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Research Article

Chrysin, a Natural Flavonoid Attenuates Cognitive Dysfunction and Neuronal Loss Associated with Amyloid β (25-35) - Induced Oxidative Stress: An Experimental Model of Alzheimer's Disease

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ABSTRACT

Alzheimer's disease (AD) is the most common form of dementia in elderly. AD is characterized with loss of hippocampal and cortical neurons resulting in memory and cognitive impairment. In our study intracerebroventricular injection of $A\beta_{25-35}$ induced the neurodegeneration in rats. Administration of $A\beta_{25-35}$ (10µg/rat) resulted in poor memory retention in behavioral tasks but does not show significant difference in motor deficit, which was assessed using Rota-rod test. $A\beta_{25-35}$ administration also caused marked oxidative stress as denoted by significant increase in the levels of thiobarbituric acid reactive substance (TBARS) and acetylcholine esterase (AChE), decrease in the activities of glutathione peroxidase (GPx), glutathione reductase (GR), reduced glutathione (GSH), superoxide dismutase (SOD), catalase (CAT) and Vitamin C (Vit C). Administration of Chrysin (CN) at doses 25 and 50 mg/kg body weight restored memory impairment observed in the $A\beta_{25-35}$ induced rats. Treatment with CN could mitigate oxidative damage, as evident by decreased levels of TBARS, AChE and restoration in the activities of antioxidant enzymes. Histopathological sections of the hippocampal region showed the extent of neuronal loss in $A\beta_{25-35}$ induced rats and its restoration upon administration of CN. From the results we suggest that CN might have neuroprotective effect in alleviating $A\beta_{25-35}$ induced oxidative stress in rats.

Keywords: Alzheimer's disease, $A\beta_{25-35}$, Chrysin, Antioxidant status, Neuroprotection.

INTRODUCTION

Alzheimer's disease (AD) is an age-related neurodegenerative disorder with progressive cognitive dysfunction and characterized by presence of senile plaques in the brain.[1] The pathological cleavage of amyloid precursor protein (APP) is responsible for the accumulation of amyloid-β (Aβ) proteins, aggregating into fibrillar oligomers and generating amyloid deposits that, in turn, form the senile plaques.[2,3]

Oxidative stress has been implicated as a major cause of neurotoxicity in a number of neurodegenerative disorders including AD. Oxidative damage in AD may be a direct result of Aβ. Markers of oxidative DNA damage, including mitochondrial DNA damage, have been localized to amyloid plaque affected areas in the AD brain[4] and also the generation of lipid peroxidation products and the lipo-peroxidation of membranes are noted in amyloid plaques.[5]

Intracerebroventricular (ICV) injection of Aβ causes prolonged impairment of brain glucose and energy metabolism by desensitization of neuronal insulin receptors.[6] ICV injection of Aβ has been used for the animal model of AD.[7,8]

$A\beta_{25-35}$ is considered the shorter toxic fragment exerting neurotoxic effects similar to those produced by $A\beta_{1-40/42}$, such as learning and memory impairment, neuronal apoptosis, cholinergic dysfunction, and oxidative stress.[9,10]

The flavonoids are a diverse family of chemicals commonly found in fruits and vegetables. Flavonoids are plant polyphenolic compounds, which comprise several classes including flavonols, flavanones and flavanols. Chrysin (5,7-dihydroxyflavone) is a natural flavonoid extracted from many plants, honey and propolis.[11,12] Several studies have shown that chrysin (CN) has multiple biological activities, such as anti-inflammation, anti-oxidation and vasorelaxation effects.[13,14] CN effectively inhibits expression of the key pro-inflammatory enzymes, including inducible nitric oxide synthase and cyclooxygenase-2.[15] Recent studies suggest that CN can protect neurons from oxidative insults and apoptosis. CN dose-dependently inhibited tunicamycin-induced neuronal cell death in SH-SY5Y cells via inhibition of mitochondrial apoptosis pathway.[16,17]

Therefore, we conclude that CN, with the anti-inflammatory and antioxidant properties, may exert beneficial effect on improving cognitive deficits in rats. In this preliminary study we hypothesized that CN may act as a neuroprotectant against Aβ induced cognitive dysfunction. To test our hypothesis, we used the $A\beta_{25-35}$ aggregate infused by ICV injection in rat model of AD, which has been commonly used in recent studies,[18,19] and evaluated the efficacy of CN, against $A\beta_{25-35}$ induced oxidative stress in rats.

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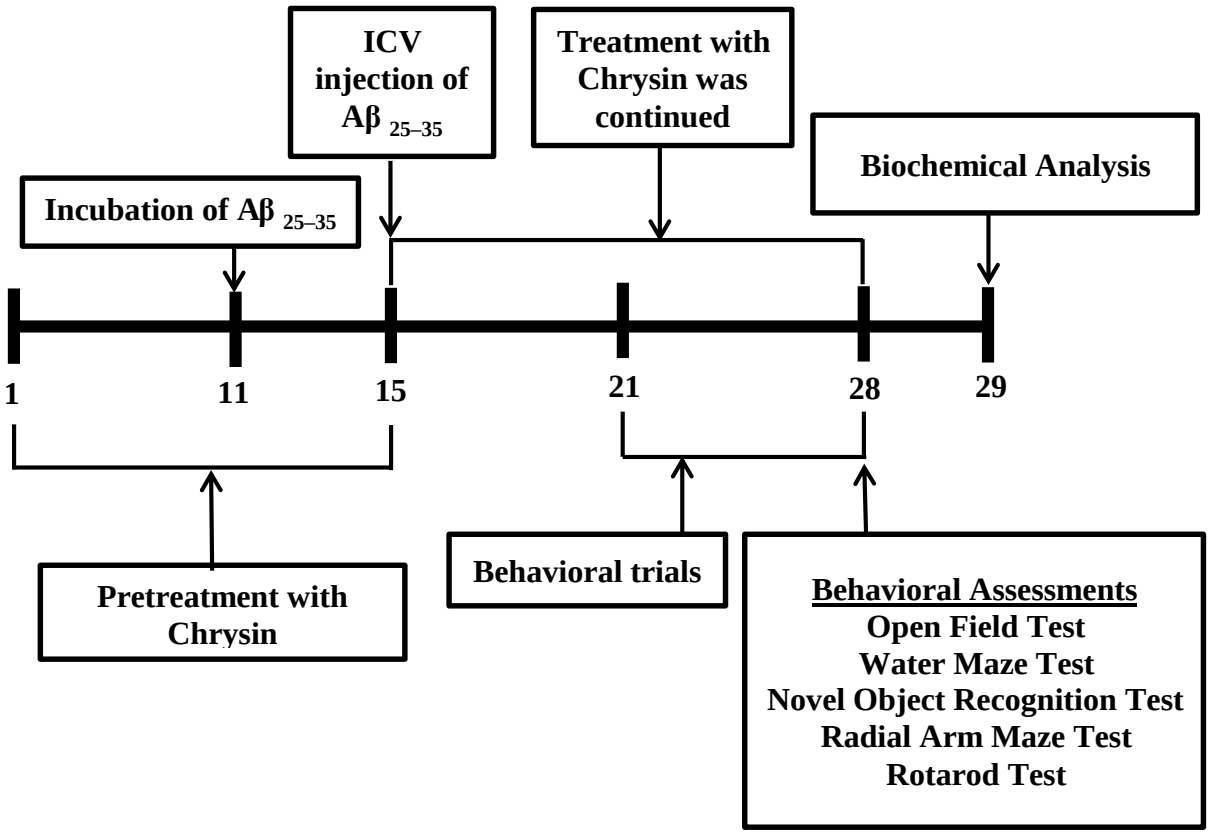

Fig. 1: Experimental design of the study

MATERIALS AND METHODS

Chemicals and Reagents

$A\beta_{25-35}$ was purchased from Sigma Aldrich. Glutathione reductase, glutathione (GSH)-reduced form, 5,5′-dithiobis-2-nitrobenzoic acid (DTNB), 1-chloro-2,4-dinitrobenzene (CDNB) were purchased from SRL. β - Nicotinamide adenine dinucleotide phosphate reduced (NADPH) was purchased from CDH. All other chemicals used were of analytical grade.

Animals

Male Wistar rats weighing 250 – 300g were obtained from Central Animal House, Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai - 113, Tamil Nadu, India. Rats were housed separately in polypropylene cages and fed standard pellet diet, kept under hygienic conditions. Rats were kept on a 12 hr light and dark cycles with free access to water *ad libitum*. All experiments and protocols described in the present study were approved by the Institutional Animal Ethics Committee (IEAC) of Dr. ALMPGIBMS, University of Madras, Taramani campus, Chennai - 113, Tamil Nadu, India.

Surgery and ICV administration of $A\beta_{25-35}$ peptide

Aggregates of $A\beta_{25-35}$ were prepared by incubating the peptide at a concentration of 2µg/µl in distilled water for 4 days at 37^{o}C prior to administration, as described by Delobette et al. to form fibril-like structures and globular amporphous aggregates.[20] For ICV injection through a Hamilton syringe, the animals were anesthetized with Ketamine (80 mg/kg i.p.) and Xylazine (10 mg/kg i.p.). They were then stereotaxically injected directly into the right lateral ventricle at coordinates (AP: -1 mm, L: ±1.5 mm, and DV: -3.5 mm) according to Paxinos and Watson.[21]

Drugs and treatments

The animals were randomly divided into five groups of 6 animals each. Group I, control group received distilled water 1 mL/kg; Group II, induced group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) [22] ; Group III, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (25 mg/kg) [23] ; Group IV, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (50 mg/kg) [23] ; Group V, drug alone group, received CN (50 mg/kg). $A\beta_{25-35}$ (10µg/rat) was administered through ICV route to all the animals in Group II, III and IV on 15th day. The CN dissolved in 2% DMSO were administered to all the animals in Group III, IV and V by oral gavage once a day for 14 days prior to $A\beta_{25-35}$ injection and continued up to 28 days. Figure 1 shows the experimental design of the study.

Post-operative care

Recovery of anesthesia took approximately 4–5 h. The rats were kept in a well-ventilated room at 25±3°C in individual cages until they gained full consciousness; they were then housed together in groups of 4 animals per cage. Food and water was kept inside the cages for the first week, allowing animals' easy access, without physical trauma due to overhead injury. Animals were then treated normally food, water and the bedding of the cages were changed twice per week as usual.

Behavioral studies

All the behavioral studies were performed at room temperature in a calm room without any outside

Table 1: Exploratory Behavior

Groups	Exploratory Behavior (number)		
	Head dippings	Rearing	Line crossings
Group I	10.2 ± 2.16	23.5 ± 2.3	35.7 ± 1.79
Group II	3.9 ± 0.4**	10.2 ± 1.72**	14.2 ± 0.87**
Group III	7.1 ± 0.89*	14.9 ± 1.05*	27.8 ± 1.13*
Group IV	9.7 ± 0.7**	22.2 ± 0.67**	34.3 ± 1.82**
Group V	10.4 ± 2.27	22.7 ± 1.53	35.3 ± 0.83

Table 2: Escape Latency

Groups	Escape Latency (Seconds)
Group I	35.33 ± 1.09
Group II	63.18 ± 1.35**
Group III	45.14 ± 1.62*
Group IV	38.19 ± 1.15**
Group V	35.24 ± 1.24

Table 3: Preference index

Groups	Preference index (%)	Recognition index (%)
Group I	52.34 ± 1.06	67.35 ± 1.32
Group II	48.67 ± 0.83	50.07 ± 1.05**
Group III	51.14 ± 1.25	58.86 ± 1.57*
Group IV	50.89 ±0.95	65.49 ± 1.13**
Group V	51.73 ± 1.12	68.04 ± 1.28

interference. All the experiments were performed between 10.00 am and 6.00 pm.

Open field test

In order to control for possible effects on locomotor activity, animals were explored twice, with a 24 h interval, to a 40cm x 50cm x 60cm open field whose brown linoleum floor was divided into 16 equal squares by white lines. In both sessions, animals were placed in the rear left square and left to explore it freely for 5 min during which time the number of line crossings, rearing and head dippings were counted.[24]

Water maze task

The Morris water maze was performed as described previously by Morris.[25] The experimental apparatus consisted of a circular water tank (diameter-100 cm; height=35 cm), containing water at 28˚C to a depth of 15 cm and rendered opaque by adding powdered milk. A platform (diameter 4.5 cm; height 14.5 cm) was submerged 0.5 cm below the water surface and placed at the midpoint of one quadrant. The platform remains fixed in the position during the training session. Each animal was subjected to four consecutive trials during which they were allowed to escape on to the hidden platform and allowed to remain there for 20 seconds. Escape latency time to locate the hidden platform in water maze was noted as an index of acquisition or learning. In case the animal was unable to locate the platform within 120 seconds, it was lifted out and placed on the platform for 20 seconds. After several trials, the test was conducted on the 14th day after the injection of Aβ peptide. On the 14th day the platform was removed and the time spent by each animal in target quadrant searching for the hidden platform was noted as an index of retrieval and measured.

Table 5: Rota-rod responses

Groups	Rota-rod (Seconds)
Group I	135.25 ± 33.04
Group II	143.15 ± 43.67 ^{NS}
Group III	130.12 ± 39.03 ^{NS}
Group IV	132.28 ± 35.43 ^{NS}
Group V	135.19 ± 31.24

Novel object recognition test

The novel object recognition test was performed 7–14 days after the $A\beta_{25–35}$ injection, according to a previous study,[26] with minor modifications. The task consisted of three sessions: habituation, training and retention. Each animal was individually habituated to the box (30 x 30 x 30 high cm), with 10 min of exploration in the absence of objects for 3 days (habituation session). During the training session, two objects were placed in the middle of the box. An animal was then placed midway at the front of the box, and total time spent exploring the two objects was recorded for 10 min. During the retention session, animals were placed back into the same box 24 h after the training session, in which one of the familiar objects used during training was replaced with a novel object. The animals were then allowed to explore freely for 5 min, and the time spent exploring each object was recorded. Throughout the experiments, the objects were used in a counterbalanced manner in terms of their physical complexity and emotional neutrality. A preference index, a ratio of the amount of time spent exploring any one of the two objects (training session) or the novel object (retention session) over the total time spent exploring both objects, was used to measure cognitive function.

Radial arm maze training

Habituation session: During RAM training the animals was food deprived to about 80% of their *ad libitum* body

Table 4: acquisition and reacquisition

Groups	Acquisition		Re-acquisition	
	No. of trials (out of 10)	Latency (in seconds)	No. of trials (out of 10)	Latency (in seconds)
Group I	8.47 ± 0.47	473.4 ± 12.53	9.3 ± 0.25	473.2 ± 12.13
Group II	8.82 ± 0.21	440.2 ± 21.22	3.2 ± 0.14**	1157.5 ± 22.73**
Group III	8.71 ± 0.32	419.3 ± 14.87	5.7 ± 0.23*	879.7 ± 17.73*
Group IV	8.84 ± 0.52	478.2 ± 23.12	6.9 ± 0.23**	568.2 ± 14.96**
Group V	8.42 ± 0.24	472.5 ± 18.84	8.9 ± 0.17	472.3 ± 16.82

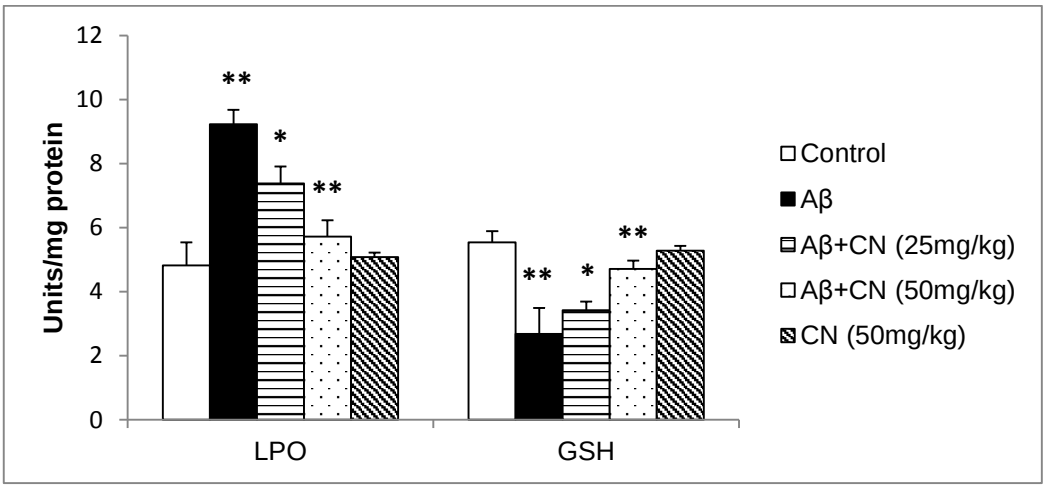

Figure 2: Graphical representation of results with LPO and GSH

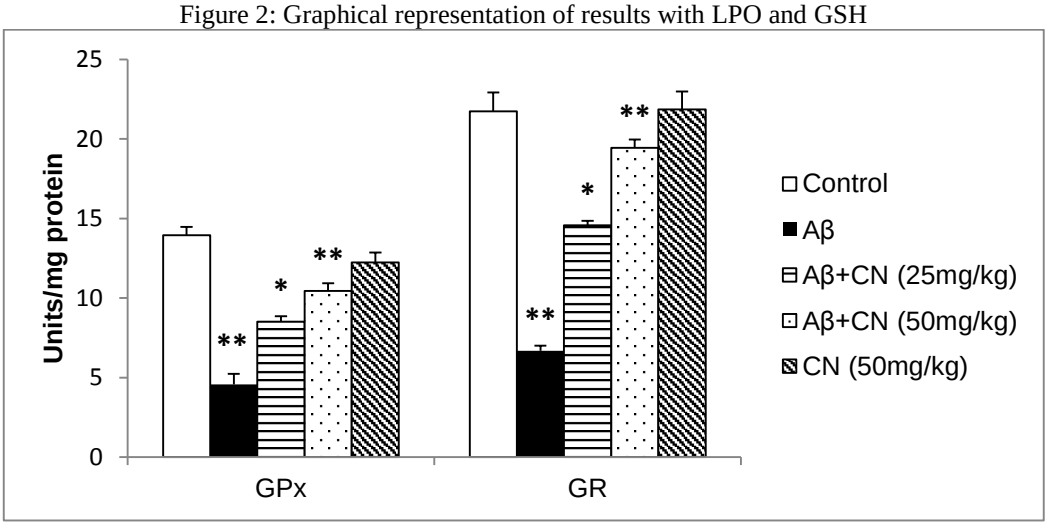

Figure 3: Graphical representation of results with with GPx and GR

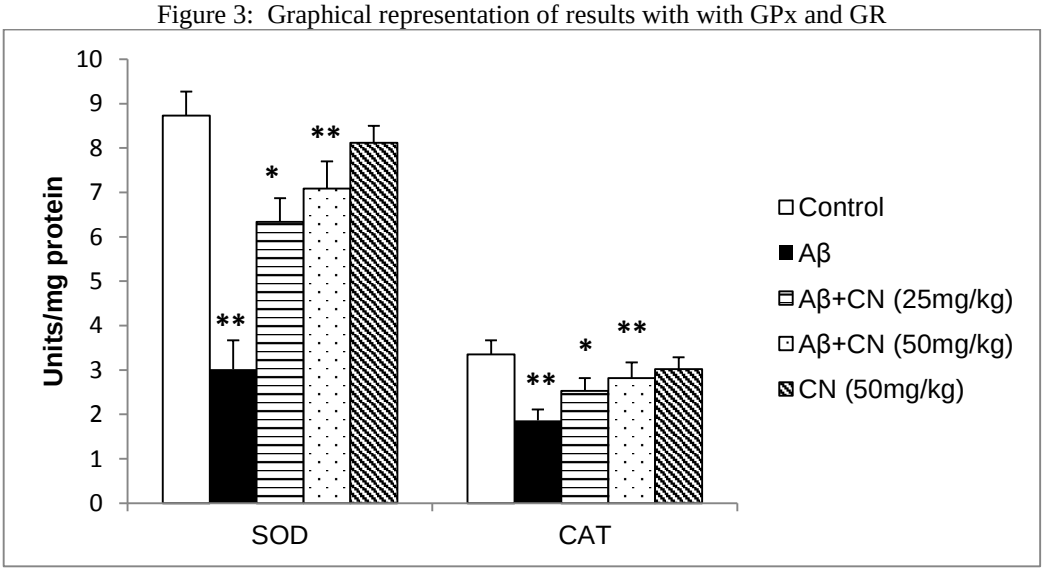

Figure 4: Graphical Representation of results with SOD and CAT

weight and trained for 5 days to run on a radial arm maze. (Brown, wooden, 60x10 cm arms) extending from an octagonal central platform. The maze was kept in the centre of a dimly lit room with many posters and objects hanging on the wall. The animals were placed in the center of the maze with all 8 arms accessible and baited. The rats were removed from the maze after visiting all the arms. Arms were re-baited only after the animal left the arm and the maze was cleaned with 50% alcohol solution between animals. Only animals reaching this criterion were trained on the memory tasks. Entry into an arm previously visited within any daily trial was scored as an error.[27,28] Following habituation session, the animals were trained for 10 trials per day on RAM task. Retention trials were performed once on the 14th day post-surgery.

Rota-rod accelerating test

The

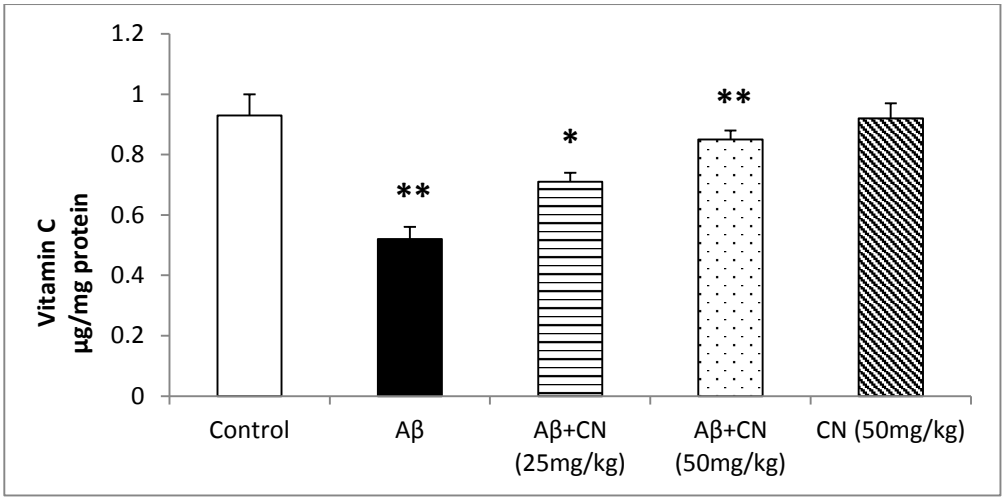

Figure 5: Graphical representation of results with Vitamin C

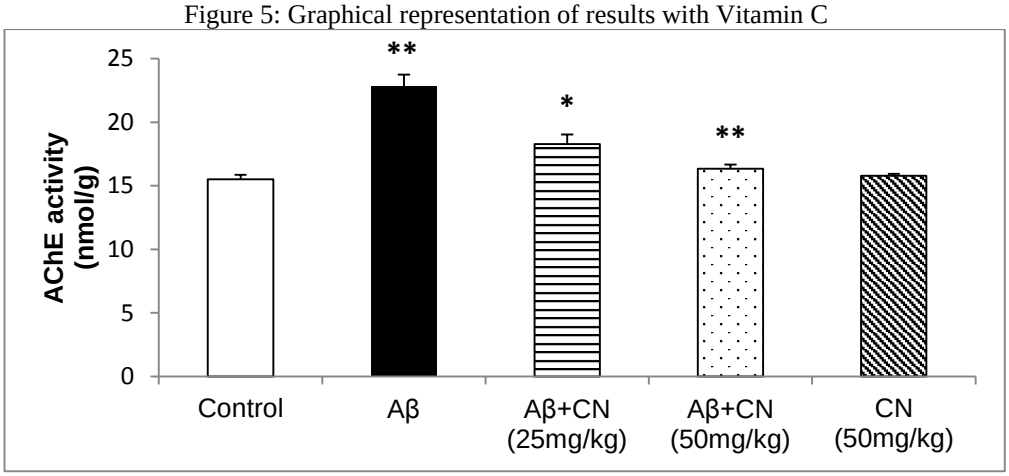

Figure 6: Graphical representation of results with with AChE activity

Rota-rod accelerating test was also performed to each animal. It was to examine the possible defects in neuromuscular coordination that might occur on the chemically treated rats.[29] Before the stereotaxic surgery, each rat was placed in a rota-rod apparatus and subjected to accelerating test. The rat was placed on the rotating rod (at the slowest speed, 4 rpm) for 2 minutes. The rats that could not hold on the rod for more than 2 minutes were excluded from the further experiments, including stereotaxic surgery and chemical treatment. For the qualified rats that were used in chemical treatment, starting from the 7th day after surgery, the rats were trained per day as described above for 2 days. At day 14 after surgery, the rotational speed of the rod was then switched to its maximum speed of 40 rpm, and the length of the time rats could grasp at the rod was measured. The test score is the average number of seconds that rats could hold onto the rod per trial. The variation in rota-rod performance among rat groups was used to evaluate the impairment of the motor coordination.[30]

Biochemical Studies

After the experimental period of 28 days, all the animals were sacrificed and their brains were removed quickly and their hippocampus were collected and rinsed with ice cold 0.9% NaCl. The hippocampus were then transferred to the ice cold 0.1 M phosphate buffer (pH 8) and homogenized.

Assay for thiobarbituric acid reactive substance (TBARS)

method of Utley et al.[31] was modified for the estimation of lipid peroxidation. Briefly, 0.2 ml homogenate was pipetted in Eppendorf tube and incubated at 37±1°C in a metabolic water bath shaker for 60 min at 120 strokes up and down; another 0.2 ml was pipetted in an Eppendorf tube and placed at 0°C incubation. After 1 h of incubation, 0.4 ml of 5% TCA and 0.4 ml of 0.67% TBA was added in both samples (i.e., 0°C and 37°C). The reaction mixture from the vial was transferred to the tube and centrifuged at 3500×g for 15 min. The supernatant was transferred to another tube and placed in a boiling water bath for 10 min. Thereafter, the test tubes were cooled and the absorbance of the color was read at 535 nm. The rate of lipid peroxidation expressed as nmol of thiobarbituric acid reactive substance formed/min/mg protein.

Assay for reduced glutathione content

Glutathione was measured according to the method of Ellman.[32] The equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.1 ml of this supernatant, 2 ml of phosphate buffer (pH 8.4), 0.5 ml of 5,5-dithiobis (2-nitrobenzoic acid) and 0.4 ml of double distilled water were added. The mixture was vortexed and the absorbance was read at 412 nm within 15 min. The concentration of reduced glutathione was expressed as µg/g tissue.

Determination of glutathione reductase activity

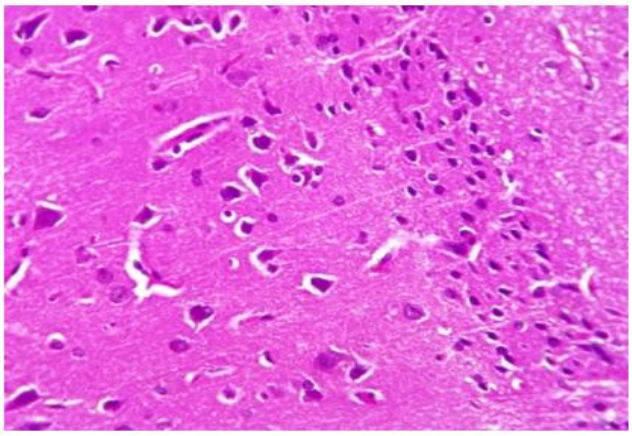

Fig. 7a: Transverse section of hippocampus of rat brain showing normal histo-architecture (H&E, 400x)

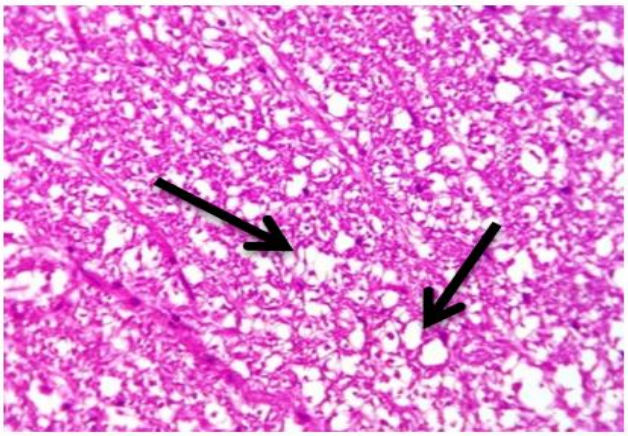

Fig.7b Transverse section of hippocampus of rat brain showing severe vacuolar degeneration of the neuronal cells (Arrows) (H&E, 400x)

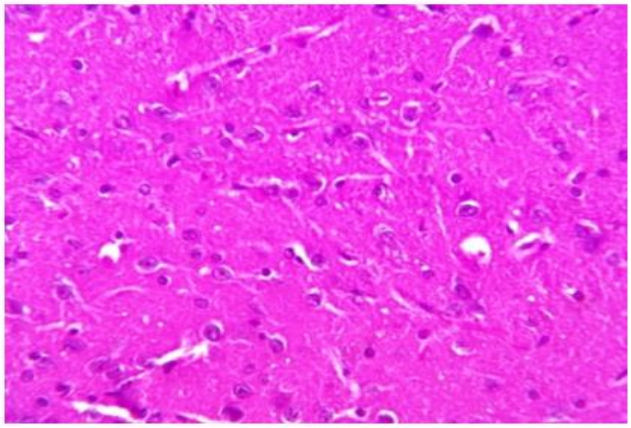

Fig. 7c: Transverse section of hippocampus of rat brain showing mild neuronal cell loss (Arrow) when treated with 25mg/kg body weight dosage of CN (H&E, 400x)

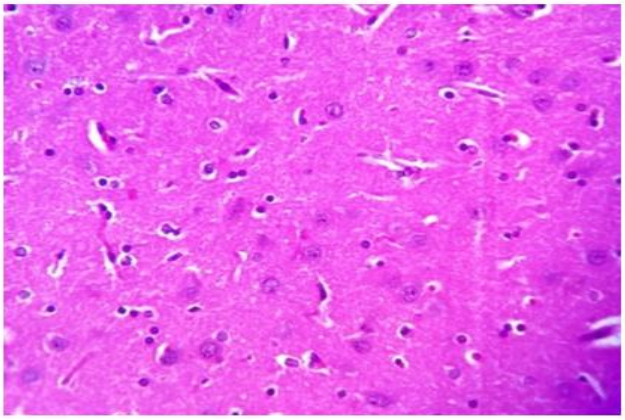

Fig. 7d: Transverse section of hippocampus of rat brain showing decreased degeneration and improved neuronal configuration which is much more significant when compared with 25mg/kg body weight (H&E, 4000x)

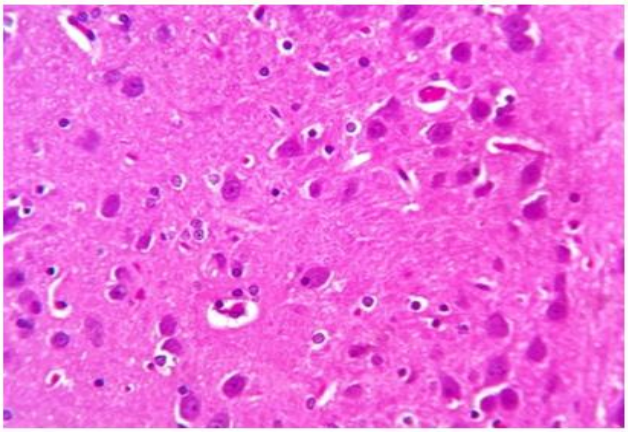

Fig. 7e: Transverse section of hippocampus of rat brain showing normal morphology which resembles that of the control (H&E, 400x)

GR activity was measured by the method of Carlberg and Mannervik.[33] The assay system consisted of 0.1 M PB (pH 7.6), 0.5 mM EDTA, 1 mM GSSH, 0.1 mM NADPH and PMS (0.1 ml) in a total volume of 2.0 ml. The enzyme activity was quantitated at room temperature by measuring the disappearance of NADPH at 340 nm and was calculated as nmol NADPH oxidized/min/mg protein.

Determination of glutathione peroxidase activity

GPx activity was measured at 37 °C by the coupled assay method of Wheeler et al.[34], in which oxidation of GSH was coupled to NADPH oxidation, catalyzed by GR. The reaction mixture consisted of 0.2 mM H_2O_2, 1 mM GSH, 1.4 unit of GR, 1.43 mM NADPH, 1 mM sodium azide, PMS (0.1 ml) and PB (0.1 M, pH 7.0) in a total volume of 2.0 ml. The disappearance of NADPH at 340 nm was recorded at room temperature. The enzyme activity was calculated as nmol NADPH oxidized/min/mg protein.

Determination of superoxide dismutase activity

Superoxide dismutase activity was measured according to the method described by Marklund and Marklund,[35] with some minor modifications. To 1 ml of homogenate 0.25 ml of ethanol and 0.15 ml of chloroform was added and kept in a mechanical shaker for 15 mins and centrifuged. To 0.5 ml of supernatant, 2.0 ml of pyrogallol was added. Changes in optical density 0,1,2,3 mins at 420 nm were read in spectrophotometer. Control tubes containing 0.5 ml of water were also treated in a similar manner against a buffer blank. The enzyme activity was expressed as units/mg protein. One unit is equivalent to the amount of SOD required to inhibit 50% of pyrogallol auto-oxidation.

Determination of catalase activity

Catalase activity (CAT) was assayed by the method of Aebi.[36] Briefly, the assay mixture consisted of 0.05 M phosphate buffer (pH 7.0), 0.019 M H_2O_2, and 0.05 ml PMS in a total volume of 3.0 ml. Changes in absorbance were recorded at 240 nm. Catalase activity was calculated in terms of nmol H_2O_2 consumed/min/mg protein.

Estimation of Ascorbic acid (Vitamin C)

Vitamin C was measured by the method of Oayama.[37] To 0.5 ml of homogenate, 0.5 ml of water and 1 ml of TCA were added, mixed thoroughly and centrifuged. To 1 ml of the supernatant, 0.2 ml of DTC reagent was added and incubated at 37 °C for 3 hrs. Then 1.5 ml of sulfuric acid was added, mixed well and the solutions were allowed to stand at room temperature for another 30 minutes. The color developed was read at 520 nm. Spectrophotometrically the level of ascorbic acid is expressed as µg /mg protein.

Acetyl cholinesterase activity

AchE is a marker of extensive loss of cholinergic neurons in the forebrain. The AchE activity was assessed by the Ellman method.[38] The assay mixture contained 0.05 ml of supernatant, 3 ml of sodium phosphate buffer (pH 8), 0.1 ml of acetylthiocholine iodide and 0.1 ml of DTNB (Ellman reagent). The change in absorbance was at 412 nm and the results were expressed as micromoles of acetylthiocholine iodide hydrolyzed/min/mg protein.

Determination of protein

Protein was determined by the method of Lowry.[39]

Histopathology

The animal from each group were anesthetized with Ketamine (80 mg/kg i.p.) and Xylazine (10 mg/kg i.p.). The brain was carefully removed without any injury after opening the skull. The collected brain was washed with ice cold normal saline and fixed in 10% formalin. Paraffin embedded sections were processed in alcohol-xylene series and stained with haematoxylin-eosin dye. The sections were examined microscopically for histopathological changes in the hippocampal zone.

Statistical analysis

Data represents mean ± S.D. Statistical comparisons were performed by one way analysis of variance (ANOVA) followed by student 't' test using SPSS 10 version. If ANOVA analysis indicated significant differences, Tukey's post-hoc test was performed to compare mean values between treatment groups and control. A value of $P<0.01$ was considered as statistically significant.

RESULTS

Behavioral Study

Effect of CN on $A\beta_{25-35}$ induced changes in Open Field test

ICV infusion of $A\beta_{25-35}$ (10µg/rat) showed significant ($P<0.01$) decrease in head dipping ($P<0.01$), rearing ($P<0.01$) and line crossings ($P<0.01$) when compared to that of control group and these were found to be significantly increased ($P<0.05$) in CN (25 mg/kg) and ($P<0.01$) in CN (50 mg/kg) in a dose-dependent manner. The exploratory behavior of CN alone (50 mg/kg) was similar to that of control group. (Table: 1)

Data represents mean ± S.D (n = 6 in each group). Group I, control group received distilled water 1 mL/kg; Group II, induced group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat); Group III, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (25 mg/kg); Group IV, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (50 mg/kg); Group V, drug alone group, received CN (50 mg/kg). $A\beta_{25-35}$ (10µg/rat) was administered via ICV route to all the animals in Group II, III and IV on 15th day. The CN dissolved in 2% DMSO were administered to all the animals in Group III, IV and V by oral gavage once a day for 14 days prior to $A\beta_{25-35}$ injection and continued up to 28 days. $^{**}P<0.01$; $^{*}P<0.05$; Group II compared with Group I; Group III (Aβ+CN (25mg/kg)), Group IV (Aβ+CN (50mg/kg)) were compared with Group II by one way ANOVA with Tukey's post hoc test.

Effect of CN on $A\beta_{25-35}$ induced changes in Morris Water Maze test

When compared with the escape latency of control group, $A\beta_{25-35}$ induced group took significantly more time to find the hidden platform ($P<0.01$) on all days. Whereas CN treated group took significantly shorter time ($P<0.05$) in CN (25 mg/kg) and ($P<0.01$) in CN (50 mg/kg) in a dose-dependent manner to reach the platform compared to that of the $A\beta_{25-35}$ induced group. No significant change was seen with CN alone (50 mg/kg) treated group. (Table: 2)

Data represents mean ± S.D (n = 6 in each group). Group I, control group received distilled water 1 mL/kg; Group II, induced group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat); Group III, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (25 mg/kg); Group IV, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (50 mg/kg); Group V, drug alone group, received CN (50 mg/kg). $A\beta_{25-35}$ (10µg/rat) was administered via ICV route to all the animals in Group II, III and IV on 15th day. The CN dissolved in 2% DMSO were administered to all the animals in Group III, IV and V by oral gavage once a day for 14 days prior to $A\beta_{25-35}$ injection and continued up to 28 days. $^{**}P<0.01$; $^{*}P<0.05$; Group II compared with Group I; Group III (Aβ+CN (25mg/kg)), Group IV (Aβ+CN (50mg/kg)) were compared with Group II by one way ANOVA with Tukey's post hoc test.

Effect of CN on $A\beta_{25-35}$ induced changes in Novel Object Recognition test

Visual recognition memory was assessed using a novel object recognition test. Compared with the training session, $A\beta_{25–35}$ induced group showed significantly less frequent exploratory behavior ($P<0.01$) to a novel object than a familiar object when compared to the control group. Whereas CN treated group showed significantly more frequent exploratory behavior to a novel object ($P<0.05$) in CN (25 mg/kg) and ($P<0.01$) in CN (50 mg/kg) in a dose-dependent manner when compared to that of $A\beta_{25–35}$ induced group. No difference was seen with CN alone (50 mg/kg) treated group. (Table: 3)

Data represents mean ± S.D (n = 6 in each group). Group I, control group received distilled water 1 mL/kg; Group II, induced group, received an ICV injection of $A\beta_{25–35}$ (10µg/rat); Group III, treatment group, received an ICV injection of $A\beta_{25–35}$ (10µg/rat) and CN (25 mg/kg); Group IV, treatment group, received an ICV injection of $A\beta_{25–35}$ (10µg/rat) and CN (50 mg/kg); Group V, drug alone group, received CN (50 mg/kg). $A\beta_{25–35}$ (10µg/rat) was administered via ICV route to all the animals in Group II, III and IV on 15th day. The CN dissolved in 2% DMSO were administered to all the animals in Group III, IV and V by oral gavage once a day for 14 days prior to $A\beta_{25–35}$ injection and continued up to 28 days. $^{**}P<0.01$; $^{*}P<0.05$; Group II compared with Group I; Group III (Aβ+CN (25mg/kg)), Group IV (Aβ+CN (50mg/kg)) were compared with Group II by one way ANOVA with Tukey's post hoc test.

Effect of CN on $A\beta_{25–35}$ induced changes in Radial Arm Maze test

Prior to surgery, all rats acquired the RAM task and were making approximately 9 correct choices (>90% accuracy) in their first 4 arms selections (acquisition). ICV infusion of $A\beta_{25–35}$ (10µg/rat) produced significant ($P<0.01$) impairments in the RAM performance (re-acquisition) compared to control group. Also, induction with $A\beta_{25–35}$ exhibited less accurate performance than the control group. Treatment with CN (25mg/kg and 50mg/kg) improved RAM performance dose-dependently. The RAM performance of CN alone (50 mg/kg) was similar to that of the control group. (Table: 4)

Data represents mean ± S.D (n = 6 in each group). Group I, control group received distilled water 1 mL/kg; Group II, induced group, received an ICV injection of $A\beta_{25–35}$ (10µg/rat); Group III, treatment group, received an ICV injection of $A\beta_{25–35}$ (10µg/rat) and CN (25 mg/kg); Group IV, treatment group, received an ICV injection of $A\beta_{25–35}$ (10µg/rat) and CN (50 mg/kg); Group V, drug alone group, received CN (50 mg/kg). $A\beta_{25–35}$ (10µg/rat) was administered via ICV route to all the animals in Group II, III and IV on 15th day. The CN dissolved in 2% DMSO were administered to all the animals in Group III, IV and V by oral gavage once a day for 14 days prior to $A\beta_{25–35}$ injection and continued up to 28 days. $^{**}P<0.01$; $^{*}P<0.05$; Group II compared with Group I; Group III (Aβ+CN (25mg/kg)), Group IV (Aβ+CN (50mg/kg)) were compared with Group II by one way ANOVA with Tukey's post hoc test.

Effect of CN on $A\beta_{25–35}$ induced changes in Rota-rod test

The neuromuscular function was assessed using a Rota-rod test. There was no much significant (NS) alteration among the differently treated rat groups. However $A\beta_{25–35}$ induced group showed moderate (NS) motor deficit when compared to the control group. (Table: 5)

Data represents mean ± S.D (n = 6 in each group). Group I, control group received distilled water 1 mL/kg; Group II, induced group, received an ICV injection of $A\beta_{25–35}$ (10µg/rat); Group III, treatment group, received an ICV injection of $A\beta_{25–35}$ (10µg/rat) and CN (25 mg/kg); Group IV, treatment group, received an ICV injection of $A\beta_{25–35}$ (10µg/rat) and CN (50 mg/kg); Group V, drug alone group, received CN (50 mg/kg). $A\beta_{25–35}$ (10µg/rat) was administered via ICV route to all the animals in Group II, III and IV on 15th day. The CN dissolved in 2% DMSO were administered to all the animals in Group III, IV and V by oral gavage once a day for 14 days prior to $A\beta_{25–35}$ injection and continued up to 28 days. $^{**}P<0.01$; $^{*}P<0.05$; ^{NS} Non significant; Group II compared with Group I; Group III (Aβ+CN (25mg/kg)), Group IV (Aβ+CN (50mg/kg)) were compared with Group II by one way ANOVA with Tukey's post hoc test.

Biochemical Studies

Effect of CN on $A\beta_{25–35}$ induced changes in the contents of TBARS and Reduced glutathione (GSH)

The content of TBARS was elevated significantly ($P<0.01$) in the $A\beta_{25–35}$ induced group as compared to the control group (Figure 2). The increased TBARS level was significantly restored ($P<0.05$) in CN (25mg/kg) and ($P<0.01$) in CN (50mg/kg) in a dose-dependent manner when compared with that of the $A\beta_{25–35}$ induced group. No significant change was observed in CN alone (50mg/kg) treated group as compared to the control group. On the other hand, the content of reduced glutathione (GSH) in the hippocampus was depleted significantly ($P<0.01$) in the $A\beta_{25–35}$ induced group when compared with that of the control group and its depleted level was restored significantly ($P<0.05$) in CN (25mg/kg) and ($P<0.01$) in CN (50mg/kg) in a dose-dependent manner compared to that of the $A\beta_{25–35}$ induced group. No significant change was observed in control and CN alone (50mg/kg) treated groups.

Data represents mean ± S.D (n = 6 in each group). Group I, control group received distilled water 1 mL/kg; Group II, induced group, received an ICV injection of $A\beta_{25–35}$ (10µg/rat); Group III, treatment group, received an ICV injection of $A\beta_{25–35}$ (10µg/rat) and CN (25 mg/kg); Group IV, treatment group, received an ICV injection of $A\beta_{25–35}$ (10µg/rat) and CN (50 mg/kg); Group V, drug alone group, received CN (50 mg/kg). $A\beta_{25–35}$ (10µg/rat) was administered via ICV route to all the animals in Group II, III and IV on 15th day. The CN dissolved in 2% DMSO were administered to all the animals in Group III, IV and V by oral gavage once a day for 14 days prior to $A\beta_{25–35}$ injection and continued up to 28 days. $^{**}P<0.01$; $^{*}P<0.05$; Group II compared with Group I; Group III (Aβ+CN (25mg/kg)), Group IV (Aβ+CN (50mg/kg)) were compared with Group II by one way ANOVA with Tukey's post hoc test. Thiobarbituric acid reactive substances (TBARS); Reduced glutathione (GSH).

Effect of CN on $A\beta_{25-35}$ induced changes in the activity of antioxidant enzymes (GPx, GR, SOD and CAT)

Figure 3 shows the activities of GPx, and GR in the hippocampus of control and experimental rats. The activity of these two enzymes were significantly decreased (P<0.01) in the $A\beta_{25-35}$ induced group, as compared to that of the control group. Their activities were significantly increased (P<0.05) in CN (25mg/kg) and (P<0.01) in CN (50mg/kg) in a dose-dependent manner. No significant change was observed in control and CN alone (50mg/kg) treated groups.

Data represents mean ± S.D (n = 6 in each group). Group I, control group received distilled water 1 mL/kg; Group II, induced group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat); Group III, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (25 mg/kg); Group IV, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (50 mg/kg); Group V, drug alone group, received CN (50 mg/kg). $A\beta_{25-35}$ (10µg/rat) was administered via ICV route to all the animals in Group II, III and IV on 15th day. The CN dissolved in 2% DMSO were administered to all the animals in Group III, IV and V by oral gavage once a day for 14 days prior to $A\beta_{25-35}$ injection and continued up to 28 days. **P<0.01; *P <0.05; Group II compared with Group I; Group III (Aβ+CN (25mg/kg)), Group IV (Aβ+CN (50mg/kg)) were compared with Group II by one way ANOVA with Tukey's post hoc test. Glutathione peroxidase (GPx); Glutathione reductase (GR).

Figure 4 shows the effect of CN on the activities of SOD and CAT in the hippocampus. The activity of SOD and CAT was found to be significantly reduced (P<0.01) in the $A\beta_{25-35}$ induced group, as compared to the control group. The decrease in SOD activity was significantly restored (P<0.05) in CN (25mg/kg) and (P<0.01) in CN (50mg/kg) in a dose-dependent manner, as compared to the $A\beta_{25-35}$ induced group. No significant change was observed in control and CN alone (50mg/kg) treated groups.

Data represents mean ± S.D (n = 6 in each group). Group I, control group received distilled water 1 mL/kg; Group II, induced group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat); Group III, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (25 mg/kg); Group IV, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (50 mg/kg); Group V, drug alone group, received CN (50 mg/kg). $A\beta_{25-35}$ (10µg/rat) was administered via ICV route to all the animals in Group II, III and IV on 15th day. The CN dissolved in 2% DMSO were administered to all the animals in Group III, IV and V by oral gavage once a day for 14 days prior to $A\beta_{25-35}$ injection and continued up to 28 days. **P<0.01; *P <0.05; Group II compared with Group I; Group III (Aβ+CN (25mg/kg)), Group IV (Aβ+CN (50mg/kg)) were compared with Group II by one way ANOVA with Tukey's post hoc test. Superoxide dismutase (SOD); Catalase (CAT).

Effect of CN on $A\beta_{25-35}$ induced changes in the levels of Vitamin C

Figure 5 shows the levels of Vitamin C in control and experimental rats. $A\beta_{25-35}$ induced group showed a significant decrease (P<0.01) in the level of Vitamin C when compared to the control group. The Vitamin C levels were significantly increased (P<0.05) in CN (25mg/kg) and (P<0.01) in CN (50mg/kg) in a dose-dependent manner, as compared to the $A\beta_{25-35}$ induced group. No significant difference was observed in control and CN alone (50mg/kg) treated groups.

Data represents mean ± S.D (n = 6 in each group). Group I, control group received distilled water 1 mL/kg; Group II, induced group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat); Group III, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (25 mg/kg); Group IV, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (50 mg/kg); Group V, drug alone group, received CN (50 mg/kg). $A\beta_{25-35}$ (10µg/rat) was administered via ICV route to all the animals in Group II, III and IV on 15th day. The CN dissolved in 2% DMSO were administered to all the animals in Group III, IV and V by oral gavage once a day for 14 days prior to $A\beta_{25-35}$ injection and continued up to 28 days. **P<0.01; *P <0.05; Group II compared with Group I; Group III (Aβ+CN (25mg/kg)), Group IV (Aβ+CN (50mg/kg)) were compared with Group II by one way ANOVA with Tukey's post hoc test. Vitamin C (Vit C).

Effect of CN on $A\beta_{25-35}$ induced changes in the activity of acetylcholine esterase (AChE)

Figure 6 shows the activity of acetylcholine esterase in control and experimental rats. The activity of AChE was found to be significantly increased (P<0.01) in the $A\beta_{25-35}$ induced group, as compared to the control group and were significantly decreased (P<0.05) in CN (25mg/kg) and (P<0.01) in CN (50mg/kg) in a dose-dependent manner, as compared to the $A\beta_{25-35}$ induced group. No significant change was observed in control and CN alone (50mg/kg) treated groups.

Data represents mean ± S.D (n = 6 in each group). Group I, control group received distilled water 1 mL/kg; Group II, induced group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat); Group III, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (25 mg/kg); Group IV, treatment group, received an ICV injection of $A\beta_{25-35}$ (10µg/rat) and CN (50 mg/kg); Group V, drug alone group, received CN (50 mg/kg). $A\beta_{25-35}$ (10µg/rat) was administered via ICV route to all the animals in Group II, III and IV on 15th day. The CN dissolved in 2% DMSO were administered to all the animals in Group III, IV and V by oral gavage once a day for 14 days prior to $A\beta_{25-35}$ injection and continued up to 28 days. **P<0.01; *P <0.05; Group II compared with Group I; Group III (Aβ+CN (25mg/kg)), Group IV (Aβ+CN (50mg/kg)) were compared with Group II by one way ANOVA with Tukey's post hoc test. Acetylcholine esterase (AChE).

Histological studies

Figure 7: Photomicrographs showing the morphology of hippocampus in control and ICV $A\beta_{25-35}$ induced rats. Figure 7a (Control): Transverse section of hippocampus of rat brain showing normal histo-architecture (H&E, 400x). Figure 7b ($A\beta_{25-35}$ induced): Transverse section of hippocampus of rat brain showing severe vacuolar degeneration of the neuronal cells (Arrows) (H&E, 400x).

Figure 7c (Aβ + CN 25mg/kg body weight): Transverse section of hippocampus of rat brain showing mild neuronal cell loss (Arrow) when treated with 25mg/kg body weight dosage of CN (H&E, 400x). Figure 7d (Aβ + CN 50mg/kg body weight): Transverse section of hippocampus of rat brain showing decreased degeneration and improved neuronal configuration which is much more significant when compared with 25mg/kg body weight (H&E, 4000x). Figure 7e (CN 50mg/kg body weight alone): Transverse section of hippocampus of rat brain showing normal morphology which resembles that of the control (H&E, 400x).

Figure 7 shows the effect of CN in $A\beta_{25-35}$ induced histological changes in the hippocampus of control and experimental rats. Figure 7a (Control): Transverse section of hippocampus of rat brain showing normal histo-architecture (H&E, 400x). Figure 7b ($A\beta_{25-35}$ induced): Transverse section of hippocampus of rat brain showing severe vacuolar degeneration of the neuronal cells (Arrows) (H&E, 400x). Figure 7c (Aβ + CN 25mg/kg body weight): Transverse section of hippocampus of rat brain showing mild neuronal cell loss when treated with 25mg/kg body weight dosage of CN (H&E, 400x). Figure 7d (Aβ + CN 50mg/kg body weight): Transverse section of hippocampus of rat brain showing decreased degeneration and improved neuronal configuration which is much more significant when compared with 25mg/kg body weight (H&E, 4000x). Figure 7e (CN 50mg/kg body weight alone): Transverse section of hippocampus of rat brain showing normal morphology which resembles that of the control (H&E, 400x).

DISCUSSION

In the present study, we examined the neuroprotective effect of CN on $A\beta_{25-35}$ induced cognitive deficit in rats. $A\beta_{25-35}$ is most toxic Aβ fragment that has been detected in the brain of AD patients.[40,41,42] $A\beta_{25-35}$ is the core fragment of full-length Aβ and possesses many of the characteristics of the full-length Aβ peptide, including aggregative ability and neurotoxic properties such as learning and memory impairment, morphological alterations and cholinergic dysfunction.[40,41,43,44]

ICV administration of $A\beta_{25-35}$ resulted in significant cognitive impairment as observed by behavioral tests and caused marked oxidative stress as indicated by significant increase in the levels of thiobarbituric acid reactive substance (TBARS) and acetylcholine esterase (AChE), decrease in the levels of Vitamin C, glutathione (GSH), glutathione peroxidase (GPx), glutathione reductase (GR), superoxide dismutase (SOD) and catalase (CAT) activities.

Cognitive decline has been shown to be accompanied by increase in oxidative stress.[45,46] The open field test evaluates the exploratory behavioral activities and it is assessed by head dipping, rearing and line crossings. $A\beta_{25-35}$ induced rats showed decreased exploratory behavior due to cognitive dysfunction which was significantly attenuated on treatment with CN at doses 25 and 50 mg/kg body weight.

The impairment of memory formation is caused by damage in the hippocampus and associated areas of the temporal cortex. The deposition of Aβ first forms in temporal cortical regions including the hippocampus. [47] In the present study, a single ICV injection of $A\beta_{25-35}$ to rats induced a significant impairment of learning and memory in Morris water maze test. From the results obtained it is found that ICV administration of $A\beta_{25-35}$ shows increase in escape latency. This was thereby decreased gradually by treating the animals with CN, thus preventing the memory damage caused by $A\beta_{25-35}$. Novel Object Recognition test evaluates the memory acquisition and recalling.[48] The hippocampus is crucial for memory recall and recognition. ICV administration of $A\beta_{25-35}$ causes damage to the hippocampus thereby leading to memory impairment. Treatment with CN was able to increase the recognition index. Hence CN dose-dependently and significantly prevented the impairment of recognition memory induced by $A\beta_{25-35}$.

The cognitive impairment was evaluated by RAM task. The impairment in RAM task can be associated with a significant alteration of various neurotransmitter levels in different regions of the brain. Earlier studies have shown that activation of locus ceruleus promotes learning and memory.[49] In RAM task $A\beta_{25-35}$ induced rats showed increased latency time due to cognitive deficits, whereas treatment with CN reverted back the changes significantly. Rota-rod test was used to assess motor functions. No significant effect was observed on motor functions following $A\beta_{25-35}$ administration.

Free radicals play a crucial role in the pathogenesis of AD. MDA is the most abundant individual aldehyde resulting from lipid peroxidation and can be considered as a marker of lipid peroxidation. Lipid peroxidation can be used as an index for measuring the damage that occurs in membranes of tissue as a result of free radical generation.[50,51] In our study $A\beta_{25-35}$ induction caused an increase in the levels of LPO. Treatment with CN, on the other hand restored back the levels of LPO. These results suggest that $A\beta_{25-35}$ induced memory impairment is related to an accumulation of oxidative stress in the hippocampus which was thereby attenuated on treatment with CN.

SOD is responsible for catalyzing the conversion of superoxide anions into hydrogen peroxide.[52,53] Which is further decomposed to water and oxygen by CAT.[54] The activities of SOD and CAT were found to be significantly diminished in the hippocampus of $A\beta_{25-35}$ induced rats. Thereby treatment with CN was found to prevent the decrease in the activities of SOD and CAT. This suggests that the neuroprotective effect of the CN might be due to its antioxidant activity.

Glutathione (GSH) is the major non-protein thiol antioxidant in mammalian cells and it is considered to be the main intracellular redox buffer. GSH protects cellular protein-thiols against irreversible loss, thus preserving protein function. One of the most important GSH-dependent detoxifying processes involved is (GPx), which plays a central role in the removal of hydrogen and organic peroxides and leads to the formation of oxidized glutathione (GSSG). GSSG is reduced back to its thiol

form (GSH) by the ancillary enzyme glutathione reductase (GR), leading to the consumption of NADPH, which is mainly produced in the pentose phosphate pathway. It was suggested that the inhibition of GSH synthesis leads to an increase in Aβ induced cell death and intracellular Aβ accumulation.[55] The decreased level of GSH, GPx and GR in $A\beta_{25–35}$ induced animals indicates that there is an increased generation of free radicals. CN treatment was able to restore the levels of GSH, GPx, GR and thereby causes a significant decrease in the generation of free radicals.

Antioxidant such as beta carotene and vitamins C and E may protect cells from the type of damage that leads to aging in the brain and tissues. Both vitamin C and E are antioxidants which are likely to reduce oxidative stress and injury in the central nervous system; this may reduce the Aβ plaque deposition in the neuronal cells. Ascorbic acid is useful for recycling tocopherol and recycles oxidized transition metals back to their reduced forms.[56] In our study, with regards of non-enzymatic antioxidant we have estimated the level of vitamin C present in the hippocampus. $A\beta_{25–35}$ induced group showed a significant decline in the level of vitamin C which was significantly restored on treatment with CN.

AChE is an acetylcholine hydrolyzing enzyme that is responsible for the termination of cholinergic response.[57] In the present study, AChE activity in the hippocampus was significantly increased in rats treated with $A\beta_{25-35}$. The activity of AChE depends largely on the membrane characteristics, since the enzyme is membrane bound. Barbosa et al. 2002 [58] suggested that Aβ peptides induce Ca^{2+} influx that leads to increased activity of AChE which is attributed to Ca^{2+} mediated oxidative stress. The increase in AChE activity due to $A\beta_{25-35}$ induction was thereby attenuated on treatment with CN.

Several histopathological findings of previous studies showed neuronal degeneration in the hippocampal region of $A\beta_{25–35}$ induced rat brain.[59,60] In our study the histopathological changes caused by $A\beta_{25–35}$ induction showed severe vacuolar degeneration and neuronal cell. Treatment with CN showed less sign of degeneration in a dose dependent manner.

Despite numerous studies on the beneficial effects of CN in various neurotoxicity models, its therapeutic potential in ameliorating learning and memory impairment associated with Alzheimer's disease has not been well delineated. Therefore the present study indicated that treatment with CN could ameliorate the cognitive impairment in $A\beta_{25–35}$ induced animals and attenuated oxidative stress, suggesting that CN improves cognitive function and also being an antioxidant restoring the levels of antioxidant enzymes.

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