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Intracerebroventricular administration of colchicine produces cognitive impairment associated with oxidative stress in rats

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Abstract

Oxidative stress has been implicated in neurodegenerative disorders including the Alzheimer's disease (AD). Central administration of colchicine is known to cause cognitive impairment in rats and is likened to sporadic AD in humans. However, it is not known whether this cognitive impairment is associated with free radical generation. Therefore, in the present study, the effect of intracerebroventricular colchicine was studied on paradigms of learning and memory behavior and the markers of oxidative stress in rats. Adult male Wistar rats (200–250 g) were injected with colchicine (intracerebroventricular) bilaterally (15 μg/rat; 7.5 μg/site) on the first day. The learning and memory behavior was assessed using passive avoidance paradigm, elevated plus maze and closed field activity test on Days 13, 14 and 21. The parameters of oxidative stress were assessed by measuring the malondialdehyde (MDA), glutathione, superoxide dismutase (SOD) and catalase levels in brain tissue on Day 21 of the colchicine injection. The rats developed significant learning and memory impairment as indicated by deficit in behavioral paradigms. There was a significant elevation in MDA levels and decrease in levels of glutathione. No significant difference was observed in SOD and catalase levels. Thus, the study demonstrates that central administration of colchicine causes impairment in learning and memory with associated increase in oxidative stress.

Keywords: Alzheimer's disease; Colchicine; Learning and memory; Oxidative stress; Rat

1. Introduction

Alzheimer's disease (AD) is a complex neurodegenerative disease characterized by progressive decline in memory, language and other cognitive functions. Although AD looks rather uniform in terms of both the morphological end stage in brain and clinical feature, there is no evidence that this neurodegenerative disorder originates by one single cause. Several etiologic hypotheses have been advanced for AD, viz. genetic defect, appearance of neurofibrillary tangles, altered amyloid precursor processing, deficiency of neurotropic factors, mitochondrial defect, trace element neurotoxicity, energy metabolism deficit and oxidative stress (Gajdusek, 1985; Martinez et al., 1994; Shigenaga et al., 1994).

Evidence is provided that a smaller proportion of 5–10% of all Alzheimer cases is caused by genetic abnormalities of chromosomes leading to early AD. In contrast, the greater proportion of all Alzheimer cases (90–95%) are found to be of late onset and sporadic in origin (Hoyer, 2000). Sporadic dementia of Alzheimer type (SDAT) is shown to be associated with microtubule dysfunction and characterized by the appearance of specific cytoskeletal cellular abnormalities, which is associated with cognitive impairment (Fu et al., 1986; Grundke-Iqbal et al., 1986; Iqbal et al., 1986; Matsuyama and Jarvick, 1989; Perry et al., 1978). Experimentally, in animals, it has been demonstrated that central administration of microtubule disrupting agents can result in cell death associated with cognitive impairment, which resemble the microtubule dysfunction in AD (Flaherty et al., 1989; Sofroniew et al., 1986; Tilson and Peterson, 1987; Tilson et al., 1988).

Colchicine, as a microtubule-disrupting agent (James and Dennis, 1981), produces marked destruction of hippocampal granule cells, mossy fibers and septohippocampal pathways (SHC; a cholinergic link between medial septum and vertical limb of diagonal band). It induces neurofibrillary degeneration by binding to tubulin, the principal structural protein of microtubule (McClure, 1972; Wilson and Fried-

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kin, 1966; Walsh et al., 1986), which is associated with loss of cholinergic neurons and decrease in acetylcholine transferase, thereby resulting in impairment of learning and memory (Kevin et al., 1989; Dwaine and Thomas, 1990). Thus, the intracerebroventricular colchicine model is relevant to SDAT in humans as both are characterized by a progressive deterioration of cognitive functions, microtubule destruction and decrease in ChAT activity (Bensimon and Chermat, 1991; Nakagawa et al., 1987).

Oxidative stress due to increase in free radical generation of impaired endogenous antioxidant mechanism is an important factor that has been implicated in neuronal damage and in AD, and cognitive defects seen in elderly (Pratico and Delanty, 2000; Cantuti et al., 2000). Evidences indicate that the hippocampal infusion of colchicine increased the glutamate (GLU)/gamma amino butyric acid (GABA) ratio in the cortex of the mice brain (Yu et al., 1997), and also the nitric oxide (NO) by increase in the NADPHd-positive neurons in the different areas of hypothalamus of guinea pig (Laurence et al., 2000). This relative increase in GLU activity and NO (Coyle and Puttafarcken, 1993) may cause oxidative stress and brain damage. However, free radical generation and consequent oxidative stress leading to the cognitive impairment in colchicine model is not known. Therefore, the present study was designed to investigate the effect of intracerebroventricular administration of colchicine on parameters of oxidative stress, namely malondialdehyde (MDA), glutathione, superoxide dismutase (SOD) and catalase along with the effect on learning and memory in rats.

2. Materials and methods

2.1. Animals

Studies were carried out using male Wistar rats weighing 200–250 g. They were obtained from the central animal house facility of All India Institute of Medical Sciences, New Delhi and stock bred in the departmental animal house. The rats were group-housed in polyacrylic cages (38×23×10 cm) with not more than four animals per cage and maintained under standard laboratory conditions with natural dark and light cycle (approximately 14-h light/10-h dark cycle) and room temperature 25±1 °C. They were allowed free access to standard dry diet (Golden Feeds, India) and tap water ad libitum. All the behavioral procedures were carried out between 09:00 and 13:00 h. All procedures described were reviewed and approved by the Institutional Committee for Ethical Use of Animals.

2.2. Intracerebroventricular administration of colchicine and schedule for behavioral test

Animals were randomly divided into two groups of 10 animals each. Prior to surgery, rats were anaesthetized by chloral hydrate (240 mg/kg ip in 4% solution) and positioned in stereotaxic apparatus. A sagittal incision was made in the scalp and two holes were drilled through the skull for placement of the injection cannula into the lateral cerebral ventricles. The first group received intracerebroventricular artificial cerebrospinal fluid (ACSF) 10 μl/site (ACSF: 147 mM NaCl, 2.9 mM KCl, 1.6 mM $MgCl_2$, 1.7 mM $CaCl_2$ and 2.2 mM dextrose) and the second group administered intracerebroventricular colchicine (7.5 μg in 10 μl/site; Kee Pharma, India) dissolved in ACSF, bilaterally into the lateral cerebral ventricle with the help of microsyringe. The stereotaxic coordinates for intracerebroventricular injection were 0.8 mm posterior to bregma, 1.8 mm lateral to sagittal suture and 3.6 mm beneath the cortical surface. To ensure diffusion of the administered drug, the cannula was left in place for a period of 2 min following injection. Behavioral tests were carried out on Days 13, 14 and 21, and markers of oxidative stress were estimated after the last behavioral testing on Day 21 of the colchicine injection.

2.3. Behavioral test

2.3.1. Passive avoidance learning

Memory retention deficit was evaluated by step through passive avoidance apparatus. The apparatus consists of equal size light and dark compartments (30×20×30 cm). A 40-W lamp was fixed 30 cm above its floor in the center of the light compartment. The floor consisted of metal grid connected to shock scrambler. The two compartments were separated by a trap door that could be raised to 10 cm. On Day 13 after colchicine injection, rats were placed in the light compartment and the time lapse, before each animal entered the dark compartment and had all four paws inside it, was measured in seconds and termed as initial latency (IL). Immediately after the rat entered the dark chamber with all the four paws inside the dark chamber, the trap door was closed and an electric foot shock (0.8 mA) was delivered for 3 s. Five seconds later, the rat was removed from the dark chamber and returned to its home cage. Rats that had an IL of more than 60 s were excluded from further experiments. Twenty-four hours and 8 days after the IL (i.e., Days 14 and 21, respectively, after colchicine injection), the latency time was again measured in the same way as in acquisition trial and termed as first retention latency (1st RL) and second retention latency (2nd RL), but the foot shock was not delivered and the latency time was recorded to a maximum of 600 s. To improve the reliability and validity of the foot shock avoidance test, the grid as well as the rat paw were moistened with water before foot shock as this is known to reduce the wide inter animal variability in paw skin resistance of the rats (Mayer et al., 1990).

2.3.2. Elevated plus maze

The plus maze consists of two opposite open arms (50×10 cm), crossed with two closed arms of the same dimensions with 40-cm high walls. The arms were con-

nected with a central square (10×10 cm). On Day 13 after the colchicine injection, rats were placed individually at one end of an open arm, facing away from the central square. The time taken for the rat to move from the open arm and enter into one of the closed arms was recorded as initial transfer latency (ITL). Animal was allowed to explore the maze for 30 s after recording ITL and returned to its home cage. After 24 h and 8 days of ITL, the rat is placed similarly on the open arm and retention latency is noted again and termed as first retention transfer latency (1st RTL) and second retention transfer latency (2nd RTL) (Sharma and Kulkarni, 1992).

2.3.3. Closed field activity

Spontaneous locomotor activity was assessed on Days 13, 14 and 21 of colchicine administration. Each animal was observed over a period of 5 min in a square (30 cm) closed arena equipped with infrared light sensitive photocells using a digital photoactometer (Techno India) and values expressed as counts/5 min. The apparatus was placed in a darkened, light and sound attenuated and ventilated testing room with other behavioral testing apparatus (Lannert and Hoyer, 1998).

The sequence of the behavioral tests conducted in the retest phase on Days 13, 14 and 21 was passive avoidance behavior, followed by transfer latency in plus maze, and finally the locomotor activity.

2.4. Biochemical tests

Following the behavioral testing, the animals were decapitated under ether anesthesia and the brains quickly removed, cleaned with ice-cold saline and stored at −80 °C.

2.4.1. Tissue preparation

Brain tissue samples were thawed and homogenized with ice-cold 0.1 M phosphate buffer (pH 7.4) 10 times (w/v) (Annadora and Michel, 1995). Aliquots of homogenates from rat brain were separated and used to determine protein, lipid peroxidation and glutathione. Whereas the remaining homogenates were centrifuged at 15,000 rpm for 60 min and the supernatant was then used for enzyme assays. Catalase activity was determined immediately after sample preparation and SOD was determined within 24 h. Protein concentration was determined according to Lowry et al. (1951) using purified bovine serum albumin as standard.

2.4.2. Measurement of lipid peroxidation

MDA, a measure of lipid peroxidation, was measured as described by Jainkang et al. (1990). The reagents, 1.5 ml acetic acid (20%, pH 3.5), 1.5 ml thiobarbituric acid (0.8%) and 0.2 ml sodium dodecyl sulphate (8.1%), were added to 0.1 ml of processed tissue samples, then heated at 100 °C for 60 min. The mixture was cooled with tap water and 5 ml of *n*-butanol:pyridine (15:1), 1 ml of distilled water was added. The mixture was vortexed vigorously. After centrifugation at 4000 rpm for 10 min, the organic layer was separated and absorbance was measured at 532 nm using a spectrophotometer. The concentration of MDA is expressed as nmol/g tissue.

2.4.3. Measurement of reduced glutathione

Glutathione was measured according to the method of Ellman (1959). The equal quantity of homogenate was mixed with 10% trichloroacetic acid and centrifuged to separate the proteins. To 0.01 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.

2.4.4. Measurement of catalase

Catalase activity was measured by the method of Aebi (1974). A total of 0.1 ml of supernatant was added to cuvette containing 1.9 ml of 50 mM phosphate buffer (pH 7.0). The reaction started by the addition of 1 ml freshly prepared 30 mM H_2O_2. The rate of decomposition of H_2O_2 was measured spectrophotometrically from the changes in absorbance at 240 nm. The activity of catalase was expressed as U/mg protein.

2.4.5. Measurement of SOD

The SOD activity of the brain tissue was analyzed by the method described by Kakkar et al. (1984). Assay mixture contained 0.1 ml of sample, 1.2 ml of sodium pyrophosphate buffer (pH 8.3, 0.052 M), 0.1 ml phenazine methosulphate (186 μM), 0.3 ml of 300 μM nitroblue tetrazolium and 0.2 ml NADH (750 μM). Reaction started by the addition of NADH. After incubation at 30 °C for 90 s, the reaction was stopped by the addition of 0.1 ml glacial acetic acid. Reaction mixture was stirred vigorously and shaken with 4 ml of *n*-butanol. The mixture was allowed to stand for 10 min, centrifuged and butanol layer was separated. Color intensity of the chromogen in the butanol was measured spectrophotometrically at 560 nm. The concentration of SOD was expressed as U/mg protein.

2.4.6. Statistical analysis

The data were processed by one-way analysis of variance (ANOVA) followed by posttest and individual comparisons using Student's *t*-test (two-tailed).

3. Results

3.1. Passive avoidance test

The mean IL determined on Day 13 did not differ significantly between the control and colchicine-treated group, and all the rats entered the dark chamber within

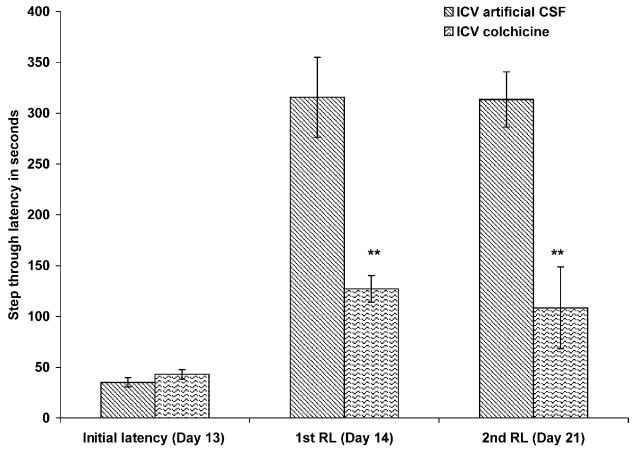

Fig. 1. Effect of intracerebroventricular colchicine on retention latency of passive avoidance paradigm in rats. The columns show mean values of latency in seconds and the bar represents S.E.M. On the abscissa, 1st RL 24 h after IL; 2nd RL 8 days after IL. **P<.001 significant decrease in the retention latency in colchicine-treated rats as compared to control rats.

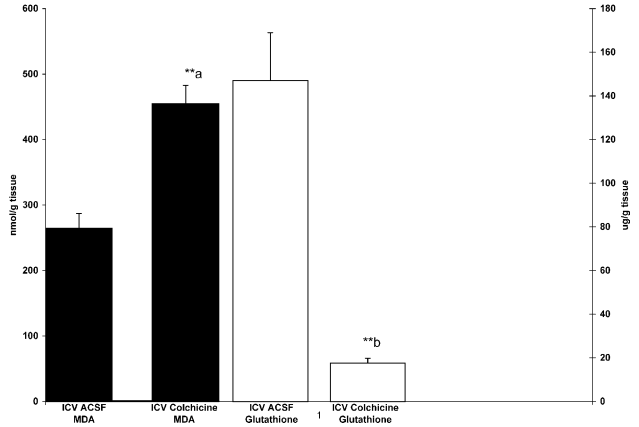

Fig. 3. Effect of intracerebroventricular colchicine on MDA and glutathione levels in brain tissue (colchicine was administered 21 days before estimation). The columns show mean values and the bar represents S.E.M. Black and empty columns represent MDA and glutathione values, respectively. (a) **P<.001 significant increase in the level of MDA in colchicine-treated rats compared to control rats. (b) **P<.001 significant decrease in the level of glutathione in colchicine-treated rats compared to control rats.

60 s. However, pretraining injection of colchicine significantly (P<.001) decreased the mean retention latency (1st RL and 2nd RL) as compared to control group on Days 14 and 21, respectively (Fig. 1).

3.2. Elevated plus maze

There was no significant difference in the ILs on Day 13 of the control and colchicine-treated group. In control group, the retention transfer latency was significantly (P<.001) decreased on Days 14 (1st RTL) and 21 (2nd RTL). However, in colchicine-treated group, there was insignificant change in retention transfer latency as compared to IL on Days 14 and 21, respectively (Fig. 2).

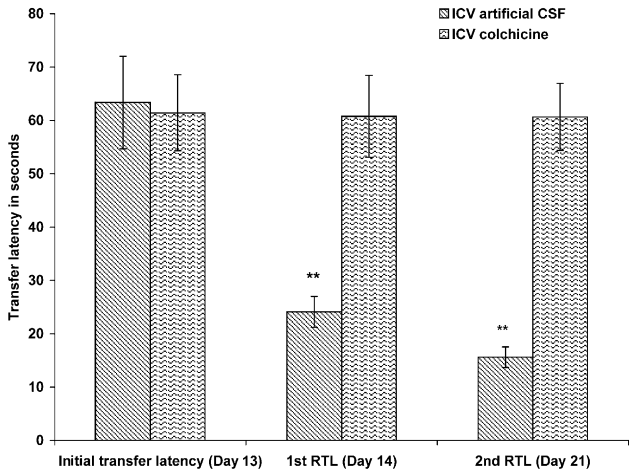

Fig. 2. Effect of intracerebroventricular colchicine on retention transfer latency in elevated plus maze paradigm in rats. The columns show mean values of retention transfer latency in seconds and the bar represents S.E.M. On the abscissa, 1st RTL 24 h after IL; 2nd RTL 8 days after IL. **P<.001 significant decrease in the retention transfer latency in control rats as compared to colchicine-treated rats.

3.3. Closed field activity

The spontaneous locomotor activity when measured on Days 13, 14 and 21 was not significantly different between the control group (172.8±25.8, 166.2±15.3 and 157±14.4 counts/5 min) and colchicine (171.6±11.2, 160.6±10.1 and 164.5±8.6 counts/5 min)-treated group on these three occasions.

3.4. Measurement of parameters of oxidative stress

Twenty-one days after intracerebroventricular colchicine, brain MDA, glutathione, SOD and catalase levels were estimated. There was a significant (P<.001) rise in MDA levels as compared to the control group and correspondingly a significant (P<.001) decline in the levels of reduced glutathione in the colchicine-treated group as compared to the control group (Fig. 3). However, the levels of catalase did not differ significantly between the control (8.8±3.7 U/mg protein) and colchicine-treated (12.7±3.3 U/mg protein) rats. Also, there was insignificant difference between the SOD levels in the control group (5.8±0.5 U/mg protein) and the colchicine-treated group (3.8±1.0 U/mg protein).

4. Discussion

Central administration of colchicine, like intracerebroventricular, intradentate and to the nucleus basalis magnocellu-

laris, has been characterized by a progressive deterioration of learning and memory, microtubule dysfunction and decrease in cholinergic turnover. This is associated with loss of cholinergic neurons in brain due to inhibition of fast axoplasmic flow or direct toxic effect on cholinergic terminals (Dwaine and Thomas, 1990; Walsh et al., 1989; Tilson et al., 1988). Therefore, it has been considered to be a relevant animal model of AD (Perry et al., 1978). In the present study, colchicine was injected directly into the lateral cerebral ventrices, so that the region in close proximity to several populations of cholinergic neurons (medial septum, horizontal and vertical limb of the diagonal band, striatum) would allow a direct evaluation of the cholinotoxicity. It was reported that the intracerebroventricular injection of colchicine significantly decreased the number of cholinergic neurons in the medial septum/vertical limb of the diagonal band, which project to the hippocampus and synapse on granule cells, pyramidal cells and interneurons. Therefore, it was expected that the intracerebroventricularly administered colchicine would preferentially act on the cholinergic neurons (Dwaine and Thomas, 1991).

Colchicine has been reported to mediate cascade of actions. The central administration of colchicine elevates GLU/GABA ratio in cortex of mice brain (Yu et al., 1997). The relative increase in the GLU activity exerts neurotoxic effect by generating the hydroxyl radicals (Hammer et al., 1993). Secondly, Laurence and coworkers reported an increase in the NADPHd-positive neurons in the different hypothalamic area after central administration of colchicine, which indirectly suggest increase in production of NO synthase and NO in the brain (Laurence et al., 2000). The released NO can cause neurotoxicity by multiple mechanisms: (a) by interacting with superoxide anions and resulting in peroxynitrite formation, which has cytotoxic activity; (b) by nitrosylation of enzymes, e.g., phosphokinase-C and glyceraldehydes-3-phosphate dehydrogenase, which results in inhibition of glycolysis; (c) by causing DNA mutation and strand breaking, which in turn stimulates polyADP-ribosylation of proteins, and ultimately causing massive depletion of cellular energy (Wallis et al., 1993). The glucose/energy metabolism impairment in the brain has been reported to impair the ability to scavenge the free radicals (Beal, 1995; Mattson, 1994) and cause neuronal damage (Sims and Pulsinelli, 1987). Also, the deficit in cerebral regulation of glucose is known to cause impairment of learning and memory (Mayer et al., 1990). Thus, it was our contention that the central administration of colchicine causes increase in free radical generation and the consequent oxidative stress leads to cognitive impairment.

In rats treated with colchicine, there was a reduction in retention latency as seen in passive avoidance test both on Days 14 and 21 after colchicine administration, suggesting steady rather than a transient cognitive impairment in colchicine-treated group. The results from elevated plus maze paradigm also showed that the control animals had significantly shorter retention transfer latencies on both Days 14 and 21. However, in colchicine-treated rat, there was no difference in the IL and retention transfer latency at any time period tested. Thus, the results from passive avoidance and elevated plus maze paradigms reflect poorer acquisition and retention of memory after intracerebroventricular colchicine administration. The findings are similar to that reported by other workers (Dwaine and Thomas, 1990; Walsh et al., 1989; Yu et al., 1997). There was insignificant difference between the locomotor activity of colchicine-treated and the control rats. This excludes the possibility that any CNS depressant/stimulant activity of the drug may have contributed to severe deficits in passive avoidance behavior and elevated plus maze test after the intracerebroventricular colchicine administration.

Free radicals are normal products of cellular aerobic metabolism. However, when the production of free radicals increases or defense mechanism of the body decreases, they cause cellular dysfunction by attacking at the polyunsaturated sites of the biological membranes leading to lipid peroxidation (Gupta and Sharma, 1999). The increase in levels of MDA, a marker of lipid peroxidation, in our study indicates increased free radical generation in the colchicine-treated rats. Also, there was a simultaneous significant decrease in the reduced glutathione levels. Glutathione is an endogenous antioxidant present majorly in the reduced form within the cells. It reacts with the free radicals and prevents the generation of hydroxyl radicals, which is the most toxic form of free radicals. During this defensive process, reduced glutathione is converted to oxidized form with the help of the enzyme glutathione peroxidase. The decreased level of reduced glutathione seen in our study indicates that there was an increased generation of free radicals and the reduced glutathione was depleted during the process of combating oxidative stress (Reiter, 2000; Schulz et al., 2000). The level of SOD was found to be decreased in the colchicine-treated rats, however, it was not significant. This could be because glutathione pathway may be playing a major role in combating oxidative stress in the brain. The insignificant change in the levels of catalase might be due to the fact that the catalase activity in the rat brain is very low as reported by Moreno and Meganni (1992) and may not be the key enzyme of intracellular antioxidant defense system in the brain. Thus, the present study demonstrates that intracerebroventricular administration of colchicine causes cognitive impairment, which is associated with oxidative stress in brain.

Similarly, considerable work has been done by researchers proposing intracerebroventricular streptozotocin as a model of AD (Duelli et al., 1994; Lannert and Hoyer, 1998; Hoyer et al., 2000; Blokland and Jolles, 1993; Sharma and Gupta, 2001). Recently, we have demonstrated that the intracerebroventricular streptozotocin is known to cause oxidative stress in the brain along with cognitive impairment (Sharma and Gupta, 2001). However, the

oxidative stress in intracerebroventricular streptozotocin model may be due to the impairment of glucose metabolism in the brain (Sharma and Gupta, 2001; Prickaerts et al., 1999). Whereas the oxidative stress in intracerebroventricular colchicine-treated rats may be due to increase in the GLU/GABA ratio or due to the increase in NO production in the brain. Since it has been demonstrated that increase in GLU can trigger the formation of reactive oxygen species (ROS), these increased ROS in turn may further release GLU, thus, forming a loop. This viscous cycle may lead to the free radical induced neurotoxicity (Bondy, 1995) in colchicine-treated rats. Since neuronal injury itself can induce free radical generation, it is difficult to establish whether this is a primary or secondary event. Even if free radical generation is secondary to other initiating causes, they are deleterious and a part of cascade of events that can lead to neuron death (Markesbery, 1997; Pratico and Delanty, 2000). Therefore, it is difficult to ascertain whether the oxidative stress seen in the present study is a primary or secondary effect. Destruction of microtubule, increase in oxidative stress and the associated cognitive impairment make intracerebroventricular colchicine model suitable to evaluate CNS active drugs related to cytoskeletal abnormalities and antioxidant property for treatment of AD.

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