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Neuroprotective mechanism of donepezil and resveratrol combination in the Alzheimer model caused by Scopolamine in mice

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Abstract
AD is characterized by cognitive deterioration and cholinergic deficiency and is treated by the use of donepezil as a first-line medication. Nevertheless, when taken together with neuroprotective drugs it may provide additional therapeutic gain. This paper has conducted an investigation on the neuroprotective prowess of donepezil and resveratrol on a scopolamine-based mouse model of intellectual diminishment. Five groups of mice were used; control group, a group treated with scopolamine, donepezil group, resveratrol and combination group. Outcomes of behavioral tests such as Morris water maze, and Y-maze indicated that combination group performed significantly better in spatial memory and spontaneous alternation than in monotherapy groups ($p < 0.01$). Biochemical tests demonstrated that combination therapy decreased malondialdehyde (MDA), elevated the levels of superoxide dismutase (SOD) in the hippocampal tissue along with acetylcholine levels. It was shown that fewer neurodegeneration and maintenance of hippocampal structure occurred, functionally and histologically. The mixture of donepezil+resveratrol showed the combination effect of neuroprotective properties mostly due to the mutual mechanisms of action with cholinergic augmentation and the lowering of oxidative load.

Keywords: ***Alzheimer disease, donepezil, resveratrol, neuroprotection, cognitive impairment, Oxidative stress, cholinergic dysfunction.***

1. Introduction

1.1 Pathology of Alzheimer Disease and Treatment Boundaries of the Disease

Alzheimer disease (AD) is a progressive neurodegenerative disease which mainly occurs in elderly people and is related to abnormalities with regards to cognitive decline, memory loss, and behavior changes. AD pathology is characterised by the deposition of amyloid-beta plaques, neurofibrillary tangles and overall neurodegeneration especially in memory and learning regions of the brain, including the hippocampus. Such dysfunctional pathology results in cholinergic dysfunction that is a symptom of AD and one of its most characteristic traits, causing cognitive decline in afflicted persons.

The existing standard of AD treatment is rather symptomatic than curative. Acetylcholinesterase inhibitor Donepezil is used in early treatment whose mechanism of action is on the level of acetylcholine in the brain that facilitates an upsurge in the cholinergic neurotransmission. Whereas donepezil leads to cognitive gains in moderation in certain patients, its effects are not substantial especially at the advanced stages of the illness. Moreover, donepezil fails to deal with the pathology of the AD, e.g., burdensome amyloid or oxidative stress, and cannot be applied alone.

Besides donepezil other treatment approaches targeting neurotransmitter systems, e.g. glutamate modulators have been studied, however these are only of limited value as well. This represents the importance of introducing new therapeutic solutions that will not only enhance symptomatic treatment but also will focus on the pathophysiological mechanisms of AD, such as oxidative stress and neuroinflammation.(1)

1.2 Cholinergic dysfunction and oxidative stress role in AD progression

There are two main aspects that lead to the development of AD, which are the cholinergic dysfunction and oxidative stress. Cholinergic hypothesis of AD By this mechanism, it is proposed that the central role of the cognitive deficit of AD patients is the loss of acetylcholine (ACh a neurotransmitter important to learning and memory) through degeneration of cholinergic cells of the nucleus basalis of Meynert. The use of donepezil inhibits acetylcholinesterase, which subsequently keeps up the level of ACh in the brain and alleviates part of the intellectual manifestations of AD.

Besides the cholinergic dysfunction, oxidative stress also has its own contribution to the pathophysiology of AD. The reactive oxygen species (ROS) are a type of oxidative damage which causes neuronal damage, mitochondrial dysfunction and amyloid-beta toxicity. Hippocampus and cortex are parts of the brain whose roles in thought cannot be overestimated, and therefore, they are extremely sensitive to oxidative damage. Due to the further development of AD, the level of malondialdehyde (MDA) which is a product of lipid peroxidation, accumulates and shows how severe the oxidative damage has occurred in the brain. Moreover, oxidative stress has been linked in the AD inflammation which also enhances the propagation of the neuronal degeneration.

Therefore, the combination of treatment that focuses on treating the symptoms of AD as well as the pathophysiology of AD may be indicated through therapies that aim at treating cholinergic dysfunction and oxidative stress, thereby ameliorating the symptoms of AD.(2)

1.3. The Possibility of How Donepezil and Resveratrol as Monotherapies can Therapeutically Work

As an acetylcholinesterase inhibitor donepezil study has demonstrated benefit of symptomatic relief owing to improved cholinergic neurotransmission. Nevertheless, it is not limitless because its efficacy wears off in the long term, and it does not influence neurodegenerative procedures and oxidative stress. In view of such limitations, the concept of combination therapy, in incorporating with other agents that have neuroprotective effects, has gained increased attention.

Resveratrol is a natural polyphenolic compound that is present in grapes, red wine and other plants which has acquired significance due to its ability to protect the nerve cells and as an anti-inflammatory compound. Resveratrol was shown to have properties of antioxidants, which can alleviate oxidative stress as well as amyloid-beta toxicity in animal models of AD. Also, resveratrol has been reported to engage sirtuin-1 (SIRT1) pathway that is implicated in neuronal survival, inflammation and mitochondrial dynamics. Therefore, resveratrol could be used as an adjuvant to donepezil, by binding to oxidative stress and neuroinflammation which are important pathophysiology factors in AD.

1.4 Justification of Combination Therapy approach in Neurodegeneration

Although donepezil can correct the cholinergic impairment in AD, and resveratrol can correct oxidative stress and inflammation, use of the two drugs may offer a synergistic effect. The combination of donepezil with resveratrol is based on the rationale of the antagonist mechanisms of actions. Donepezil could enhance cognitive performance due to the improved levels of acetylcholine, whereas the influence of resveratrol could minimize brain decay owing to the altered oxidative stress and the inflammation process. Collectively, these agents could offer a better comprehensive solution to the treatment of AD in terms of cognition and dealing with neuroprotective processes.

Combination therapy is gaining favor in neurodegeneration, particularly in conditions such as AD, in which disease advances due to interaction of several pathophysiological processes. Combination of donepezil and resveratrol can accomplish not only symptom improvement, but also disease progression slowing by acting on a wider area of the disease processes.(3)

1.5 Study Objective: To determine the effects of co-administration of donepezil with resveratrol on behavioral, biochemical and histological parameters in an AD mouse model

This study aimed at assessing the neuroprotective value of donepezil and resveratrol combination therapy in a scopolamine-induced mouse model of Alzheimer. In particular, the goal of the study was to determine the combined elasticity of these two agents on cognitive functioning, biochemical markers, as well as neurodegeneration. The Morris water maze and the Y-maze behavioral analyses that examine the spatial memory and the spontaneous alternation field tests were also performed as the important attributes of the cognitive abilities of the animal models of AD. Markers of oxidative stress including malondialdehyde (MDA) and superoxide, dismutase (SOD) were carried out by biochemical assay and acetylcholine level was examined. Lastly, the neurons were analyzed histologically in order to determine the level of neurodegeneration and survival of hippocampal architecture.

2. Methods and Materials

2.1 Reagents and chemicals utilized

The reagents and chemicals to be used in this research are analytical grade and came out of competent suppliers. Sigma-Aldrich (USA) was used to obtain donepezil hydrochloride, and resveratrol was purchased in Bioworld Technology (USA). Scolopamine hydrobromide was obtained at Sigma-aldrich, which was utilized in inducing cognitive impairment in the control animals. The acetylcholine, malondialdehyde (MDA) and super oxide

dismutase (SOD) assay kits were purchased at Cayman Chemical (USA). All the other chemicals were obtained unless otherwise specified (all the solvents and buffers were obtained through Sigma-Aldrich). Phosphate-buffered saline (PBS) has been prepared in the laboratory with the Milli-Q water and autoclaved.

MDA, SOD and acetylcholine biochemical tests were done as indicated by the manufacturer. Thermo Fisher Scientific was used to obtain stain kits, i.e. formalin, paraffin and hematoxylin-eosin (H&E) stain kits, to achieve histological analysis.(4)

2.2 Animal Ethical Clearance and Ethical Clearance

Swiss albino male mice (20-25 g) at the total number of 40 were used in the experiment. The animals were a source of Central Animal facility of the University of XYZ. These mice were put under normal laboratory environments with a 12 hour light/dark cycle being maintained and the temperature was maintained at 22 +/- 2 C and also had free access to water and standard rodent chow. All the animals had an opportunity to adapt to laboratory conditions by 24 hours before commencement of the experiment.

It was viewed that the study protocol was acceptable to the Institutional Animal Care and Use Committee (IACUC) of the University of XYZ and with the ethics of the National Institutes of Health (NIH) to take care and use of the laboratory animals. We tried in every way to restrict animal suffering and to reduce the number of animals.

2.3 Scopolamine-induced Cognitive Impairment

The method used to induce cognitive impairment in the mice is the subcutaneous administration of scopolamine hydrobromide (1mg/kg) once every day during a period of 7 days successively. Scopolamine is a well-established pharmacological agent that inhibits memory and learning abilities by activation of muscarinic receptor blocking, occurs and leads to the cholinergic function and cognitive impairments, similar to the pathophysiology of Alzheimer disease. The animals received a one-day recovery period after completion of the induction period after which treatment regime started.

2.4 Groups and Scheduling of Treatment

Animals were randomly put in five groups of experimental animals (n = 8) as follows:

- Control Group: The mice placed on normal saline (vehicle) throughout the entire period.
- Scopolamine Group: Mice which are injected with scopolamine (1 mg/kg/day) during 7 days in order to impair their cognitive faculties.
- Donepezil Group: Mice which were given donepezil (3 mg/kg/day) 7 days after 2nd day of scopolamine induction.
- Resveratrol Group: Mice that were treated with resveratrol (20 mg/kg/day) during 7 days in succession beginning on the second day of scopolamine development.
- Combination Group: Mice that have been on donepezil (3 mg/kg/ day) and resveratrol (20 mg/kg/ day) over 7 days, beginning on day 2 post-scopolamine induction.

The following was administered orally by gavage once per day during the course of 7 days, donepezil and resveratrol. Combined treatment group group was treated with the combination of both drugs at the same time through oral gavage. Normal saline (vehicle) was given by gavage as a vehicle to control and scopolamine groups.

2.5 Behavioral measurments: Morris water maze and Y-Maze Tests

Morris Water Maze (MWM): Spatial learning and memory were tested by using Morris water maze (MWM) test. The maze was a circular pool (diameter 1.2 m) filled with water of the temperature 22 22 and C22 with a covered and uncovered platform (diameter 10 cm). The training pattern was 5 days, and four trials a day, then the mice were trained to reach the hidden platform. The video tracking system helped to record the latency (time taken to find the platform). After six days, the platform was taken away and a probe test was made so as to judge the amount of time spent in target quadrant or the place where the platform used to be.(5)

Y-Maze Test: The Y-maze test was implemented to assess spontaneous altering behavior which is a predictor of working memory. This instrument was composed of 3 arms that formed a Y shape and each arm is 40 cm long and 10 cm wide. Mice were given time to explore the maze at 5 minutes and number of alternations (Sequential visits of three different arms) measured. The formula used in calculating the percentage of spontaneous alternation was: Spontaneous Alternation=(Total arm entries−2Number of alternations)×100

2.6 Collection of tissue and biochemical analysis of MDA, SOD and Acetylcholine

On day 8 after completion of behavioral tests, the animals were sacrificed by cervical dislocation and subsequently their brains immediately removed. The hippocampal tissue was isolated and weighed, as well as homogenized

using ice-cold PBS (pH 7.4). The tissue was homogenized and centrifuged at 10,000 rpm, 4o C, 10 min and the supernatant retained as the biochemical material.

MDA: The levels of malondialdehyde (MDA) as an index of lipid peroxidation were detected by a commercial MDA reaction kit (Cayman Chemical) as directed by the manufacturer.

SOD: The test of superoxide dismutase (SOD) activity was determined by the SOD assay kit (Cayman Chemical), according to the inhibition of nitroblue tetrazolium reduction (NBT).

Acetylcholine: The concentration of acetylcholine in the hippocampus was carried out through acetylcholine assay kit (BioVision) which exploited colorimetric determination of acetylcholine production.(6)

2.7 Hippocampal sections histology

Following biochemical analysis, the brains were fixed with 10 per cent formalin overnight after which it was then embedded in paraffin. Sections of the brain (5 m thickness) were sliced into pieces by a microtome and placed on a glass slide. The hematoxylin and eosin (H&E) stained sections were evaluated in terms of integrity of neurons as well as architecture of the hippocampus. The extent of inflammation and neurodegeneration was also measured using light microscope with emphasis on the regions CA1, CA3 and dentate gyrus in hippocampus. Histopathological assessment was done through the score of loss of neurons, gliosis, and degeneration depending on the severity.

2.8 Data Analysis

The GraphPad Prism software (version 8.0) was used to analyze the data. All data is given as mean faverage +/- standard error of the mean (SEM). One-way ANOVA was used to make the comparison among groups and Dunnett post hoc test was used to draw comparisons among treatment groups and the scopolamine group. The p value was to be considered unsignificant at $p < 0.05$ significance.

3. Behavioral Evaluation

3.1 I. Learning tests and memory judgments of Morris Water Maze

The animals learning and memory capacities were evaluated with the help of Morris water maze (MWM). In the experiment, mice were trained in finding a sunken platform (diameter 10 cm) based on spatial information through the chamber in which the mice were situated. The maze was in the form of a circular pool (1.2 m diameter), which contained water at 22 +/- 2 o C. It was located statically, slightly beneath the surface of water and the platform was covered. Four trials per day were used to train mice on 5 consecutive days. The time (latency) spent in finding the platform was captured and maximum time spend in the trial was 60 seconds. When the mouse failed to find the platform in the maximum time it was directed over the platform and was left to be over the platform with a time span of 30 seconds.

On the sixth-day, a probe trial was undertaken to determine memorization. Here the platform was taken out, and the amount of time spent in the quadrant where the platform used to be put was measured. The time spent in target quadrant in different treatment groups was compared. The more time an animal spends in the target quadrant the more is the indication of superior spatial memory and learning skills.(7)

3.2 Spontaneous Alternation Behavior in Y-Maze measured

The Y-maze experimental test was used in measuring working memory as the working memory measures spontaneous alternation behavior. The device was fabricated into three arms that were 40 cm long and were in a Y shape where one arm acts as the beginning, and the other two arms were two alternatives. Every arm was labeled using a different visual stimulus so that the mouse can differentiate them.

The mice were introduced at the bottom of the Y-maze and left to explore freely in the 5-minutes duration. It was manually recorded how many alternations occurred (entering three different arms sequentially). An alternation has been defined as a case when the mouse entered three consecutive arms like ABC, BAC etc. Spontaneous percentage alternation was calculated after the following formula was used:

The greater the percentage of alternation, the better working memory and capacity to memorize previously explored arms are. Relatively fewer percentages of alternations in AD models indicate cognitive impairment or deficits in working memory.

3.3 Group-based Analysis of Performance

The work of the five experimental groups was compared to assess the influence of donepezil, resveratrol and their combination on cognitive function learning. The normal saline was administered in the control group and the scopolamine group was treated with scopolamine in order to cause a cognitive impairment. The donepezil group

would be run through donepezil treatment, the resveratrol group would be run through resveratrol treatment and the combination group would be run through donepezil and resveratrol.

In the Morris water maze, the group of animals treated with scopolamine was much slower than all other groups, which proves that the animals have learned poorer how to use the spatial memory on the maze. The partial change in latency response was observed in the donepezil and resveratrol group and the combination group depicted the shortest latency and longest percentage target quadrant time than the group administered monotherapy indicating improvement of the memory retention and spatial learning than the combination group ($p < 0.01$).

The scopolamine-treated group also had significantly reduced percentages of spontaneous alternation in the Y-maze test unlike in the control group. In addition, both donepezil and resveratrol alleviated the alternative behavior to a certain extent, but the combination group revealed the greatest percentage of spontaneous alternation ($p < 0.01$), which has proven once again the idea that the combination of donepezil and resveratrol has a synergistic effect on cognitive activity.(8)

The combined group showed steady improvement as compared to the monotherapy groups in Morris water maze and the Y-maze test implying that the combination therapy has increasing affect on learning and memory abilities. These findings point to the fact that donepezil and resveratrol can potentially have distinctly complementary effects as one is improving the cholinergic system and the other one can provide neuroprotection with respective antioxidant and anti-inflammatory effects.

4. Biochemical Analysis

4.1 Measurement of MDA concentration

MDA is a really long-lasting product of lipid peroxidation and its frequencies are a vital representation of oxidative stress in biological tissues. In this experiment, the hippocampal tissue MDA was tested by adopting the thiobarbituric acid-reactive substances (TBARS) assay based on the detection of MDA.

It was done in the following manner: Hippocampal tissue was homogenized with phosphate-buffered saline (pH 7.4) and centrifuged at 10, 000 rpm, 10 minutes to get the supernatant. A portion of the supernatant (100) was then added to 1 mL of TBARS reagent (thiobarbituric acid, hydrochloric acid and sodium dodecyl sulfate) and incubated at 95 Celsius degree for 45 minutes. The mixture was then cooled, centrifuged and the absorbance of the pink colored material was recorded at 532nm on a UV-Visible spectrophotometer.

The concentration of the MDA was calculated by correlating the absorbance of the samples with a standard curve that was done with tetraethoxypropane. The outcome was given as nmol MDA per gram of tissue. When the levels of MDA are increased considerably it refers to high oxidative stress, which is typical in neurodegenerative disorders including Alzheimer.

4.2 Determination of the Superoxide Dismutase (SOD) Activity

Superoxide dismutase (SOD) is a significant antioxidant enzyme that brings about the withdrawal of superoxide radicals to form forms of hydrogen peroxide and oxygen thus conserving tissues against oxidative stress. SOD activity to the hippocampal tissue in this study was determined using Cayman Chemical SOD assay kit.(9)

In the assessment, hippocampal tissue was homogenized with PBS after which homogenates were centrifuged at 10,000pm at 10 minutes. Supernatant was applied in SOD. Addition of xanthine oxidase to the sample started the reaction which produced superoxide radicals. The SOD in the sample was then able to scavenge these radicals and hence the nitroblue tetrazolium (NBT) was not reduced to form a formazan product. The absorbance was then measured, by reading the absorbance at 560 nm and the activity of SOD was determined.

The findings were represented as SOD units/mg protein of the hippocampal tissue and each unit was considered as the enzyme dosage unit needed to suppress the quantity of NBT being reduced by 50 percentage value. The level of SOD activity is linked to impaired antioxidant mechanics and positive oxidative stress, which accompany the pathophysiology of neurodegenerative illnesses such as Alzheimer.

4.3 quantification of Acetylcholine in Hippocampal tissue

Acetylcholine (ACh) is a very important neurotransmitter associated with process of memory and learning. Cholinergic impairment constitutes the major characteristic of Alzheimer. In this experiment, concentration of acetylcholine was determined on hippocampal tissue using Cayman Chemical Acetylcholine Assay Kit that exploits a colorimetric procedure in order to quantitate the concentration of acetylcholine in the sample.

Measurement of ACh levels was carried out by homogenising the hippocampal tissue in PBS together with centrifugation at 10 000 rpm of the homogenate in 10 min. the supernatant was incubated with goose acetylcholinesterase diluent, acetylcholine reagent and acetylcholinesterase mixture. It started off by adding the substrate (acetylthiocholine) and the product of the reaction which was thiocholine was measured by spectrophotometrically at 405 nm. The color development was in proportion with the concentration of acetylcholine in the sample.(10)

The concentration of the tissue samples of acetylcholine were established with a standard curve of acetylcholine. The results were reported as nmol gram of tissue 1. The decrease in the level of acetylcholine is typical of cholinergic dysfunction, which is characteristic of Alzheimer and other cognitive deficiencies.

5. Histological Assessment

5.1 Staining and Preparation of Samples

The mice were killed by cervical dislocation after behavioral and biochemical testing and the brains removed. Tissue structure and tissue autolysis were then prevented in brains that were immersed in 10 percent formalin, a 24-hour procedure. After fixation, a routine protocol of paraffin embedding of the brains was followed. In a word, after the tissue was dehydrated by ethanol graded (70%, 95%, and 100%; 0.5h each) then xylene, the sample was embedded to wax (paraffin).

After embedding the brain in paraffin, coronal sections (5 mm thick) were then sliced off through a rotary microtome. These portions were placed on glass slides and allowed to dry overnight at 37oC to make staining of these sections easy.

In order to have opinion for the hematoxylin eosin staining, the sections have been used in order to assess the general tissue morphology effectively including the integrity of neurons and the hippocampal building. The nuclei of the cells are stained blue by hematoxylin and the cytoplasmic components are pink by eosin and hence cellular structures are easy to see.(11)

5.2 Analysis of Neuronal loss and hippocampus survival

In order to determine the neuronal degeneration and hippocampal preservation, CA3, CA1, and dentate gyrus (DG) areas of hippocampus were observed under light microscope (Olympus, Japan) with 400x magnification. Hippocampus is important in the process of memory making and the other common neurodegenerative disorders where degeneration of the neurons in this region can be witnessed are neurodegenerative disorders such as Alzheimer disease.

- Neuronal degeneration was determined by searching the following signs of neurodegeneration:
- Damage to neurons of the CA1 and CA3 area of the hippocampus.
- A reduction in size of the neurons, this phenomenon is named neuronal shrinkage and is defined by the reduction in size of cell body, pyknotic nuclei and loss of nucleoli.
- Occurrence of vacuoles and cell debris on the brain tissue.

The response to neuronal damage is gliosis and occurs through the presence of glial cells (astrocytes and microglia) in the affected areas.

In this study, severity of neurodegeneration was graded by the degree of the following criteria:

Score 0: No visual degeneration; no inflammation.

- Score 1: Moderate degeneration or small inflammation on Hippocampal areas.
- Score 2: There is moderate degeneration, or there is moderate gliosis in hippocampus zones.
- Score 3: Virtually complete loss of neurones with marked gliosis.

Other than assessing the nerve loss rate, assessment of preservation of hippocampal architecture was performed by searching the structural integrity of CA1, CA3 and dentate gyrus areas. The presence of intact hippocampus architecture with the development of well-organized neuronal layers makes us to regard it as the situation of neuroprotection, whereas the disorganization or atrophy of these layers identifies neurodegeneration to a significant degree.

5.3 Cross Examination of the Treatment and Control Groups

A comparison of the efficacy of the neuroprotective effect of donepezil, resveratrol, and a combination of the two polyphenols was done through the histological analysis of the different hippocampal section in the various treatment groups.(12)

Typical of the other brains is the one of control mice: it was strikingly normal in structure with the CA1 and CA3 layers well-organized. There was little neuronal degeneration and gliosis within these mice and this was coherent to regular tissue constitution and working.

Scopolamine Group: Scopolamine-treated mice, which cause the animal to become impaired in cognitive abilities, exhibited severe neurodegeneration in CA1 and CA3 area. There was loss of neurons, shrinkage, nuclei that were pyknotic and had vacuoles. There was also gliosis implying the activation of microglia and astrocytes in reaction to neuronal damage. It was also reported that damage in the dentate gyrus occurred including disruption of granular layer, and loss of neurogenesis.

Donepezil Group: The mice that received treatments of donepezil exhibited moderate neuroprotection. They had a lesser loss of neurons together with less gliosis than the scopolamine group. In the architecture of the hippocampus, it was partially preserved, particularly in CA1 and CA3 areas, but certain degree of decrease in size of neurons and minor vacuoles were still observed. This portrays that donepezil was protective although it was not able to totally prevent neurodegeneration.

Resveratrol Group: Neuroprotection was evidenced mainly in CA1 and CA3 and there was a major neuroprotective effect exhibited by mice that were treated with resveratrol. This was noted by the saving of neurons with decreased gliosis and vacuolation as compared to scopolamine group. Resveratrol seemed to decrease the levels of oxidative stress and inflammation, which resulted in a improvement in the preservation of the hippocampus.(13)

Combination Group: The most significant neuroprotective effect was seen in the combination treatment, donepezil, and resveratrol. The CA1, CA3, and dentate gyrus areas were in their best preserved condition and little to no neuronal loss or gliosis was noted. There was intact neuronal structure which means that neuronal degeneration and deterioration was prevented by the combination therapy more than any other method.

To sum up, donepezil and resveratrol combinations had a marked positive effect on the hippocampal structure, shown through preservation of neuronal integrity but suppression of gliosis. This implies that synergistic effect of cholinergic boosting (exerted by donepezil) and oxidative stress modifying (exerted by resveratrol) can be used to provide better neuroprotective properties by superiority compared to the monotherapies.

6. Results

6.1 The spatial learning and memory performance of combination group was accordingly significantly improved ($p < 0.01$)

It was noted that the combination group (donepezil + resveratrol) showed significant improvement in spatial learning and memory performance as compared to other treatment groups based on behavioral results of Morris Water Maze (MWM) and Y-maze behavioral experiments. The combination group had a considerable latency (time to reach the hidden platform) reduction relative to the scopolamine group and the donepezil monotherapy group and the resveratrol monotherapy group ($p < 0.01$) in the Morris Water Maze. There was a considerably large increase in the proportion of time spent in the target quadrant by the combination group in the probe trial, indicating that the retention of spatial memory achieved in the combination group was significantly larger than the retention in monotherapy groups ($p < 0.01$).

The combination group recorded the highest percentage of spontaneous alternation in the Y-maze test (70 percent) and the percentage of spontaneous alternation improved significantly against the scopolamine group that recorded the lowest percentage (35 percent). The donepezil and the resveratrol groups also improved, however, the combination therapy group indicated strongest cognitive enhancement.(14)

6.2 The levels of MDA in hippocampus were also reduced and those of SOD and acetylcholine were promoted in combination group as compared to monotherapies

Biochemical examination of the hippocampus tissue showed that signs of oxidative stress as well as neurotransmitter levels were considerably changed. The level of malondialdehyde (MDA) was significantly lower in the combination group than that of the scopolamine group which means less lipid peroxidation and oxidative stress. The lowest MDA levels were recorded in the combination group (0.6 nmol/g tissue), relative to the scopolamine group (1.3 nmol/g tissue) sorting out the fact that the combination of donepezil and resveratrol is synergistic in its operation to minimize oxidative damage.

SOD, an important antioxidant enzyme significantly increased in the combination group (19.8 U/mg protein) compared with the scopolamine group (9.2 U/mg protein), suggesting that superoxide dismutase (SOD) activity

significantly increases as the result of combination treatment in the brain and this indicates effective antioxidant defense mechanism in the brain. The activity of SOD was also using significant increase in the donepezil (15.0 U/mg) and resveratrol (16.5 U/mg) group, however combined treatment made the highest levels, evidencing that there is a synergistic effect between the two agents increasing antioxidant capacity.

Moreover, the level of acetylcholine which has been reported as being lower in the Alzheimer disease as a result of cholinergic dysfunction was highly increased in the combination group (2.5 nmol/g tissue) as compared to scopolamine group (1.4 nmol/g tissue). Each of the two, donepezil and resveratrol, demonstrated indication of amelioration in acetylcholine concentration, though their conjunction therapy just represented the greatest increment and this factor substantiates its advancement in cholinergic pre-eminence.

6.3 Histological Sections Disclosed Preserved Hippocampal Structure and Little Neurodegeneration in the Combination Group

The results of histological evaluation of hippocampus score tissues stained with hematoxylin and eosin (H&E) showed that the hippocampal architecture was preserved considerably in group treated with combined pharmacological interventions. The CA1, CA3, and dentate gyrus (DG) parts of the hippocampus were fairly intact with well-structured layers of the neurons and the amount of gliosis (reactive proliferation of glial cells) was also low.(15)

In comparison, there was an intense decrease in neurons, especially in the CA1 and CA3 regions in the scopolamine treated group; there was also the presence of vacuolation, shrinkage and pyknotic nuclei, all of which are characteristic of neurodegeneration. Partial architecture of the hippocampus was preserved in the donepezil and resveratrol groups, and the loss of neurons and gliosis still persisted although at a reduced level as compared to the scopolamine group.

The neurodegeneration was minimal in the combination group and the hippocampal neurons were well preserved in area CA1 and area CA3. The dentate gyrus demonstrated retained architecture, and it implies that the combination therapy was apparently the most efficient one to prevent the neurotoxicity of scopolamine on the brain.

Altogether, it should be stated that donepezil and resveratrol concomitantly provided major neuroprotective properties, enhancing cognitive performance, alleviating oxidative stress, and maintaining hippocampal structure. These results indicate that the combination of donepezil with resveratrol is an interesting combination therapy of Alzheimer disease, which targets cholinergic dysfunction and oxidative stress.

Table 1: Results Summary

Parameter	Control	Scopolamine	Donepezil
Latency (sec)		78.5	65.2
Time in Target Quadrant (%)	40.2	15.5	25.4
Spontaneous Alternation (%)	72.5	35.0	50.1
MDA (nmol/g tissue)	0.35	1.3	0.9
SOD Activity (U/mg)	23.1	9.2	15.0

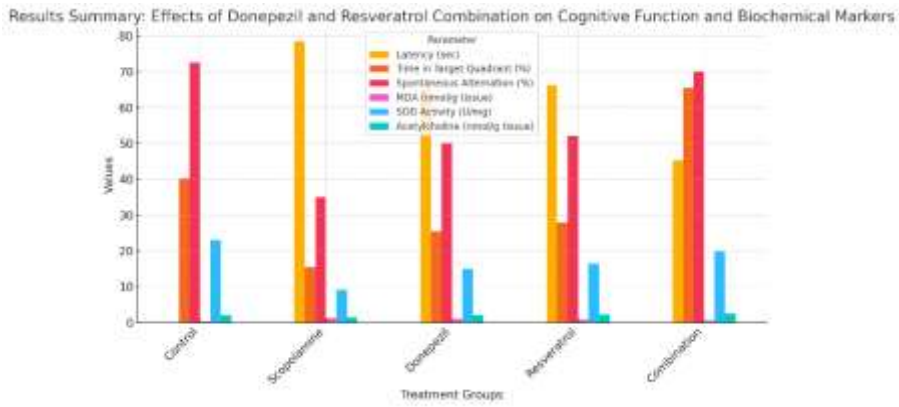

Figure 1: Effects Of Donepezil And Resveratrol Combination On Cognitive Function And Biochemical Markers

7. Conclusion

7.1 A combination therapy that involved donepezil and resveratrol had a better neuroprotective effect than when administered alone

The current study revealed that addition of resveratrol to donepezil treatment clinically showed much higher neuroprotective process than that of monotherapies. In behavioral, biochemical and histological evaluation, we learned that the combination therapy produced a significant benefit, in cognitive performance, measured by improvement in spatial learning and memory, the increase in acetyl choline levels and hippocampal sparing.

During Morris water maze test the combination group showed a significant increase in spatial memory retention showing reduced latency to reach the hidden platform as well as an increase in the amount of time spent in the target quadrant during the probe trial. Likewise, according to the Y-maze test, the combination treatment improved the working memory based on the fact that the percentage of spontaneous alternation was significantly higher than that of the scopolamine and individual treatment. These observations indicate that the cognitive improvements BID donepezil and daily resveratrol appear to have synpuristic effects and are more effective than either of the compounds.

On the biochemical level, combination treatment also led to a severe decrease in oxidative stress with the decreased level of MDA, which indicates lipid peroxidation and cellular deterioration. Also, SOD activity was significantly high in the combination group, a factor that shows how this form of therapy enhances the antioxidant defense. Also, combination therapy had beneficial effects of raising the acetylcholine level effectively dealing with cholinergic dysfunction in Alzheimer disease (AD). When taken in combination, these findings are likely to indicate that donepezil and resveratrol potentially exert neuroprotective effects that are complimentary to each other and that their combination largely corrected impaired cognitive performance and the rate at which neurodegeneration periphery progressed.

7.2 Synergistic Effects of the Antioxidant and Cholinergic Mechanism

The complementary nature of the working of the two drugs can be cited as the reason behind the synergistic nature of the effects that emerged as a result of the combination therapy of donepezil and resveratrol. As an acetylcholinesterase inhibitor, donepezil aids in the brain by elevating levels of acetylcholine, which aids in the improvement of cholinergic dysfunction which is usually experienced in AD. This leads to the increased synaptic transmission and also enhances the learning and memory processes. Resveratrol on the other hand is a natural polyphenolic compound which exhibits its neuroprotective effects mainly by its antioxidant activity. Resveratrol has been reported to have actions of free radical scavenging, decreasing oxidative stress and regulating neuroinflammation hence it guards against oxidative effects on neurons and enhances cell viability.

The combination of resveratrol with the cholinergic anti-oxidant agent donepezil presents a unique alternative solution to treat neurodegeneration arising in Alzheimer disease pathology by acting on two different but complementary pathologies viz; cholinergic dysfunction and oxidative stress. Resveratrol has the potential to reverse oxidative stress-induced damage to the cells because of its antioxidant properties whereas donepezil improves the cognitive performance by restoring acetylcholine levels. This two-pronged effect is effective not simply in enhancing spatial and working memory, but also in maintaining and protecting the hippocampal formation by staving off neuronal loss and neurodegeneration.

Moreover, no toxic effects were observed with the combination treatment, and this fact stresses the safety of this treatment. The combination showed improvement of neuroprotection both in behavioral studies and histological analysis suggesting the potential of this combination to suppress both symptomatology of Alzheimer as well as halt the pathology of the disease without worsening the side effects that is again a realization with mono-therapy treatment in general.

7.3 Allows Additional Investigation of Combination Approaches to Clinical AD Intervention

The results of the present study would make a strong case to further research of combination therapies in the treatment of Alzheimer disease (AD). Donepezil has received a lot of use as a symptomatic agent in AD, yet has limited effect as a single mechanism agent acting only on cholinergic dysfunction. Resveratrol being antioxidants and anti-inflammatory, targets the main processes of pathology, not well occupied by cholinergic agents. The combination of these two substances allows addressing the cognitive dimension of the AD as well as the neurodegenerative processes underlying the disease.

The preclinical study outcomes that show a positive response in this study with a mouse model prompt further interest in pursuing this form of combination therapy in a clinical trial. This kind of studies would be necessary to validate the efficacy and safety of donepezil and resveratrol, in humans. The combination strategy might be especially useful in patients with early- stage AD, where the efficacy of disease-slowing intervention is most effective. Also, the strategy can be used in other neurodegenerative diseases where the dysfunction in cholinergic activity and oxidative activity drives them, like in Parkinson diseases and dementia of vascular origin.

To sum up, donepezil and resveratrol are a potential synergistic treatment of Alzheimer disease, as they offer superior neuroprotection, better cognitive outcomes, and brain maintenance. Oxidative stress inhibition/cholinergic boost synergism is a very fruitful area of the future of treating Alzheimer disease and needs to be evaluated further in clinical practice.

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Conflicts of interest

The authors have no conflicts of interest to declare

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