dark cycle, 22±2 °C and relative humidity of 45-65%. The animals were allowed free access to laboratory chow diet and water *ad libitum*. The protocol used in this study for the use of animals was approved by the Sinil Pharmaceutical Animal Ethical Committee (SI-2012-04). At

the end of the study, the animals were euthanized and brains were removed, and all efforts were made to minimize their suffering.

Experimental design

The experimental protocol followed in this study was according to previously described by Goverdhan *et al*[17]. with minor modifications. Scopolamine (Sigma, St. Louis, MO, USA) 1.4 mg/kg was dissolved in distilled water and injected intraperitoneally (i.p.) into 7 hours fasted mice. Followed by oral administration of vehicle/ drug to the following groups of mice- Group 1: Vehicle (CMC 1%), Group 2: AD model (CMC 1%), Group 3: Positive control (donepezil 5 mg/kg bw) Group 4: VDDS (VDDS 30.66 mg/kg bw) (Table 1). VDDS dose was calculated according to the daily recommended dose of each VDDS component. VDDS/donepezil was administered once daily for 12 days.

In vivo memory enhancing effects of VDDS

Rectangular maze test

Before the start of maze test, all the animals were trained to the experiment by familiarizing with the rectangular maze chamber for 2 days. Transfer latency (time taken to reach the reward chamber) was recorded where the lower scores of assessment indicate efficient learning while higher scores indicate poor learning in animals. The time taken by the animals to reach the reward chamber from the entry was noted on day 3 to 9[17].

Hot plate test

Each group animals were administered the appropriate vehicle/drug and after 1 hour, the escape latencies were tested using a hot plate. Latency to lick their paws at 55°C hot plate was recorded in each mouse on 10, 11 and 12th day. After the experiment, all animals were placed back to their respective cages[18].

Tissue preparation

After behavioral test, mice were deeply anaesthetized using CO_2 and sacrificed by cervical dislocation. Brain tissues were immediately collected. Each brain was homogenized in extraction buffer (PRO-PREPTM Intron Biotechnology, Korea). Protein concentration was determined using standard assay kit (Pierce, Rockford, IL). All samples were stored at -20 °C until further analyses.

Ex vivo estimation of $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in the brain homogenate

Mouse $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in the brain homogenate were analyzed by ELISA kits according to the manufacturer's instruction (Wuhan EIAab Science Co.,Ltd, China). All the assays were performed in triplicate for each sample.

Ex vivo estimation of AChE enzyme levels in the brain homogenate

AChE enzyme levels in the brain homogenate were determined using standard assay kit (AAT Bioquest (Amplite), Inc., USA). The absorbance was read at 410 nm after 10 minutes of incubation.

Ex vivo estimation of antioxidant levels in the brain homogenate (NO and SOD)

NO and SOD levels in the brain homogenate were estimated using Promega and Dojindo assay kits, respectively. Individual blanks were prepared for correcting the background absorbance for each assay. Each sample was assayed in triplicate. NO activity is expressed as µM of NO content and SOD as % of control.

Ex vivo estimation of chemokine levels in the brain homogenate (IFN-γ, MCP-1 and RANTES)

The chemokine levels in terms of IFN-γ, MCP-1 and RANTES in the brain homogenate were determined according to the manufacturer instruction manual (Invitrogen). All the three chemokine levels are expressed in terms of pg/ml.

Statistical Analyses

Data were expressed as mean ± S.D. for six animals in each group. Statistical analyses were done by one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test using KaleidaGraph v. 4.0, Synergy Software. $p < 0.05$ was considered to be statistically significant.

RESULTS

In vitro neuroprotective effect of VDDS

Each component of VDDS (Vitamin B1, B2, B3, B5, B6, B7, B9, B12, Vitamin C, CoQ10, green tea and Ginkgo biloba) was evaluated for its neuroprotective effect using human neuroblastoma SK-N-SH cells at 100 µg/ml. The neuroprotective effect of VDDS and its components is detailed in Fig. 1. The data is expressed as % cell survival. Among all components, green tea and ginkgo extract showed highest and almost equal neuroprotective effect with a cell survival of 47%. Interestingly, VDDS showed about 17% synergetic neuroprotective effect with a cell survival of 55% (Fig. 1).

In vivo Memory enhancing effects of VDDS (Rectangular maze test and hot plate test)

Rectangular maze test (Fig. 2A) was performed for total 7 days (day 3 to day 9). Oral administration of VDDS for 9 days showed drastic change in the transfer latency. On day 9 (7th day) of the rectangular maze test the transfer latency was decreased by 52% as compared to scopolamine insulated mice (Fig. 2A). The scopolamine insulted mice showed the highest latency (about 720 sec). In hot plate test (Fig. 2B), escape time on 10, 11 and 12th day was noted. On 12th day a significant decrease in the escape latency was observed in the VDDS and donepezil supplemented mice. The escape latency in scopolamine insulted mice was maximum on day 12 (9.33 sec) whereas VDDS showed noteworthy reduction in the escape latency (6.53 sec) (43%).

Ex vivo effect of VDDS on $A\beta_{1-40}$ and $A\beta_{1-42}$ levels in the brain homogenate

At the end of the study, the brain homogenate was evaluated for $A\beta_{1-40}$ and $A\beta_{1-42}$ levels. A significant increase in both the parameters was observed in the scopolamine-induced AD mice. Interestingly, the altered levels of $A\beta_{1-40}$ and $A\beta_{1-42}$ were restored significantly as compared to scopolamine insulted mice, in the VDDS and reference drug supplemented groups (Fig. 3A and B).

Ex vivo effect of VDDS on AChE levels in the brain homogenate

In Fig. 3C, AChE levels are shown. At the end of the study, a significant increase in the levels of AChE was observed in the scopolamine treated mice as compared to control rats. This deleterious effect was significantly lowered after the 12 days supplementation of VDDS. Also, effect of VDDS was comparable to reference drug, donepezil.

Ex vivo effect of VDDS on antioxidant levels in the brain homogenate

The antioxidant effect in the brain homogenate was scrutinized using NO and SOD. In scopolamine-induced dementia mice (AD mice), NO levels in the brain homogenate were significantly increased to 13.33 µM, whereas group IV showed noteworthy reduction in the NO content after 12 days of oral VDDS administration (Fig. 4A). Similarly, SOD levels were significantly increased in the orally administered VDDS or standard drug donepezil groups, as compared to scopolamine-insulted mice (Fig. 4B).

Ex vivo effect of VDDS on chemokine levels in the brain homogenate

IFN-γ, MCP-1 and RANTES levels are detailed in Fig. 5. All three AD related chemokine levels were significantly increased in the mice brain homogenate treated with only scopolamine (Group II). This adverse effect was considerably prohibited in VDDS or donepezil treated mice (Fig. 5).

Table 1: VDDS composition

Component	mg
Vitamin B1	0.4
Vitamin B2	1.6
Vitamin B3	1.6
Vitamin B5	0.4
Vitamin B6	1.6
Vitamin B7	0.02
Vitamin B9	0.016
Vitamin B12	0.04
Vitamin C	4
CoQ10	0.2
Gingko leaf extract	4.8
Green tea extract	16

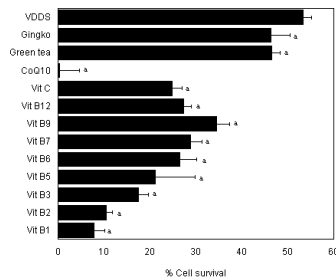**Note: Each of components used in VDDS formulation was approved by Korea Food and Drug Administration.**

Figure. 1. *In vitro* **neuroprotective effect of VDDS and its each component. SK-N-SH cells were seeded and incubated for overnight and treated with Aβ_{25-35} (100 μM), VDDS and each of its component (100 μg/ml) for 24 hours. The cellular viability was evaluated using MTT assay. The data is expressed as percentage of cell survival of the control (non-treated cells). Bars are means ± S.D where [a]$p < 0.05$ compared with VDDS.**
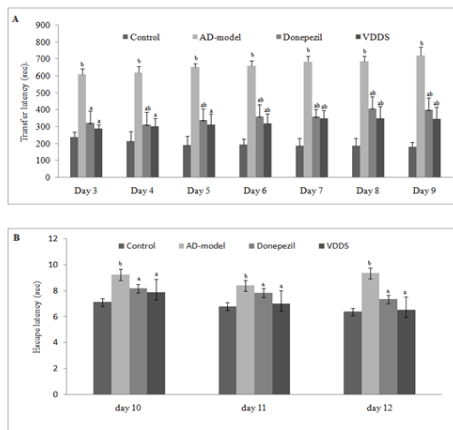

Figure 2: Memory enhancing effects of VDDS in scopolamine-induced mice (A) rectangular maze test results from day 3 to day 9. (B) hot plate results from day 10 to day 12. Data is presented as S.D. (n=6). Bar 1-Control, Bar 2-AD-model, Bar 3-donepezil, Bar 4-VDDS. [a]$p < 0.05$ compared with AD-model mice and [b]$p < 0.05$ compared with control mice
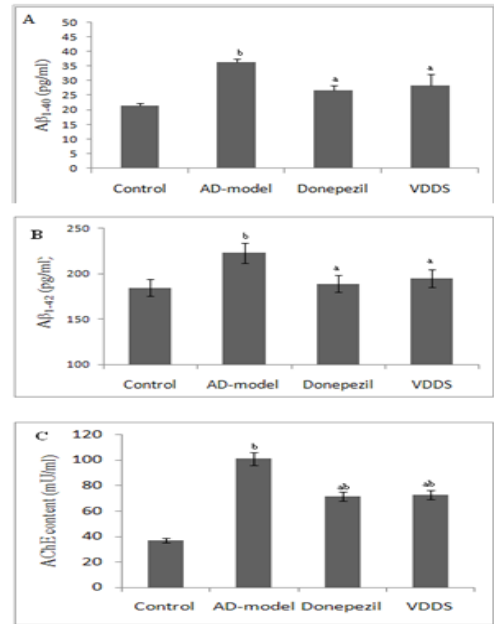

Figure3: Effect of VDDS on (A) Aβ_{1-40}, (B) Aβ_{1-42} and (C) AChE levels in the brain homogenate. Data is presented as S.D. (n=6). Bar 1-Control, Bar 2-AD-model, Bar 3-donepezil, Bar 4-VDDS. [a] $p < 0.05$ compared with AD-model mice and [b]$p < 0.05$ compared with control mice
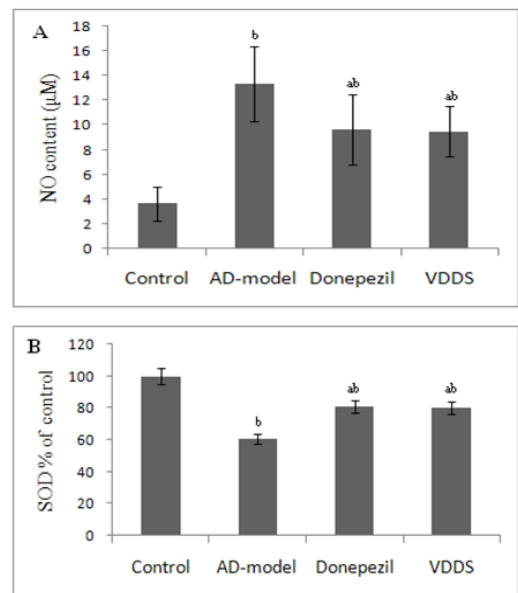

Figure 4: Effect of VDDS on (A) NO (B) SOD levels in the brain homogenate. Data is presented as S.D. (n=6). Bar 1-Control, Bar 2-AD-model, Bar 3-donepezil, Bar 4-VDDS. [a]$p < 0.05$ compared with AD-model mice and [b]$p < 0.05$ compared with control mice
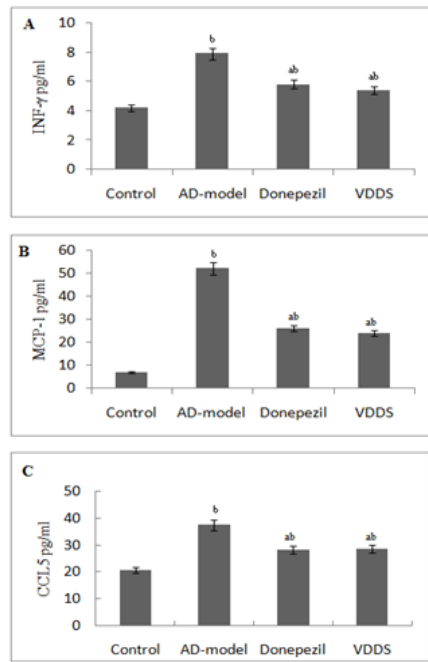
Figure 5: Effect of VDDS on (A) IFN-γ (B) MCP-1 and (C) RANTES levels in the brain homogenate. Data is presented as S.D. (n=6). Bar 1-Control, Bar 2-AD-model, Bar 3-donepezil, Bar 4-VDDS. [a] *p< 0.05 compared with AD-model mice and* [b]*p< 0.05 compared with control mice*

DISCUSSION

The present study demonstrates the protective effect of VDDS in the treatment of AD. It is clearly evident from the *In vitro* results that VDDS has significant (*p*<0.05) synergistic neuroprotective effect in human neuroblastoma, SK-N-SH cells as compared to each of its components (Fig. 1). This protective effect of VDDS in SK-N-SH cells is possibly due to potent protection against Aβ toxicity. Ongoing research on AD has shown the effectiveness of neuroprotective agents in alleviating symptoms of AD and the parallel application of such therapy in the treatment of AD can play a crucial role in slowing AD progression[8].

Scopolamine, a nonselective muscarinic cholinergic antagonist, causes cognitive deficit via increasing AChE levels[19]. Also, scopolamine-induced animal model has shown to increase levels of Aβ and elicit oxidative stress in the rodent brain[19,20]. Therefore, by virtue of the property, scopolamine is useful *In vivo* model for AD. In the current study, scopolamine-induced mice model was employed to study the effect of VDDS on memory function. *In vivo* behavioral studies showed noteworthy elevation in the transfer and escape latencies in case of both the tests (maze and hot plate) (Fig. 2A and B) in scopolamine insulted group. Also, the brain homogenate was found to have evaluated levels of Aβ$_{1-40}$, Aβ$_{1-42}$ and AChE levels in the chemically induced AD mice (Fig. 3A, B and C). Interestingly, in VDDS administrated group two memory tests in mice revealed improved learning and memory function (Fig. 2A and B), and also both the elevated levels of Aβ (1-40 and 1-42) and AChE were significantly decreased as compared to scopolamine-induced mice (Fig. 3A, B and C). Rising evidence suggest that AChE plays a vital role in the deposition of Aβ and plaque formation in AD[21,22]. Thus, it can be postulated that one of the mechanisms involved in the improvement of memory function and suppression of Aβ accumulation by VDDS might be strongly related to its AChE inhibition property. Reactive oxygen species have been found to be associated in the pathogenesis of many degenerative diseases, including human AD[23]. In this research, we evaluated the antioxidant levels in terms of NO and SOD in the brain homogenate of mice. The results (Fig. 4A and B) highlighted the ability of VDDS to defend the brain from deleterious effects of chemical (scopolamine) insult. This effect of VDDS might be strongly attributable to its free radical scavenging potential.

Also, in the present research, the brain homogenate was evaluated for various chemokine (IFN-γ, MCP-1 and RANTES). Clinical studies suggest the important role of inflammatory chemokines in the neurodegenerative cascade leading to AD[6,24]. Also, functional consequences of secretion of several pro-inflammatory chemokines have been linked with increased production of reactive oxygen species[25]. Previously, Reale *et al*. have demonstrated that the AD patients administered AChE inhibitor (pyridostigmine bromide), were found to have reduced expression and production of various chemokines including RANTES and IFN- γ[24]. Therefore, it can be suggested that the probable mechanism of VDDS in suppressing pro-inflammatory chemokines may be in parts explained by its anti-AChE and antioxidant properties.

CONCLUSIONS

VDDS treatment ameliorated cholinergic deficit, memory parameters and pro-inflammatory chemokines in the AD mice, which may find clinical application in treating neuronal deficit in the AD patients. The protective action of VDDS on AD dementia model may be attributed to its multiple effects like anticholinesterase, antioxidant and modulation of pro-inflammatory chemokines. Thus, VDDS provides a strong biochemical rationale to be a potent and safe neuroprotective, memory enhancer and free radical scavenging agent and could be used as a dietary supplement for prevention or early treatment of AD.

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