Am J Neurodegener Dis 2021;10(2):13-27
www.AJND.us /ISSN:2165-591X/AJND0125946

Original Article

Therapeutic potential of *Allium Sativum* against the $A\beta_{(1-40)}$-induced oxidative stress and mitochondrial dysfunction in the *Wistar* rats

Neetu Saini[1], Monika Kadian[1], Alka khera[2], Aanchal Aggarwal[3], Anil Kumar[1]

[1]Division of Pharmacology, UIPS, Panjab University, Chandigarh 160014, India; [2]Postgraduate Institute of Medical Education and Research, Chandigarh 160012, India; [3]National Agri Food Biotechnology Institute, Mohali 140308, Punjab, India

Received November 9, 2020; Accepted March 10, 2021; Epub April 15, 2021; Published April 30, 2021

Abstract: From the early stages of any neurodegenerative-disease mitochondrial functionality has been mortally extricated, though the exact timeline of these events is still unclear, it is likely to represent a progressive neurons-decline and cognitive-functions. Hence strategies suggested by herbal extract to restore mitochondrial functions may be a remedial approach to chronic neurodegenerative disorder like Alzheimer's disease (AD). This research was designed to evaluate if $A\beta_{1-40}$ induced oxidative stress and mitochondrial dysfunction could be inhibited by *Allium Sativum* (AS) supplementation. AD was induced by a single intra-hippocampal injection of $A\beta_{1-40}$ (5 µg/4 µl), while herbal supplementation was given orally (100, 250, 500 mg/kg body weight, daily) for 3 weeks. Morris water maze was used to assess cognitive function shows deficits in $A\beta_{1-40}$ treated animals, there is no significant alteration in locomotor function as examined by actophotometer. This was accompanied by enhancement in oxidative stress indicating by accentuated ROS and protein carbonyl levels. Concomitantly, decrease in activity of antioxidant enzymes was observed in diseased animals; as expressed by reduced superoxide-dismutase and catalase activity, as well as reduction in GSH levels and impaired mitochondrial functions. Medium dose of AS has been found effective in restoring the memory impairment along with antioxidant levels but high dose is more efficient as observed in the $A\beta_{1-40}$ treated rats. High dose of AS, on the other hand significantly ameliorates the mitochondrial-dysfunction in comparison to medium dose. Taken together, the findings reveal that AS reverses $A\beta_{1-40}$ induced brain alteration, it could be an efficient clinical mitigation action against AD growth.

Keywords: *Allium Sativum*, Alzheimer's disease, $A\beta_{1-40}$, mitochondrial dysfunction, oxidative stress

Introduction

Neurodegenerative diseases are progressive and multiplexed heterogeneous disorders of the nervous system characterized by the neuronal loss. There is lot of evidence that impairment in cellular energy is a common feature of neurodegenerative diseases, the most common of which is Alzheimer's disease (AD) [1]. However, the etiology of AD is still not fully understood [2]. The prominent pathological features include the deposition of the extra-neuronal senile plaques consisting primarily of amyloid beta protein (Aβ), intra-neuronal neurofibrillary tangles in the brain synaptic failure and neuronal decline [3, 4]. These pathological hallmarks might be triggering the mitochondrial disturbance.

Clinical and pathological studies have revealed a nexus between neuronal loss and the mitochondrial dysfunctions which further leads to memory loss in AD [5]. In addition, studies have implicated the constitutive and infrequent role of mitochondria in synaptic activity [6]. It is generally recognized that mitochondria are critical to maintain neuronal strengthen via their functions in maintaining calcium homeostasis, providing energy, and regulating the production of reactive oxygen species (ROS), as well as formation of intermediates or final products of several essential molecules [8, 9]. Possibly, mitochondrial dysfunctions impair synaptic transmission at pathological states. Indeed, the consequently occurrence of oxidative stress and faulty mitochondrial functions may incite onset of AD [10]. To treat the AD,

there are very limited studies aimed at mitochondrial dysfunction and focusing on reversing oxidative stress and cell death pathways. An alternative treatment approach provides innovative research efforts aimed at improving mitochondrial and bioenergetic functions.

It is widely believed that central administration of Aβ induces the some pathological events such as immunoreactivity [12], excessive free radical generation and oxidative damage that can be positively correlated with the extent of cognitive impairment [13]. In this study, $Aβ_{1-40}$ has been used to introduce AD in rat model. Numerous study has been reported that oligomeric Aβ cluster which is relatively soluble and diffusible, are more able to exert a toxic effect on the neuronal plasma membrane together with synapses [14]. Thus, $Aβ_{1-40}$ induced cognitive dysfunction is a relevant and more accepted model for AD in human [15]. Hence this research focused on antioxidant therapy, including oxidative stress and mitochondrial dysfunctions. Antioxidant strategies concentrate on endogenous antioxidant upregulation or the delivery of exogenous antioxidant [16].

Herbal medicines by using the plants extracts containing biologically active pharmacological compounds have shown potential intervention between neuropathological events [17]. Several herbal extracts have been under research for their ability to reduce inflammation and pathological conditions including polyphenols, flavonoids and carotenoids etc. [18]. One of the most traditional plants in herbal medicine is *Allium Sativum* L. (Liliaceae) commonly known as garlic, which is used to boost the immunity and revitalization of metabolism to defend against various disease such as cancer, diabetes and heart disease [19, 20]. In addition, studies revealed that several organosulfur compounds exert in AS, including alliin, diallyl sulfide and diallyl trisulfide were able to diminished cells in glioblastoma [20]. Some clinical studies have been proposed its therapeutic effects to liquidate brain cancer cells in patients [21].

Therefore, the present study was planned to examine the therapeutic and preventive effects of AS in the Aβ-induced AD model with an alternative treatment strategy aimed at improving mitochondrial and bioenergetic function. The improvement of cell bioenergetics in preclinical models may produce widespread positive results that could benefit people with AD.

Materials and methods

Chemicals

The chemicals used in this analysis were all of analytical grade and derived from different chemicals companies. $Aβ_{1-40}$ was procured from Sigma Chemical Co. (St. Louis, MO, USA), Merck (Mumbai, India). Hydro-alcoholic bulb extract of AS containing Allin (1.5-2.5% confirmed by HPLC) was a gift from Phyto Life Sciences Private limited (Ahmedabad, Gujarat, India).

Ethics statement

The protocols used for this study have been approved by the Committee for Institutional Animal Ethics (PU/45/99/CPCSEA/IAEC/2018/216) and were in accordance with the regulations on fair use and treatment of laboratory animals.

Treatment methods

A total 42 adult male wistar rats weighing between 200 and 250 g were procured from Panjab University's Central Animal House facility, Chandigarh, India. For a week the animals had been allow to acclimatize to the local vivarium. The animals' body weights were recorded daily and separated randomly into the following seven groups with each group having six animals:

i. Control Group: Animals were administered 1 ml of distilled water.

ii. Sham Group: Animals were intrahippocampal (i.h.) infused with vehicle.

iii. Aβ induced Group: Animals were intrahippocampal (i.h.) infused with 5 μg $Aβ_{(1-40)}$ [22] dissolved in aCSF further incubate in PBS as procedure are given below. Solutions (4 μl) were injected using a Hamilton microsyringe positioned in the injection cannula.

iv. *Per se* group (High Dose 500 mg/Kg): *Allium Sativum* treatment was given via oral administration at a dosage of 500 mg/kg body wt. 3 weeks per day. Dose will be standardized first.

v. AS (100) (Allium Sativum Low Dose 100 mg/Kg): *AS* was administered orally to the $A\beta_{(1-40)}$ treated group at the dose of 100 mg/kg body wt. 3 weeks per day.

vi. AS (250) (Allium Sativum Medium Dose 250 mg/Kg): *AS* was administered orally to the $A\beta_{(1-40)}$ treated group at the dose of 250 mg/kg body wt. 3 weeks per day.

vii. AS (500) (Allium Sativum High Dose 500 mg/Kg): *AS* was administered orally to the $A\beta_{(1-40)}$ treated group at the dose of 500/kg body wt. 3 weeks per day.

$A\beta_{(1-40)}$ adminstration

Before injection, $A\beta_{1-40}$ (Sigma-Aldrich, USA) was incubated in phosphate buffer saline at 37°C for 7 days to allow peptide assembly associated with toxicity. The incubated $A\beta_{1-40}$ solutions generally contain both fibril-like structures and different-sized oligomers. Rats that has been deeply anesthetized with pentobarbital (55 mg/kg, i.p.) and mounted in a stereotactic frame was injected with the incubated $A\beta_{1-40}$ solution into each side of the hippocampus with a standardized volume of $A\beta_{1-40}$ using the following stereotaxic coordinates: 3.6 mm posterior to bregma, 2.4 mm left/right to midline, and 2.8 mm ventral to bregma. After surgery, each rat was injected with penicillin (100,000 U) in the hind quarter muscle to prevent infection.

Neurobehavioral studies

Animals were testing for cognitive and motor functions using morris water maze and actophotometer test respectively.

Spatial memory was assessed according to the method described by Morris [23]. The animals were trained to escape from drowning by swimming to a hidden escape platform by using visuospatial navigation cues. The platform remained fixed at the same position on each day but the starting point of the animals varied in the each trial. The acquisition test is a measure of spatial reference memory, whereas, retrieval test is considered as a measure of strength of spatial memory.

Acquisition test: A water tank (140 cm in diameter and 55 cm in high) was filled with water up to 25 cm from the top. A platform (11 cm diameter) was placed at the center of one of the quadrant and submerged about 1 cm below the surface of water. All animals were subjected to four trials (acquisition) from day 16 to 19. During each trial, the animals were placed in one of the four randomly selected quadrants facing the wall of the tank. On day 20^{th}, time spent and distance covered to reach the platform was recorded using ANY-maze tracking software (Stoelting Co., Wood Dale, IL, USA).

Retrieval test: After 24 h of acquisition, the platform was removed and animals were allowed to swim for 180 s. The memory was assessed by measuring the time spent in the target quadrant (that earlier had the hidden platform) along with latency and number of entries to the target quadrant.

The spontaneous locomotor activity was monitored by actophotometer, which is a square chamber connected to photoelectric cells and the activity of the animals was recorded for 5 min. by light beams breaks passing through the chamber [24].

Sample preparation

After 21 days of respective treatments, animals in various groups were fasted overnight and sacrificed by cervical dislocation under light ether anesthesia. Their brains were removed and rinsed in ice-cold isotonic saline. The brains were dissected to separate cerebral cortex and hippocampus. Differential centrifugation method was used to isolate mitochondria with slight modifications [25]. 10% (w/v) tissue homogenates were prepared in ice-cold isolation buffer with 1.0 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) (215 mM mannitol, 75 mM sucrose, 0.1% fat-free BSA, 20 mM HEPES, 1.0 mM EGTA, and pH is adjusted to 7.2 with KOH). All procedures were performed on ice throughout the protocol. The homogenates were centrifuged at 1000 × g for 8 min at 4°C. Pellets containing nucleus were discarded and the supernatants, so called post nuclear supernatant (PNS) were collected in fresh eppendorf and then centrifuged at 10,000 × g for 10 min at 4°C. Here we recover the supernatants, so called post mitochondrial supernatant (PMS) for biochemical estimations. Pellets, thus, obtained (containing mitochondria of interest) were resuspended in 0.01% digitonin in isolation buffer and kept for incubation for 10 min on ice. After incubation with 0.01% digitonin, mitochondrial suspensions were centrifuged at 10,000 × g for 10 min at 4°C. The resulting supernatants were discard-

ed and the pellets obtained were resuspended with 2.0 ml of isolation buffer without EGTA and again spun at 12,300 × g for 10 min at 4°C. The final mitochondrial pellets were resuspended in 200 ul of isolation buffer without EGTA and immediately used for mitochondrial complexes studies.

Biochemical analysis for oxidative stress and antioxidant enzymes

The following biochemical assays were conducted immediately in the PMS to evaluate the antioxidant propensity of AS supplementation against the Aβ administration.

Reactive oxygen species (ROS): ROS that includes hydroxyl, peroxyl and other reactive oxygen radicals were quantitated by using 2', 7'-Dichlorofluorescin diacetate (DCFH-DA) as described by LeBel et al. [26]. The fluorescence emission was determined at 530 nm after excitation at 488 nm. Results were revealed as pmoles DCF/min/mg protein.

Protein carbonyls: The carbonyl content of proteins was calculated by the method of Levine et al. [27] with certain modification. In the reaction yellow colored complex was obtained, was read at 370 nm. The protein carbonyls content was expressed as nmoles carbonyl/mg protein using extinction coefficient (22.0 mM^{-1} cm^{-1}).

Superoxide dismutase (SOD): The activity of superoxide dismutase (SOD) was examined according to the method of Kono [28]. This enzyme catalyzed the liquidity of superoxide radicals to molecular oxygen and hydrogen peroxide to provide the cellular defense. The blue colored complex in the reactions (enzymatic and non-enzymatic) was followed at 560 nm for 3 min. The enzyme activity were expressed as units/mg protein (one unit of enzyme is defined as the amount of enzyme inhibiting the rate of reaction by 50%).

Catalase: Catalase (CAT) activity in the brain samples was estimated using the method described by Aebi [29]. It plays a vital role in cellular self-defense through break down of the hydrogen peroxide into water and oxygen. The down fall in the absorbance was measured at 240 nm and results expressed as µmols of H_2O_2 decomposed/min/mg protein, using molar extinction coefficient of H_2O_2 (71 M^{-1} cm^{-1}).

Glutathione: Glutathione (GSH) content was quantified by the method of Roberts and Francetic [30]. Results were expressed as nmols GSH/mg protein.

Protein content: The concentration of proteins was calculated according to the Lowry et al. method [31]. The absorbance was read at 750 nm. The values were expressed as mg protein/ml of sample using bovine serum albumin standard curve.

Estimation of mitochondrial respiratory chain enzymes

Complex I (NADH dehydrogenase): NADH dehydrogenase' activity was measured in mitochondrial preparation by the method described by King and Howard [33]. NADH dehydrogenase catalyzed reduction of cytochrome c, was measured spectrophotometrically by increase in absorbance at 550 nm for 3 min. Results were expressed as nmoles NADH oxidized/min/mg protein by using molar extinction coefficient of reduced cytochrome c at 550 nm (19.6 mM^{-1} cm^{-1}).

Complex II (succinate dehydrogenase): Succinate dehydrogenase (SDH) was assayed according to the method of King et al. [34]. SDH catalyzes the oxidation of succinate to fumarate in the presence of potassium ferricyanide, which was measured spectrophotometrically by the decrease in absorbance at 420 nm for 3 min. Results were expressed as nmoles succinate oxidized/min/mg protein, using molar extinction coefficient of ferricyanide at 420 nm (2.08 mM^{-1} cm^{-1}).

Complex IV (cytochrome oxidase): Activity of cytochrome oxidase in brain mitochondria was assayed according to the method described by Sottocasa et al. [35]. The oxidation of cytochrome c was spectrophotometrically calculated by reduction of absorbance at 550 nm for 3 min. Results were expressed as nmoles cytochrome c oxidized/min/mg protein using molar extinction coefficient of cytochrome c at 550 nm (19.6 mM^{-1} cm^{-1}) and the results were normalized to citrate synthase activity.

MTT reduction: The reduction in MTT was used to assess the overall activity of the dehydrogenases present in the mitochondrial preparation by the method of Liu et al. [36]. To appropriate amount of sample, MTT (0.5 mg/ml)

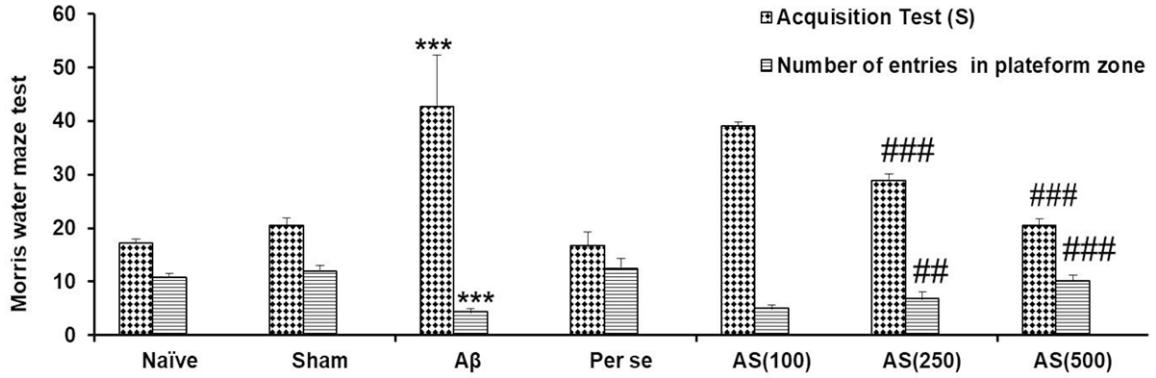

Figure 1. Effect of Allium Sativum on acquisition in $A\beta_{40}$ induced intra-hippocampal rat model of Alzheimer's disease. Values are mean ± SD; n=6; $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$ compared with sham animals. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$ compared with Aβ induced animals.

was added, mixed and incubated at 37°C for 30 min and then centrifuged to obtain blue formazan pellet. Later formazan pellet was dissolved in acidic isopropanol and the mixture was centrifuged at 2000 g for 10 min. The absorbance was measured at 595 nm. Reduction was showed as µg of formazan formed per minute mg protein.

Transmission electron microscopy: Transmission electron microscopy was done as described by Singh et al. [37]. Briefly, small pieces (1 mm × 1 mm) of brain tissues were taken and fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.3) for 12 hours at 4°C, post fixing in 1% osmium tetroxide, dehydrated in graded ethanol, cleared in propylene oxide at room temperature, and finally embedded in an EPON mixture containing Taab/812. 1 µm thick sections were cut with an ultra-microtome. Copper grids of sections were stained with alcoholic uranyl acetate and alkaline lead citrate washed gently with distilled water. Then, sections were observed under a Morgagni 268D transmission electron microscope (Fei Company, The Netherlands) at an operating voltage 80 kV.

Statistical analysis

All data are displayed as mean ± SD. Data were analyzed using one way analysis of variance (ANOVA) followed by tukey's post-hoc test for multiple pairwise comparisons between the various treated groups using Statistical Package for the Social Sciences version 20.0 for Windows (IBM, New York, NY). Statistical significance was defined as P<0.05.

Results

Effect of Allium Sativum on neurobehavioral assessment in $A\beta_{40}$ treated rats

During the acquisition test (learning), escape latency was found to be significantly increased (P<0.001) in Aβ treated animals in comparison to sham groups (**Figure 1**). However, AS supplemented Aβ treated group shows a significant reduction (P<0.001) in total time latency in comparison to Aβ treated group. Number of entries in platform zone was markedly decreased (P<0.001) in Aβ treated group which was significantly enhanced by AS treatment. AS at 100 mg/kg was showing nonsignificant results. AS supplementation at 250 mg and 500 mg perkg body weight were significantly (P< 0.01, P<0.001 respectively) able to restore Aβ induced deficits in behavior. High dose of AS is showing the more prominent effect on animal learning.

AS supplementation lacked any effect on the motor function in control and Aβ treated animals (**Figure 2**). There was no change observed in locomotor activity within the groups.

Effect of Allium Sativum on oxidative stress and antioxidant defense system in $A\beta_{40}$ treated rats

ROS levels in cortex (P<0.001) and hippocampus (P<0.001) regions, show significantly enhancement in Aβ induced animals in comparison to sham animals as depicted in **Figure 3** suggesting the oxidative insult involved in the development of AD. AS (100 mg/kg) was significantly reduced the ROS level in cortex (P<

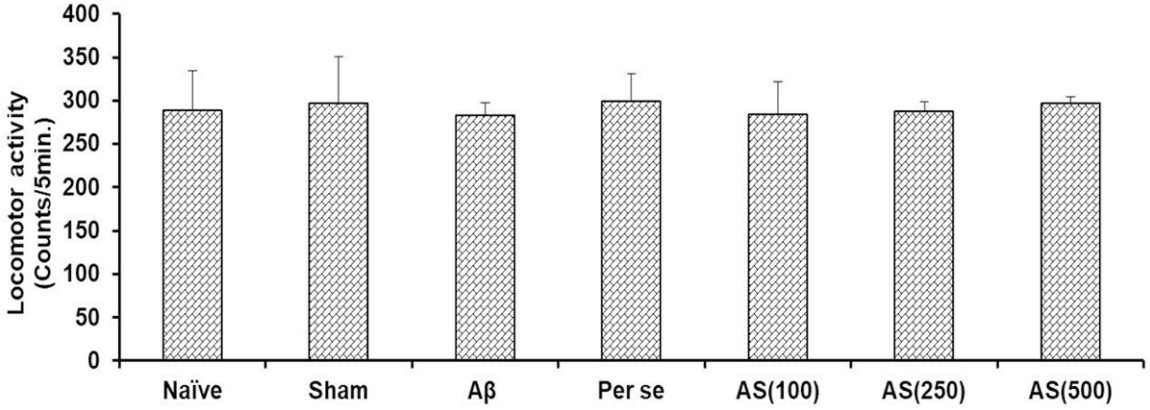

Figure 2. Effect of Allium Sativum on locomotor activity in Aβ induced intra-hippocampal rat model of Alzheimer's disease. Values are mean ± SD; n=6.

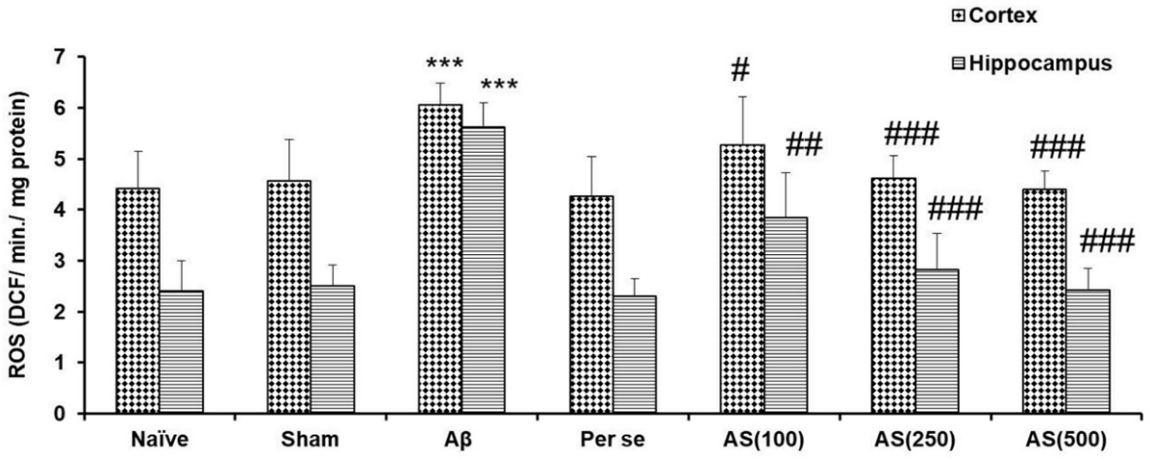

Figure 3. Effect of Allium Sativum on ROS levels in Aβ induced intra-hippocampal rat model of Alzheimer's disease. Values are mean ± SD; n=5; *P<0.05, **P<0.01 and ***P<0.001 compared with sham animals. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with Aβ induced animals.

0.05) and hippocampus (P<0.01) regions as compared to the Aβ induced animals. Treatment with AS at doses 250 mg and 500 mg per kg body weight tended to bring ROS significantly (P<0.001) towards normal values in both regions. While sham and per se group did not exhibit any significant alterations in ROS level during the experiment. However, medium and high doses were showing more effective results, but higher dose is more close to normal value.

A significant raised in protein carbonyl levels in cerebral cortex (P<0.001) and hippocampus (P<0.001) following Aβ administration was observed as compared to sham control (**Figure 4**) designating the protein oxidation that could be advanced by ROS. Sham and per se groups were ineffective during the experiment. Administration of AS (100 mg) showed the significant effect (P<0.05) in cortex region but failed to maintain any effect in hippocampus region. 250 mg/kg AS dose was found significantly (P<0.01) effective in both regions. Whereas AS at dose of 500 mg per kg body weight in Aβ induced animals significantly (P<0.001) decreased the raised protein carbonyl levels in both brain regions near to control level. Higher dose was found to be more effective among three doses.

SOD levels (in cortex P<0.01 and hippocampus P<0.001) shows significantly enhancement in Aβ induced animals in comparison to sham animals (**Figure 5**). A non-significant effect was showed at low dose in cortex region while in hippocampus region it shows the significant (P<0.05) results. AS with medium dose (250

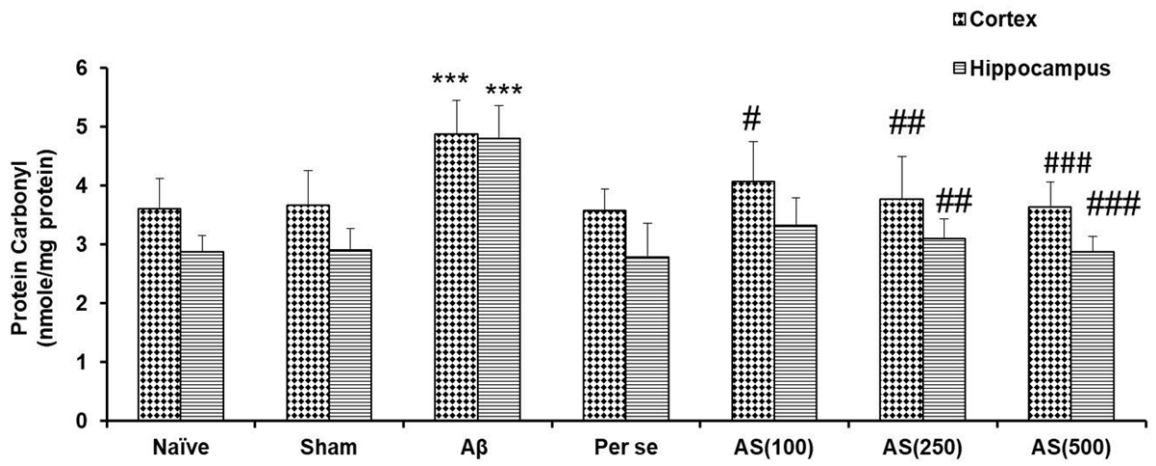

Figure 4. Effect of Allium Sativum on Protein carbonyl levels in Aβ induced intra-hippocampal rat model of Alzheimer's disease. Values are mean ± SD; n=5; *P<0.05, **P<0.01 and ***P<0.001 compared with sham animals. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with Aβ induced animals.

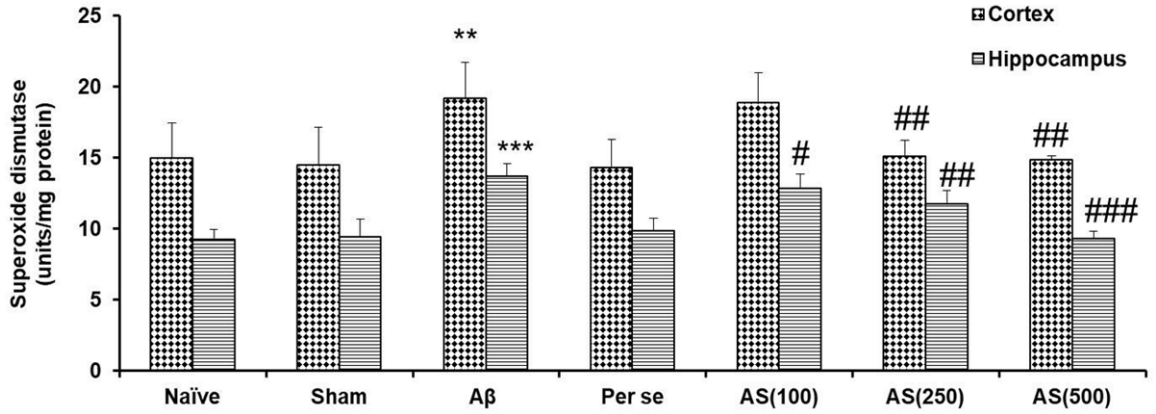

Figure 5. Effect of Allium Sativum on SOD in Aβ induced intra-hippocampal rat model of Alzheimer's disease. Values are mean ± SD; n=5; *P<0.05, **P<0.01 and ***P<0.001 compared with sham animals. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with Aβ induced animals.

mg/Kg body weight) was found significant (P<0.01) effective in both regions when compared to the Aβ induced animals. High dose (500 mg/kg body weight) was able to significantly reduced increased SOD levels in cortex (P<0.01) and hippocampus (P<0.001). Sham and per se groups didn't show any change in comparison to control.

Catalase levels shows significantly enhancement (P<0.001) in Aβ induced animals in comparison to sham animals. A similar pattern of results in cortex and hippocampus regions, was found in catalase activity as observed in the activity of SOD showing high dose (500 mg/kg body weight) of supplement was more effective on catalase levels up to the control level (**Figure 6**). There is no change found in the Sham and per se groups.

Figure 7 depicted considerable enhancement (P<0.001) in glutathione contents in brain regions as compared to sham animals following Aβ administration. Sham and per se group were showing non-significant results in comparison to control. AS supplementation at dose of 100 mg/kg body weight is showing least significant (P<0.05) results in Aβ treated animals to lower down the glutathione level. Supplementation at 250 mg/kg body weight was showing potential (P<0.01) to reduce increased level of Glutathione. Like previous results showing in this study, high dose (500 mg/kg body weight) is more effective (P<

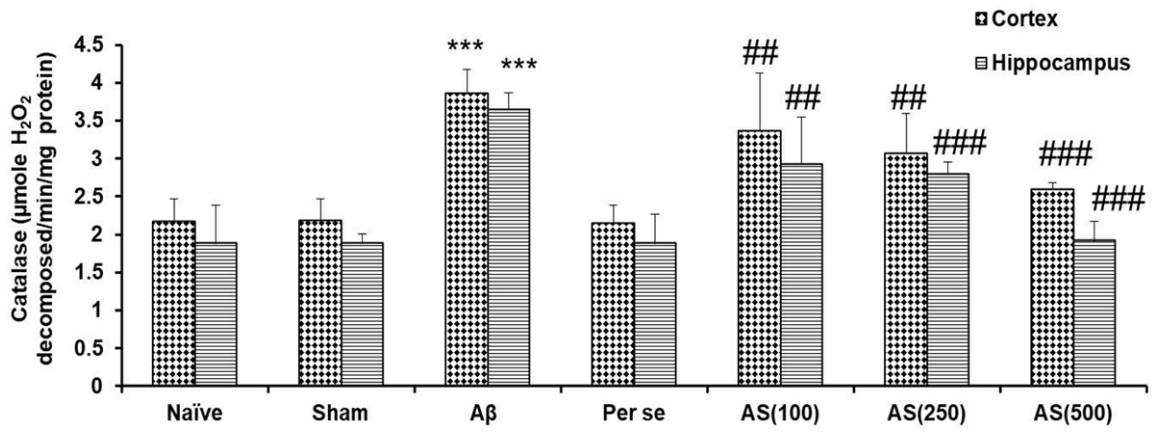

Figure 6. Effect of Allium Sativum on catalase in Aβ induced intra-hippocampal rat model of Alzheimer's disease. Values are mean ± SD; n=5; *P<0.05, **P<0.01 and ***P<0.001 compared with sham animals. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with Aβ induced animals.

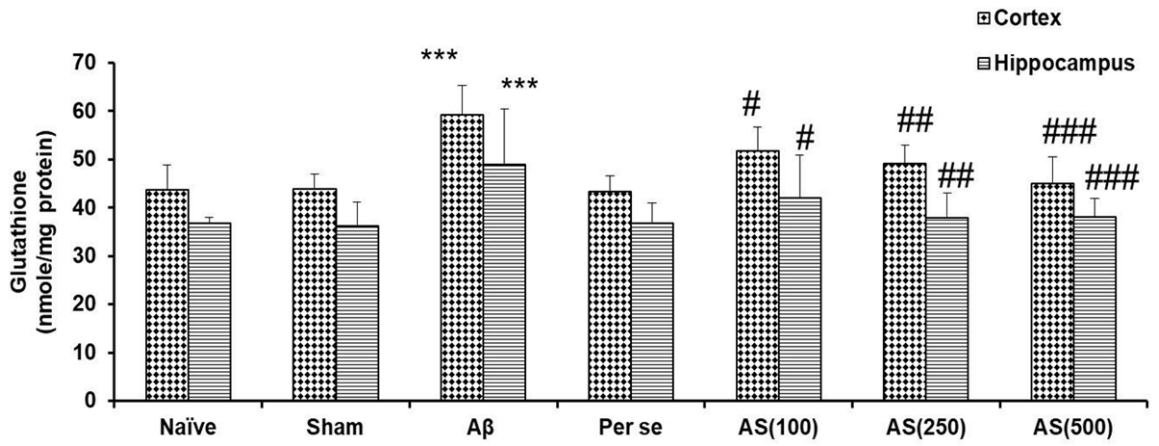

Figure 7. Effect of Allium Sativum on Glutathione content in Aβ induced intra-hippocampal rat model of Alzheimer's disease. Values are mean ± SD; n=5; *P<0.05, **P<0.01 and ***P<0.001 compared with sham animals. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with Aβ induced animals.

0.001) in case of reduction to Glutathione levels up to the control.

Effect of Allium Sativum on mitochondrial respiratory enzymes in $A\beta_{40}$ treated rats

The activity of NADH dehydrogenase (complex-1) in both regions (cortex P<0.001 and hippocampus P<0.05) was found to be significantly inhibited in Aβ administered animals in comparison to sham controls (**Figure 8**). The AS supplementations (100 mg, 250 mg and 500 mg per kg body weight) was significantly restored the activity of complex-I in cortex region. While in hippocampus region only high dose of AS was showing significant (P<0.05) result. Similar to the previous results, the high dose of AS is giving the good results among the three doses. Sham and per se groups were showing the result as control.

Succinate dehydrogenase (complex-II) showed (**Figure 9**) reduced activities (P<0.001) in the cortex and hippocampus of Aβ treated animals compared to sham animals. Sham and per se groups were showing the result as control. Low dose of AS (100 mg/kg body weight) was found not effective as compared to Aβ treated animals. Medium dose showed significant (P<0.01) result to increase the activity of succinate dehydrogenase. Analogous with previous findings high dose is showing more efficiency (P<0.001) to restore the complex-II activity in both the regions.

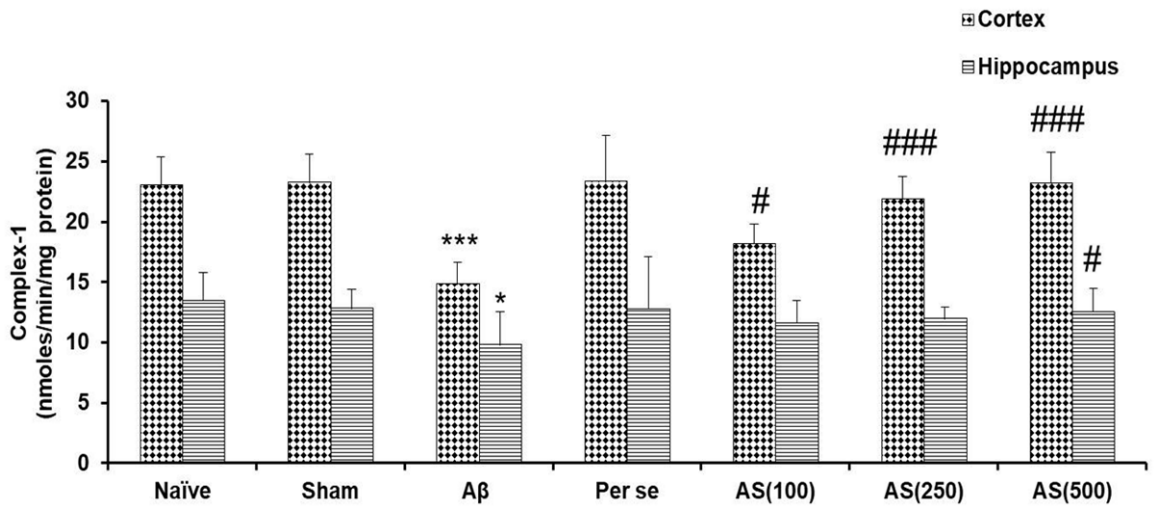

Figure 8. Effect of Allium Sativum on mitochondrial complex-I in Aβ induced intra-hippocampal rat model of Alzheimer's disease. Values are mean ± SD; n=5; *P<0.05, **P<0.01 and ***P<0.001 compared with sham animals. #P<0.05, ##P<0.01 and ###P<0.001 compared with Aβ induced animals.

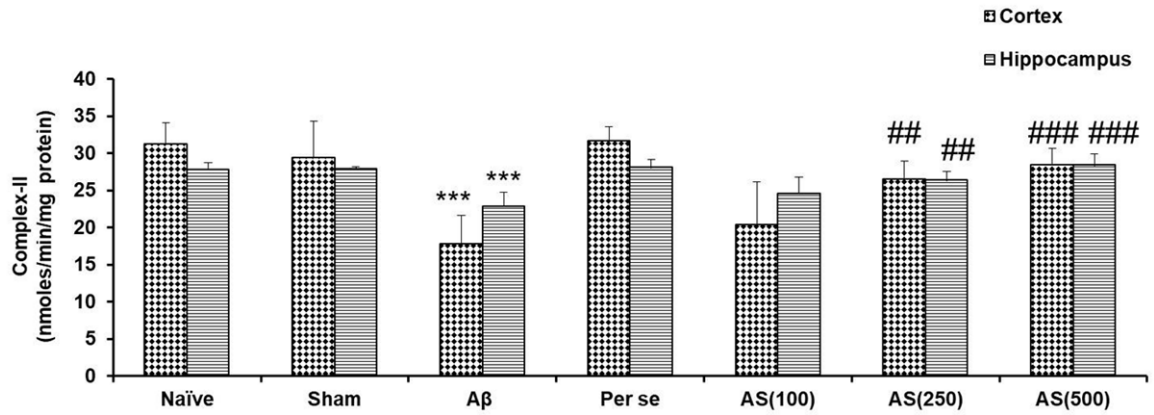

Figure 9. Effect of Allium Sativum on mitochondrial complex-II in Aβ induced intra-hippocampal rat model of Alzheimer's disease. Values are mean ± SD; n=5; *P<0.05, **P<0.01 and ***P<0.001 compared with sham animals. #P<0.05, ##P<0.01 and ###P<0.001 compared with Aβ induced animals.

A significant inhibition (P<0.001) was found in the cytochrome oxidase (complex-IV) following Aβ administration as compared to the sham control (**Figure 10**). AS (100 mg/kg body weight) supplementation was found non-effective in comparison to Aβ treated animals. Supplementations at medium and high doses of AS were significantly (P<0.001) able to maintain the mitochondrial activity in the brain regions. However 250 mg/kg AS dose failed to maintained activity in hippocampus region. The results are in agreement with the above finding; thereby showing the efficacy of AS (high dose) to ameliorate the complex-IV activity in cortex and hippocampus more efficiently. Sham and per se group were ineffective during the experiment.

MTT levels show the mitochondrial viability, were found to be significant reduced (P< 0.001) in Aβ induced animals in comparison to sham animals. AS at low dose (100 mg/kg body weight) shows no improvement in the reduction (**Figure 11**). AS (medium dose) was able to increase (P<0.001) reduced MTT levels in cortex region only. High dose (500 mg/kg body weight) of supplement is more effective (P<0.001) to ascend the reduced MTT levels up to the control level in both cortex and hippocampus regions. Sham and per se group were ineffective during the experiment.

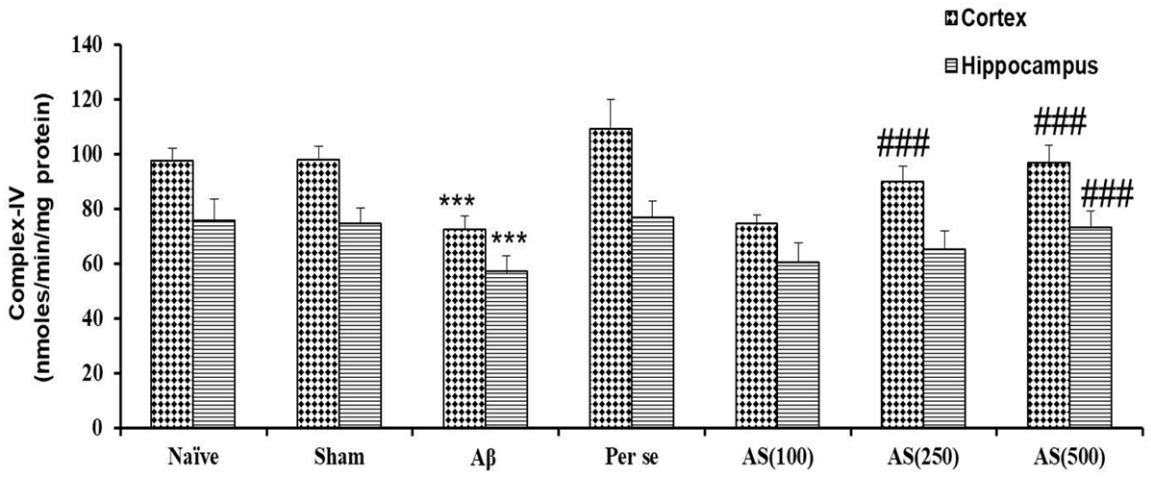

Figure 10. Effect of Allium Sativum on mitochondrial complex-IV in Aβ induced intra-hippocampal rat model of Alzheimer's disease. Values are mean ± SD; n=5; *P<0.05, **P<0.01 and ***P<0.001 compared with sham animals. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with Aβ induced animals.

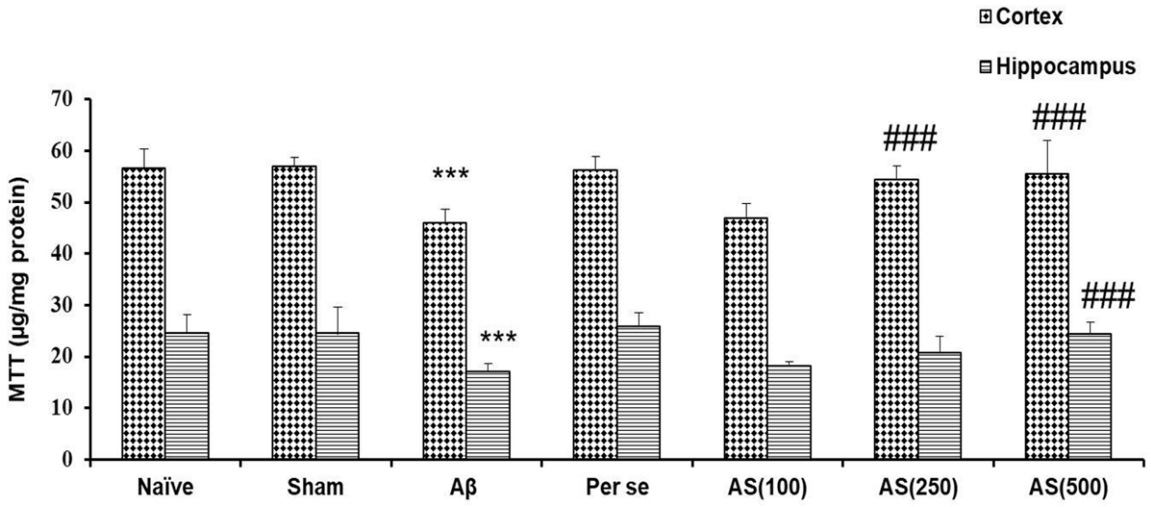

Figure 11. Effect of Allium Sativum on mitochondrial viability in Aβ induced intra-hippocampal rat model of Alzheimer's disease. Values are mean ± SD; n=5; *P<0.05, **P<0.01 and ***P<0.001 compared with sham animals. $^{\#}$P<0.05, $^{\#\#}$P<0.01 and $^{\#\#\#}$P<0.001 compared with Aβ induced animals.

Assessment of mitochondrial ultrastructure alteration in Aβ induced model examined by transmission electron microscopy

Electron microscopic study was conducted to examine the Aβ induced structural changes in the appearance of mitochondria in the brain (**Figure 12**). In the control and Sham brains, mitochondria had normal morphology and membrane integrity. The cristae revealed no indications of swelling or any injury, with homogeneous pattern of the matrix and no density changes. In the brain following Aβ administration, the mitochondria showed defaced in terms of disappearance of outer mitochondrial membrane, reduced matrix structure accompanied by increased size, swelling with poorly defined cristae and decreased matrix density suggesting gradual depletion of the energy. However, AS supplementation (high dose) is able to significantly normalize the mitochondrial appearance, indicating the protective effect of AS, which could be attributed to the role of allicin in quenching free radicals and preventing mitochondrial damage effectively.

Discussion

The present study was designed to find out the neuroprotective potential of AS supplementa-

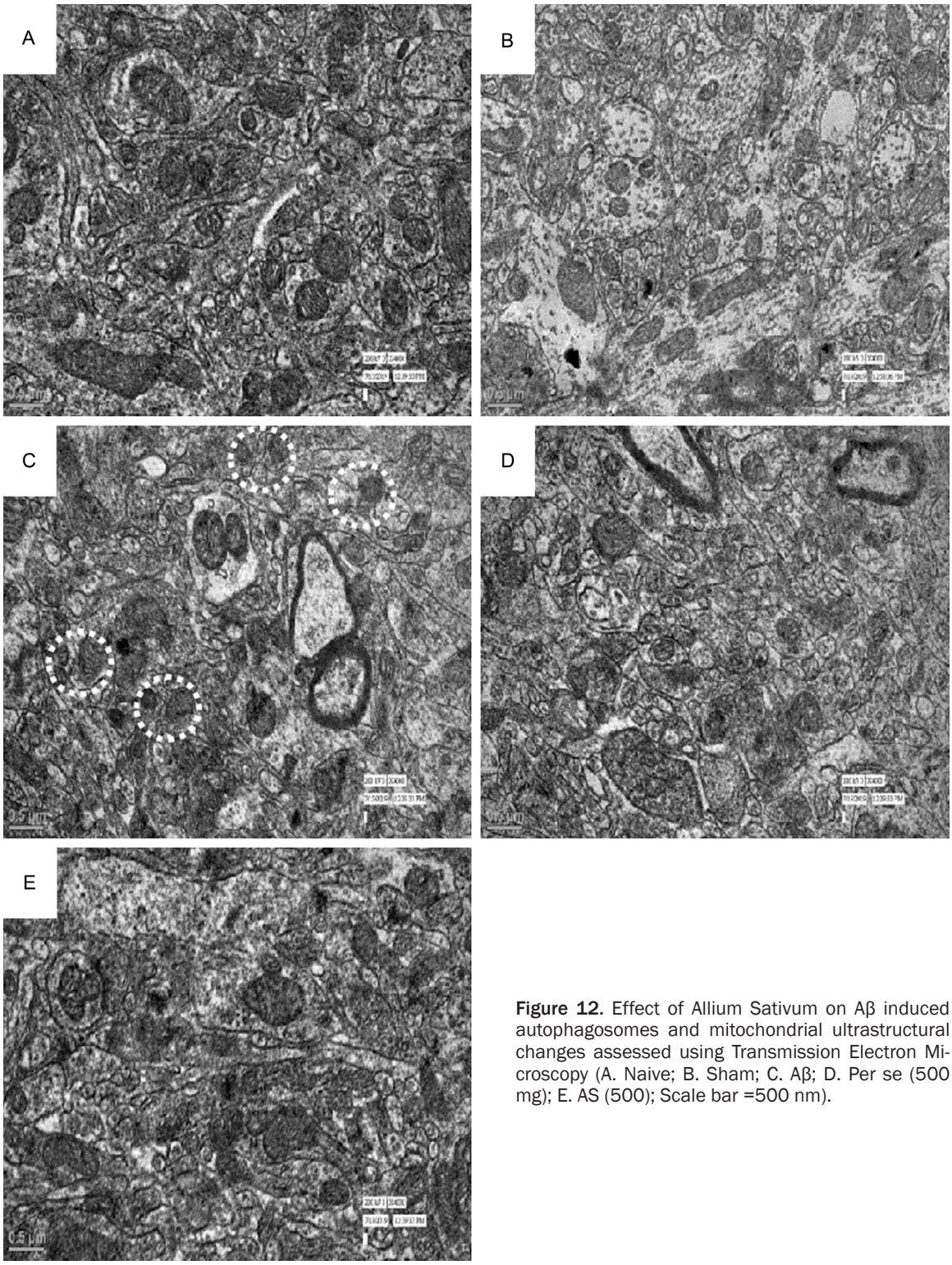

Figure 12. Effect of Allium Sativum on Aβ induced autophagosomes and mitochondrial ultrastructural changes assessed using Transmission Electron Microscopy (A. Naive; B. Sham; C. Aβ; D. Per se (500 mg); E. AS (500); Scale bar =500 nm).

tion on oxidative stress, mitochondrial dysfunction and memory impairment following Aβ administration in wistar rat. $A\beta_{1\text{-}40}$ infusions in rats are considered as a good experimental model for the Alzheimer's disease [38]. A significant decline was observed in the cognitive outcomes as measured by morris water maze test in this study. Earlier report has been sh-

owed that soluble Aβ extracted from AD brain potently inhibited long term potential (LTP) and decreased the dendritic spine formation in the rodent hippocampus [39, 40]. Another study in agreement with these findings showed soluble Aβ dimers insults their impact by perturbing glutamatergic synaptic transmission via metabotropic glutamate receptors and NMDA receptors which is essential for the induction of LTP and spine formations respectively [40, 41]. Shankar and coworkers [42] revealed in his study that insoluble amyloid plaque did not impair LTP unless solubilized into Aβ dimers, suggesting that sequester Aβ dimers are synaptotoxic. Cognitive decline has been shown to be accompanied by increase in oxidative stress in terms of ROS and protein carbonyl levels in $A\beta_{1\text{-}40}$ infused rats. It has been reported that ROS further trigger the protein oxidation via conversion amino groups of protein to carbonyl moieties, thereby increased production of the protein carbonyls [45]. Sandhir et al. [46, 47] have also reported increased level of ROS and protein carbonyl deteriorate the memory functions in colchicine induced AD.

On contrast, the results in this study indicate that AS supplementations at high dose (500 mg/kg) could be the more effective in restoration the cognitive impairment in Aβ treated animals.

ROS and protein carbonyl levels were found to be markedly decreased with medium and high dose of AS near to controls. Effect of AS (500 mg/kg) is more prominent as compared to others. These observations are in agreement with the study of Thomson et al. (2010), they treated STZ-induced diabetic rats with raw garlic extract to improving the antioxidant level. There was no significant change observed on motor functions following Aβ administration as assessed by actophotometer. The intake of AS was able to improve the behavioral abnormality through modulation of endogenous oxidative stress markers in mice brain [48, 49]. The mechanism of action behind the behavior and spatial memory enhancing effects of AS is uncertain, as its multiple active compounds such as diallyl disulphide (DAD), diallyl trisulfide (DAT), and allyl tetrasulfide (AT) have multifunctional action, making it pharmacologically complex [50]. However, the antioxidant properties of AS have been well documented which include metal ion reduction, free radical scavenging, and inhibition in lipid peroxidation as well as enhancement of antioxidant enzymes due to it containing allicin which have already been reported in literature [49, 50]. Hazzaa et al. [51] have shown in their study that DAD and DAT reduced Ca^{2+} influxes to neurons by arresting the L-type calcium channel resulting reduce excitotoxicity and neuronal death. The activities of antioxidant enzymes SOD and catalase and GSH contents were found to be significantly inhibited in the diseased group [52]. These observed declines in first defense line from oxidative stress are consistent with earlier studies and is known to be main culprit that induce the neuron demise resulting brain dysfunctioning [52, 53]. On the other hand, again high dose of AS supplementation was more able to significantly maintain the antioxidant levels in cortex and hippocampus regions in Aβ treated animals. These findings are in agreement with earlier reports indicate that AS supplementation succored animals from neurotoxicity and oxidative lesion by scavenging free radicals and stimulating antioxidant enzymes [54]. The enzymatic activities of the mitochondrial complexes-I, II, IV, and mitochondrial viability were found to be significantly reduced after administration of $A\beta_{1\text{-}40}$. Previous study has been shown engagement of free radicals such as $ONOO^-$ and NO in AD brain, directly inhibiting complexes II-III and IV activity [55]. Thus, the increased production of ROS could account, in part for the damage to mitochondrial respiratory chain enzymes observed in the present study. AD is documented for intracellular oxidative stress, lipid peroxidation, membrane instability and swelling, resulting excessive Ca^{2+} influx and mitochondrial membrane depolarization [56]. Thus, the exaggerated formation of mitochondrial oxidants may inhibit mitochondrial complexes observed in the $A\beta_{1\text{-}40}$ group. Interestingly, among the different range of AS-supplementation high dose (500 mg/kg) was found to be more significant to ameliorate $A\beta_{1\text{-}40}$ induced inhibition of mitochondrial respiratory chain complexes; decreased the swelling and structural integrity to diseased animals. These findings are in line with an earlier study, in which AS was shown to maintain mitochondrial integrity and function by bioenergetics [54, 57].

In conclusion, the results of the present study reveal that AS may prevent cognitive decline in $A\beta_{1\text{-}40}$ administered AD model through free

radical scavenging activity. AS could be maintained the thiol status and upregulate the antioxidant enzymes, resultant improvement of mitochondrial function. The outcome from this study may at least clarify in part the nootropic intervention of AS. Therefore, AS may have therapeutic potential for treatment of dementia involving oxidative stress mediated mitochondrial dysfunctions.

Acknowledgements

The financial assistance provided from Indian Council of Medical Research (ICMR) New Delhi, India, under ICMR-RA grant (No. 3/1/90/Neuro/2018-NCD-I) and ICMR-SRF grant (No. 45/35/2018-PHA/BMS), is strong accounted.

Disclosure of conflict of interest

None.

Address correspondence to: Anil Kumar, Division of Pharmacology, University Institute of Pharmaceutical Sciences, Panjab University, Chandigarh 160014, India. Tel: +91-172 253 4106; Fax: +91-172 2543101; E-mail: kumaruips@yahoo.com

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