Beneficial Effects of Donepezil on Vascular Endothelial Dysfunction-Associated Dementia Induced by L-Methionine in Rats

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(Received August 14, 2008; Accepted January 21, 2009)

Donepezil (acetyl cholinesterase inhibitor) is a mainstay of clinical intervention to contain memory deficits of Alzheimer's disease. However, its beneficial role in endothelial dysfunction-associated dementia *i. e.* vascular dementia still needs to be explored. The present study was designed to investigate the effect of donepezil on vascular endothelial dysfunction, and associated memory deficits in rats. Atorvastatin (3-hydroxy-4-methyl-glutaryl (HMG)-CoA inhibitor) was taken as the standard. Rats were administered L-methionine (1.7 g/kg per day, *p.o.*, 4 weeks and 4 days *i.e.* chronic treatment) to produce endothelial dysfunction and dementia. Serum nitrite level was estimated as a marker of endothelial function. Morris water maze (MWM) test was employed for assessment of memory. Brain tissue thiobarbituric acid reactive substances (TBARS) and reduced glutathione (GSH) were estimated to assess oxidative stress. Brain acetylcholine esterase (AChE) activity and serum total cholesterol level were also estimated. L-methionine produced endothelial dysfunction as reflected by significant decrease of serum nitrite concentration. L-methionine-treated rats performed poorly on MWM indicating impairment of memory as well. These rats also showed a significant rise in brain oxidative stress, AChE activity and serum total cholesterol levels. Donepezil (0.1 mg/kg *p.o.*) and atorvastatin (10 mg/kg *p.o.*) attenuated L-methionine-induced endothelial dysfunction. This intervention reversed L-methionine-induced rise of brain oxidative stress and AChE activity. Furthermore, atorvastatin produced a reduction of L-methionine-induced rise in serum cholesterol. The beneficial effects of donepezil may be attributed to its multiple effects and this study highlights the potential of donepezil in vascular dementia.

Key words —— endothelial dysfunction, learning, memory, L-methionine, dementia, donepezil

INTRODUCTION

Dementia is an organic brain disease defined as loss of intellectual ability of sufficient severity to interfere either with occupational functioning, usual social activities or relationship of a person in the absence of gross clouding of consciousness or motor involvement.[1] Dementia of vascular origin *i.e.* vascular dementia (VaD) has gained much attention in recent times. After alzheimer disease (AD), VaD is the second most common cause of dementia. In the vascular system, nitric oxide (NO) generated by endothelial NO synthase (eNOS) plays an important role in maintenance of vascular tone.[2] Hyperhomocysteinemia (Hhcy), or elevation of plasma total homocysteine, is an important risk factor for cardiovascular disease, stroke and vascular dementia.[3,4] Hhcy has been shown to induce endothelial dysfunction by decreasing the bioavailability of NO, and increasing vascular oxidative stress.[5,6] The decreased NO level has been demonstrated to contribute to the pathogenesis of dementia.[7]

Oxidative stress and vascular dysfunction are recognized as important contributing factors in the pathogenesis of AD and other dementia of vascular origin.[7] In AD and other neurodegenerative diseases, structural deformities in the cerebral capillaries lead to impairment of cerebral perfusion with subsequent neuronal dys-function and death.[5] The well established risk factors of endothelial dysfunction and subsequent VaD such as hypertension, history of stroke, diabetes mellitus and hypercholesterolemia are all associated with high risk of AD.

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The noted vascular dys-function (vascular deformities) in AD and common risk factor of AD and VaD suggest an overlap between AD and VaD.[8] Only limited therapeutic interventions are available to reduce the incidence of vascular endothelial dysfunction-associated memory deficits. Currently statins are being widely explored for their usefulness against endothelial dysfunction. We have recently reported that statins exert beneficial effects against memory deficits related with dementias of AD.[1,9] Donepezil (a cholinesterase inhibitor) is a mainstay of clinical intervention to contain memory deficits of AD. However, its beneficial role in endothelial dysfunction and associated dementia still needs to be explored. Therefore the present study was designed to investigate the effect of donepezil on vascular endothelial dysfunction and associated memory deficits.

MATERIALS AND METHODS

Animals —— Age-matched (6, months old) male Wistar albino rats weighing 150–200 g were employed in the present study. Animals were procured from Institute of Veterinary Science, Izat Nagar, Barielly, Uttar Pradesh, India. Rats were provided standard laboratory feed (Kisan Feeds Ltd., Chandigarh, India) and tap water *ad libitum* and were exposed to 12 hr light and dark cycle. The animals were acclimatized to the laboratory condition before experiments. The experimental protocol was duly approved by Institutional Animal Ethics Committee (IAEC) and care of the animals was taken as per guidelines of the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest Government of India (Registration No. 107/1999/CPCSEA).

Drugs and Chemicals —— Atorvastatin was a gift from Ind Swift Ltd., Mohali, Punjab, India. Donepezil was a gift from Wokhardt Ltd., Baddi, Himachal Pradesh, India. All other reagents were purchased from Merck Limited, Mumbai, India, SD Fine-Chemicals Limited, Mumbai, India, Loba Chem, Mumbai, India, and Sigma-Aldrich, U.S.A. Atorvastatin and L-methionine were suspended in 0.5% w/v of CMC whereas donepezil was dissolved in distilled water.

L-Methionine-induced Endothelial Dysfunction and VaD —— Rats were administered L-methionine (1.7 g/kg per day *p.o.*) for 4 weeks to produce Hhcy-induced endothelial dysfunction.[10] Assessment of vascular endothelial function was carried out by measuring acetylcholine-induced endothelium-dependent relaxation and sodium nitroprusside-induced endothelium-independent relaxation using isolated aortic ring preparation according to the method of Pieper[11,12] together with estimation of serum nitrite concentration.

Body weight of rats was monitored weekly. After 4 weeks, rats were subjected to Morris water maze (MWM) test for the evaluation of their memory status. The L-methionine treatment was continued during acquisition trials on MWM.

Morris Water Maze Test —— MWM test was employed to assess learning and memory of rats.[13,14] The MWM procedure is based on the principle where animals placed in a large pool of water try to escape from the water by finding an escape platform. MWM consists of a large circular pool (150 cm in diameter, 45 cm in height), filled to a depth of 30 cm with water at 28±1°C. The water was made opaque with white colored dye. The tank was divided into four equal quadrants with the help of two threads, fixed at right angles to each other on the rim of the pool. A submerged platform (10 cm^2), painted white was placed inside the target quadrants of this pool 1 cm below the surface of the water. The position of the platform was kept unaltered throughout the training session. Each animal was subjected to four consecutive trials on each day with a gap of 5 min. The rat was gently placed in the water between quadrants (Q), facing the wall of the pool with drop location changing for each trial, and allowed 120 sec to locate the submerged platform. Then, it was allowed to stay on the platform for another 20 sec. If it failed to find the platform within 120 sec, it was guided gently onto the platform and allowed to remain there for 20 sec. Escape latency time (ELT) to locate the hidden platform in the water maze was noted as an index of acquisition or learning. Each animal was subjected to four acquisition trials daily for 4 consecutive days. On day 5, the platform was removed and each rat was allowed to explore the pool for 120 sec. Mean time spent in all four quadrants was noted. The mean time spent by the animals in the target quadrant searching for the hidden platform was noted as an index of retrieval.

Acquisition Trial: Each rat was subjected to four trials on each day. A rest period of 5 min was allowed in between each trial. Four trials per day were repeated for 4 consecutive days. Starting position

on each day to conduct four acquisition trials was changed as described below and Q4 was maintained as target quadrant in all acquisition trials. Mean ELT calculated for each day during acquisition trials and day 4 ELT was used as an index of acquisition.

Day1	Q1	Q2	Q3	Q4
Day2	Q2	Q3	Q4	Q1
Day3	Q3	Q4	Q1	Q2
Day4	Q4	Q1	Q2	Q3

Retrieval Trial: On 5th day the platform was removed. Rat was placed in water maze and allowed to explore the maze for 120 sec. Each rat was subjected to four such trials and each trial was started from different quadrant. Mean time spent in all three quadrants *i.e.* Q1, Q2 and Q3 were recorded and the time spent in the target quadrant *i.e.* Q4 in search of missing platform provided an index of retrieval. The experimenter always stood at the same position. Care was taken not to disturb the relative location of water maze with respect to other objects in the laboratory serving, as prominent visual clues. All the trials were completed between 09.00 to 17.00 hr in semi sound proof laboratory.

Biochemical Parameters ——

Collection of Sample: The animals were sacrificed by cervical dislocation; thoracic aorta and brain tissue were carefully removed. Thoracic aorta was used to estimate endothelium-dependent and -independent relaxation, whereas brain tissue was subjected to various biochemical estimations.

The removed brains were homogenized in phosphate buffer (pH 7.4, 10% w/v) using Teflon homogenizer. The clear supernatant, obtained after centrifugation at 3000 rpm for 15 min, was used to estimate acetyl cholinesterase (AChE) activity, thiobarbituric acid reactive species (TBARS), reduced glutathione (GSH) and protein content.

Blood samples for biochemical estimation were collected just before sacrificing the rats. The blood was kept at room temperature for 30 min then centrifuged at 4000 rpm for 15 min to separate serum. Serum was used to estimate serum homocysteine, serum nitrite concentration and serum total cholesterol.

Estimation of Serum Homocysteine: Determination of homocysteine was carried out using HPLC (Varian Inc., Palo Alto, CA, U.S.A.) attached with fluorescent HPLC detector according to the method of Dimitrova *et al.*[15] Rats with serum homocysteine levels > 10 μM were considered to have Hhcy.

Estimation of Serum Nitrite Concentration: Serum nitrite concentration was estimated by the method of Sastry *et al.* and absorbance was measured spectophotometrically (DU 640B Spectrophotometer, Beckman Coulter Inc., Fullerton, CA, U.S.A.) at 545 nm.[16]

Estimation of Total Cholesterol: Serum total cholesterol was estimated spectrophotometrically at 540 nm by Allain's method with slight modification, using commercially available conventional diagnostic kit.[17]

Estimation of Brain AChE Activity: Whole brain AChE activity was measured by the method of Ellman *et al.*, with slight modification.[18] Change in absorbance/min of the sample was read spectrophotometrically at 420 nm.

Estimation of TBARS: Whole brain TBARS level was measured by the method of Ohkawa *et al.*, with slight modification. The absorbance was measured spectrophotometrically at 532 nm.[19]

Estimation of GSH: The whole brain GSH level was measured by the method of Beutler *et al.*, with slight modification. The absorbance was noted spectrophotometrically at 412 nm.[20]

Estimation of Brain Total Protein: Brain total protein was estimated by the method of Lowry *et al.* The absorption was read spectrophotometrically at 750 nm.[21]

Experimental Protocol: Seven groups, each comprising 10 albino Wistar rats, were employed in the present study.

Group I (*Vehicle Treated Control*): Rats were administered 0.5% w/v carboxymethylcellulose (CMC) (10 ml/kg per day *p.o.*) for 4 weeks then subjected to MWM test. Vehicle was also administered 45 min before acquisition trial conducted from day 1 to day 4 and retrieval trial conducted on day 5.

Group II (*L-Methionine Treated Chronic*): To induce Hhcy, rats were administered L-methionine (1.7 g/kg per day *p.o.*) for 4 weeks then subjected to MWM test. The treatment of L-methionine was continued (administered 45 min before) during acquisition trial conducted from day 1 to day 4. The animals were administered vehicle (0.5% w/v CMC, 10 ml/kg *p.o.*) 45 min before retrieval trial conducted on day 5.

Group III (*L-Methionine Treated Acute*): Rats were administered L-methionine (1.7 g/kg per day *p.o.*) 45 min before acquisition trial conducted from day 1 to day 4 on MWM. The animals were administered vehicle (0.5% w/v CMC, 10 ml/kg *p.o.*) 45 min before retrieval trial conducted on day 5.

Group IV (*Atorvastatin*): Rats were adminis-

tered atorvastatin (10 mg/kg per day *p.o.*) for 2 weeks then subjected to MWM test. The treatment was continued (administered 45 min before) during acquisition trial conducted from day 1 to day 4. The animals were administered vehicle (0.5% w/v CMC, 10 ml/kg *p.o.*) 45 min before retrieval trial conducted on day 5.

Group V (*Donepezil*): Rats were administered donepezil (0.1 mg/kg per day *p.o.*) for 2 weeks then subjected to MWM test. The treatment was continued (administered 45 min before) during acquisition trial conducted from day 1 to day 4. The animals were administered vehicle (distilled water, 10 ml/kg *p.o.*) 45 min before retrieval trial conducted on day 5.

Group VI (*L-Methionine Chronic + Atorvastatin Treated*): The Hhcy rats were treated with atorvastatin (10 mg/kg per day *p.o.*) for 2 weeks (3rd and 4th week of L-methionine administration) then subjected to MWM test. The co-administration of atorvastatin and L-methionine was continued (administered 45 min before) during acquisition trial conducted from day 1 to day 4. The animals were administered vehicle (0.5% w/v CMC, 10 ml/kg *p.o.*) 45 min before retrieval trial conducted on day 5.

Group VII (*L-Methionine Chronic + Donepezil Treated*): The Hhcy rats were treated with donepezil (0.1 mg/kg per day *p.o.*) for 2 weeks (3rd and 4th week of L-methionine administration) then subjected to MWM test. The co-administration of donepezil and L-methionine was continued (administered 45 min before) during acquisition trial conducted from day 1 to day 4. The animals were administered vehicle (distilled water, 10 ml/kg *p.o.*) 45 min before retrieval trial conducted on day 5.

Statistical Analysis —— The results are expressed as mean ± standard error of means (S.E.M.) The data obtained from various groups were statistically analyzed by one-way analysis of variance (ANOVA) followed by Tukey's multiple range test. $P < 0.05$ was considered statistically significant.

RESULTS

Effect of Vehicle/Atorvastatin/Donepezil and L-Methionine on ELT and Time Spent in Target Quadrant Using MWM

Vehicle-treated (0.5% w/v CMC, 10 ml/kg *p.o.*) rats showed a downward trend in their ELT. There was a significant fall in day 4 ELT, when compared versus day 1 ELT of these rats (Table 1), reflecting normal learning ability. On day 5 a significant rise in time spent in target quadrant (TSTQ) was observed when compared versus time spent in other quadrants (Fig. 1), also reflecting normal retrieval.

Administration of atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day)/donepezil (0.1 mg/kg *p.o.*, 2 weeks and 4 day) did not show any significant effect on ELT and TSTQ as compared versus vehicle-treated rats (Table 1 and Fig. 1). L-methionine (1.7 g/kg *p.o.*, 4 weeks and 4 day *i.e.* chronic) administration produced a significant increase in day 4 ELT when compared versus day 4 ELT of vehicle control (Table 1) indicating impairment of acquisition. L-methionine administration also produced a significant decrease in TSTQ when compared versus TSTQ of vehicle control animals (Fig. 1), also indicating impairment of memory. However, acute administration of L-methionine did not produce any significant effect on day 4 ELT as well as TSTQ of control animals.

Effect of Atorvastatin/Donepezil on L-Methionine-induced Impairment of Learning and Memory Using MWM

Administration of atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day)/donepezil (0.1 mg/kg *p.o.*,

Table 1. Effect of Atorvastatin, Donepezil on L-Methionine-induced Changes in Day 4 ELT, Using MWM

Groups	Treatment	Dose (kg/day, *p.o.*)	ELT (day 1) in sec	ELT (day 4) in sec
I	Control	10 ml (0.5% w/w CMC)	81.5 ± 4.5	20.2 ± 2.2[a)]
II	L-Methionine (c)	1.7 g	93.8 ± 4.2	49.9 ± 2.4[b)]
III	L-Methionine (a)	1.7 g	83.6 ± 3.8	21.7 ± 2.1
IV	Atorvastatin *per se*	10 mg	85.5 ± 4.1	22.4 ± 3.4
V	Donepezil *per se*	0.1 mg	87.8 ± 4.5	20.4 ± 2.5
VI	L-Methionine (c) + Atorvastatin	10 mg	81.9 ± 3.9	27.0 ± 2.4[c)]
VII	L-Methionine (c) + Donepezil	0.1 mg	88.2 ± 4.3	26.2 ± 2.7[c)]

L-Methionine (c) = L-Methionine chronic; L-Methionine (a) = L-Methionine acute. Each group (n = 10), represent mean ± S.E.M. *a*) $p < 0.05$ day 1 vs. day 4 ELT in vehicle control, *b*) $p < 0.05$ vs. day 4 ELT in vehicle control, *c*) $p < 0.05$ vs. day 4 ELT in L-methionine, (c) treated group.

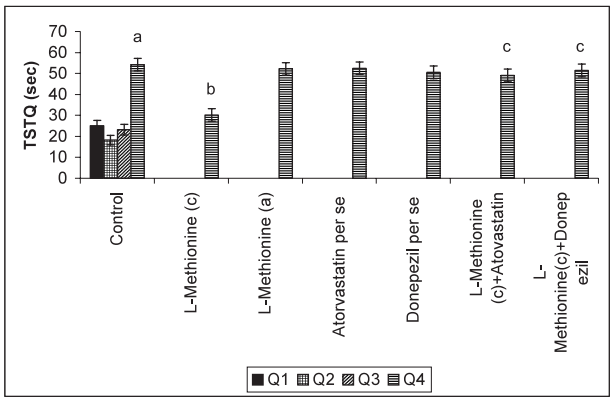

Fig. 1. Effect of Atorvastatin, Donepezil on L-Methionine-induced Changes in Day 5 Time Spent in Target (TSTQ), Using MWM

L-Methionine (c) = L-Methionine chronic; L-Methionine (a) = L-Methionine acute. Each group (n = 10), represent mean ± S.E.M. a = $p < 0.05$ Time spent in Q1, Q2, Q3, quadrant vs. Q4 quadrant in control. b = $p < 0.05$ vs. TSTQ Q4 of control. c = $p < 0.05$ vs. TSTQ Q4 of L-methionine (c) treated group.

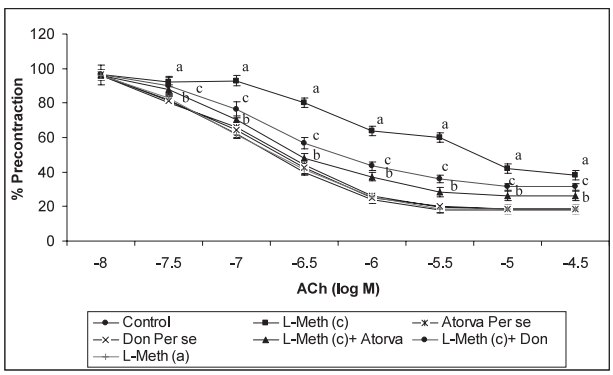

Fig. 2. Effect of Atorvastatin/Pitavastatin on L-Methionine-induced Change in Endothelium Dependent Relaxation Using Isolated Rat Aortic Ring Preparation

L-Meth (c) = L-Methionine chronic; L-Meth (a) = L-Methionine acute; Atorva = Atorvastatin; Don = Donepezil. Each group (n = 10), represent mean ± S.E.M. Responses are expressed as % of maximum contraction induced by phenylephrine (3×10^{-6} M). a = $p < 0.05$ Vs. control. b = $p < 0.01$ Vs. L-methionine (c) treated group. c = $p < 0.05$ Vs. L-methionine (c) treated group.

2 weeks and 4 day) significantly prevented L-methionine-induced rise in day 4 ELT, indicating reversal of L-methionine-induced impairment of acquisition (Table 1). Further treatment with these drugs also attenuated L-methionine-induced decrease in day 5 TSTQ, indicating reversal of L-methionine-induced impairment of memory (Fig. 1).

Effect of Atorvastatin/Donepezil on L-Methionine-induced Change in Endothelium-dependent Relaxation

ACh and sodium nitroprusside (SNP) in a

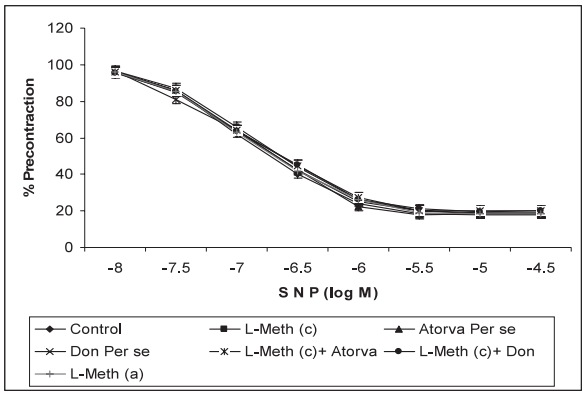

Fig. 3. Effect of Atorvastatin/Pitavastatin/L-Methionine-induced on SNP Induced Endothelium Independent Relaxation Using Isolated Rat Aortic Ring Preparation

L-Meth (c) = L-Methionine chronic; L-Meth (a) = L-Methionine acute; Atorva = Atorvastatin; Don = Donepezil. Each group (n = 10), represent mean ± S.E.M. Responses are expressed as % of maximum contraction induced by phenylephrine (3×10^{-6} M).

dose-dependent manner produced endothelium-dependent and-independent relaxation in phenylephrine (3×10^{-6} M) precontracted isolated rat aortic ring preparation. L-methionine (1.7% g/kg *p.o.*, 4 weeks and 4 day *i.e.* chronic) administration significantly ($p < 0.05$) attenuated acetylcholine-induced endothelium-dependent relaxation (Fig. 2); however, it did not affect SNP-induced endothelium-independent relaxation (Fig. 3). Treatment with atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day) and donepezil (0.1 mg/kg *p.o.*, 2 weeks and 4 day), significantly ($p < 0.01$ and $p < 0.05$) abolished the effect of L-methionine on endothelial-dependent relaxation. Atorvastatin and donepezil as well as acute L-methionine treatment did not show any effect on endothelium-dependent relaxation.

Effect of Atorvastatin/Donepezil on L-Methionine-induced Change in Serum Homocysteine Level

Chronic administration of L-methionine (1.7 g/kg *p.o.*, 4 weeks and 4 day) produced a significant ($p < 0.01$) increase in serum homocysteine when compared versus vehicle treated rats. Treatment with atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day)/donepezil (0.1 mg/kg *p.o.*, 2 weeks and 4 day) produced a significant ($p < 0.01$ and $p < 0.05$) reduction of L-methionine-induced rise in serum homocysteine level (Table 2). Atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day)/donepezil (0.1 mg/kg *p.o.*, 2 weeks and 4 day)/acute L-methionine (1.7 g/kg *p.o.*, 4 day) treatment did not show any significant effect on serum homocysteine

Table 2. Effect of Atorvastatin and Donepezil on L-Methionine-induced Changes in Serum Homocysteine

Groups	Treatment	Dose (kg/day, *p.o.*)	Serum homocysteine (μM)
I	Control	10 ml (0.5% w/w CMC)	4.25 ± 0.17
II	L-Methionine (c)	1.7 g	20.2 ± 0.88[a)]
III	L-Methionine (a)	1.7 g	4.42 ± 0.28
IV	Atorvastatin *per se*	10 mg	3.95 ± 0.21
V	Donepezil *per se*	10 mg	3.98 ± 0.24
VI	L-Methionine (c) + Atorvastatin	10 mg	12.9 ± 0.38[b)]
VII	L-Methionine (c) + Donepezil	0.1 mg	15.2 ± 0.36[c)]

L-Methionine (c) = L-Methionine chronic; L-Methionine (a) = L-Methionine acute. Each group (n = 10), represent mean ± S.E.M. *a*) $p < 0.01$ vs. serum homocysteine level of control, *b*) $p < 0.01$ vs. serum homocysteine level of L-methionine (c) treated group, *c*) $p < 0.05$ vs. serum homocysteine level of L-methionine (c) treated group.

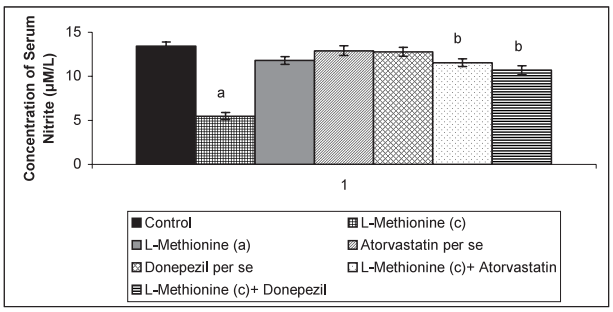

Fig. 4. Effect of Atorvastatin, Donepezil on L-Methionine-induced Changes in Serum Nitrite Level

L-Methionine (c) = L-Methionine chronic; L-Methionine (a) = L-Methionine acute. Each group (n = 10), represent mean ± S.E.M. a = $p < 0.05$ Vs. serum nitrite of control. b = $p < 0.05$ Vs. serum nitrite of L-methionine (c) treated group.

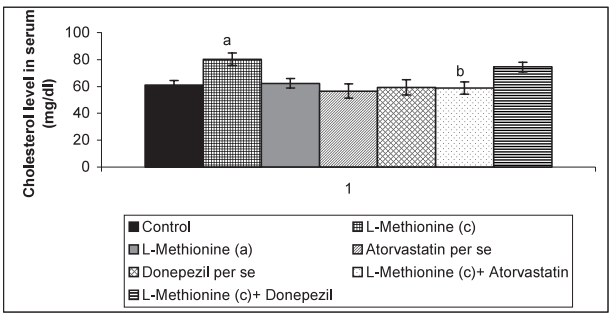

Fig. 5. Effect of Atorvastatin, Donepezil on L-Methionine-induced Changes in Serum Total Cholesterol Level

L-Methionine (c) = L-Methionine chronic; L-Methionine (a) = L-Methionine acute. Each group (n = 10), represent mean ± S.E.M. a = $p < 0.05$ Vs. serum total cholesterol of control. b = $p < 0.05$ Vs. serum total cholesterol of L-methionine (c) treated group.

level (Table 2).

Effect of Atorvastatin/Donepezil on L-Methionine-induced Change in Serum Nitrite Level

Chronic administration of L-methionine (1.7 g/kg *p.o.*, 4 weeks and 4 day) produced a significant decrease in serum nitrite when compared versus vehicle treated rats. Treatment with atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day)/donepezil (0.1 mg/kg *p.o.*, 2 weeks and 4 day) prevented L-methionine-induced decrease in serum nitrite level in a significant manner (Fig. 4). Atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day)/donepezil (0.1 mg/kg *p.o.*, 2 weeks and 4 day)/acute L-methionine (1.7 g/kg *p.o.*, 4 day) treatment did not show any significant effect on serum nitrite level (Fig. 4).

Effect of Atorvastatin/Donepezil/L-Methionine-induced Change in Total Serum Cholesterol Level

Chronic administration of L-methionine (1.7 g/kg *p.o.*, 4 weeks and 4 day) produced a significant, increase in serum total cholesterol levels of animals when compared versus vehicle control. Treatment with atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day) attenuated L-methionine-induced rise in serum total cholesterol levels (Fig. 5). However, treatment with donepezil (0.1 mg/kg *p.o.*, 2 weeks and 4 day) did not alter L-methionine-induced rise in serum total cholesterol levels in a significant manner (Fig. 5). Atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day)/donepezil (0.1 mg/kg *p.o.*, 2 weeks and 4 day)/acute L-methionine (1.7 g/kg *p.o.*, 4 day) treatment did not show any significant effect on serum total cholesterol levels when compared versus vehicle control group (Fig. 5).

Effect of Atorvastatin/Donepezil on L-Methionine-induced Change in Brain AChE activity

Chronic administration of L-methionine (1.7 g/kg *p.o.*, 4 weeks and 4 day) produced a significant increase in brain AChE activity when compared versus vehicle treated rats. Treatment with atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day)/donepezil (0.1 mg/kg *p.o.*, 2 weeks and

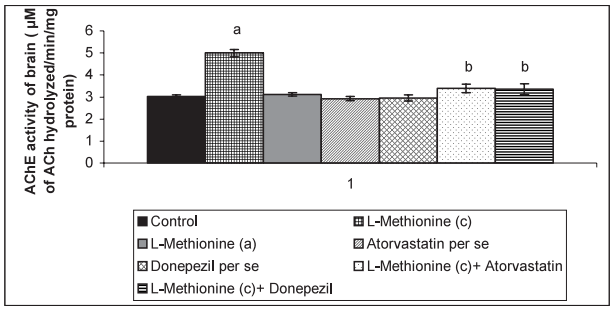

Fig. 6. Effect of Atorvastatin, Donepezil on L-Methionine-induced Changes Brain AChE Activity.

L-Methionine (c) = L-Methionine chronic; L-Methionine (a) = L-Methionine acute. Each group (n = 10), represent mean ± S.E.M. a = $p < 0.05$ Brain AChE activity of control. b = $p < 0.05$ Vs. brain AChE activity of L-methionine (c) treated group.

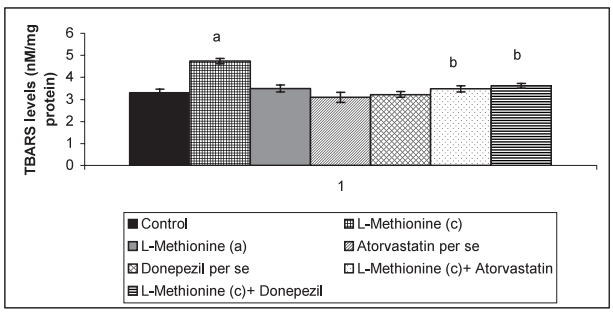

Fig. 7. Effect of Atorvastatin, Donepezil on L-Methionine-induced Changes in TBARS Level

L-Methionine (c) = L-Methionine chronic; L-Methionine (a) = L-Methionine acute. Each group (n = 10), represent mean ± S.E.M. a = $p < 0.05$ Brain TBARS level of control. b = $p < 0.05$ Vs. brain TBARS level of L-methionine (c) treated group.

4 day) significantly prevented L-methionine-induced rise in brain AChE activity. Atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day)/donepezil (0.1 mg/kg *p.o.*, 2 weeks and 4 day)/acute L-methionine (1.7 g/kg *p.o.*, 4 day) treatment did not show any significant effect on brain AChE activity (Fig. 6).

Effect of Atorvastatin/Donepezil on L-Methionine-induced Change in Oxidative Stress Levels of Brain

Chronic administration of L-methionine (1.7 g/kg *p.o.*, 4 weeks and 4 day) produced a significant increase in brain TBARS level (Fig. 7) and a decrease in the level of GSH (Fig. 8) when compared versus vehicle treated rats; hence reflecting induction of oxidative stress. Treatment with atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day)/donepezil (0.1 mg/kg *p.o.*, 2 weeks and 4 day) significantly prevented L-methionine-induced rise in oxidative stress (Figs. 7 and 8). Atorvastatin (10 mg/kg *p.o.*, 2 weeks and 4 day)/donepezil (0.1 mg/kg *p.o.*, 2 weeks and 4 day)/acute L-methionine (1.7 g/kg *p.o.*, 4 day) treatment did not show any significant effect on oxidative stress level (Figs. 7 and 8).

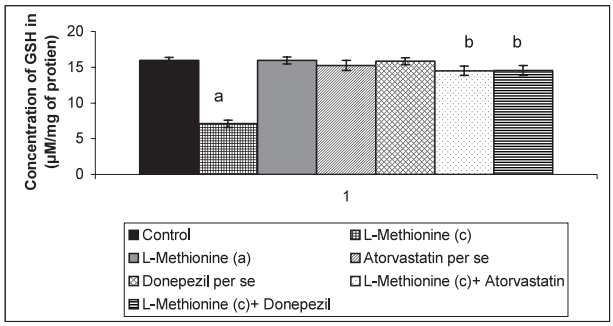

Fig. 8. Effect of Atorvastatin, Donepezil on L-Methionine-induced Changes in Brain GSH Level

L-Methionine (c) = L-Methionine chronic; L-Methionine (a) = L-Methionine acute. Each group (n = 10), represent mean ± S.E.M. a = $p < 0.05$ Brain GSH level of control. b = $p < 0.05$ Vs. brain GSH level of L-methionine (c) treated group.

DISCUSSION

The MWM employed in the present study is one of the most widely accepted models to evaluate learning and memory of animals.[13, 14] In the present investigation, control animals produced a significant decrease in day 4 ELT along with an increase in day 5 TSTQ. These results are consistent with our earlier findings[1, 9] and reports from other laboratory.[22]

Chronic but not acute L-methionine treatment for 4 weeks in the present study significantly raised serum homocysteine level, decreased serum nitrite levels and markedly attenuated Ach-induced endothelium-dependent relaxation; therefore reflecting endothelial dysfunction. L-methionine administration also produced a significant impairment of acquisition and retrieval of memory as reflected by decreased MWM performance. Moreover, an enhancement of brain AChE activity, increase in brain oxidative stress (as reflected by rise in brain TBARS and fall in GSH levels) and increase in serum total cholesterol levels were also observed. Recently, it has been reported that chronic L-methionine treatment and experimental Hhcy produce cognitive dysfunction[23, 24] and increase in brain AChE activity.[25] L-methionine-induced Hhcy is a well established model of experimental endothelial dysfunction.[10] Hhcy has been reported to induce endothelial dysfunction by decreasing the bioavailability of NO and by increasing vascular oxidative

stress.[6] Our observation also supports the above contention, since a significant fall in serum nitrite levels and an increase in oxidative stress levels (increase TBARS and decrease GSH) were noted in chronic L-methionine treated rats.

The increased level of homocysteine has been reported to produce change in structure and function of cerebral blood vessels along with oxidative stress, which play a key role in cerebral vascular dysfunction.[26] Hhcy have been shown to affect the function of cerebral circulation and L-methionine treatment is well reported to induce cerebrovascular dysfunction.[24, 26, 27] L-methionine-induced Hhcy has been reported to be involved in brain microvascular endothelial cell remodeling, which is ameliorated by administration of L-arginine (nitric oxide precursor); hence also suggesting the possibility of NO-dependent pathways in L-methionine-mediated dementia.[28] Several lines of evidence have strongly suggested a direct relationship between vascular endothelial dysfunction and dementia better known as VaD.[29, 30] Cerebral vascular endothelial dysfunction has also been shown to enhance progression of dementia of AD[30] and enhanced levels of brain AChE activity and oxidative stress have also been noted in patients with dementia of AD and other origin.[31] Hhcy has also been shown neurotoxic, and the neurotoxicity may be due to over-activation of N-methyl-D-aspartate receptors or by enhanced vulnerability of hippocampal neurons to excitotoxic insults and amyloid β-peptide toxicity.[32–34]

Methionine-rich diet in rats has been demonstrated to enhance cholesterol concentration in plasma and liver.[35] Several studies have revealed high serum cholesterol level as another important risk factor of AD.[36] Therefore chronic L-methionine-induced memory dysfunction in the present study may be attributed to its multiple effects *i.e.* decrease in serum nitrite level (endothelial dysfunction), rise in oxidative stress level, enhancement of brain AChE activity, and serum total cholesterol as well as direct neurotoxicity.

In the present study, treatment with atorvastatin (used as standard) significantly improved chronic L-methionine-induced endothelial dysfunction manifested in the terms of endothelium-dependent relaxation; increased serum nitrite levels, decreased serum homocysteine, and decreased oxidative stress (decrease TBARS and increase GSH). Statins have been demonstrated to enhance expression of eNOS in human endothelial cells.[37] They have been known to activate Akt/protein kinase B, which subsequently activates eNOS.[38] In a recent report from our laboratory, atorvastatin has been documented to improve the function of endothelium by decreasing the production of reactive oxygen species.[10] Furthermore, since the major mechanism suggested for the adverse effect of homocysteine on vascular function involves oxidative stress[39] and statins including atorvastatin have been reported to exert beneficial effect on Hhcy via their potential anti-oxidative property.[39, 40] Therefore atorvastatin-induced improvement of endothelial dysfunction may be attributed to its stimulatory effect on endothelial NO production and its antioxidative action.

In the present investigation, atorvastatin also attenuated chronic L-methionine-induced increase in AChE activity and cholesterol levels along with improvement in memory deficits. Several recent studies have reported beneficial effects of statins on memory dysfunctions-associated with dementia of AD and other origin.[41, 42] Studies involving neuroblastoma (SH-SY5Y) cells[43] and transgenic mice[44] have revealed that statins induce high expression of alpha7 nicotinic acetyl cholinergic receptors (nAChR), decrease cholinesterase activities, and increase alpha amyloid precursor proteins (APPs). Therefore suggesting involvement of these primary candidates in neuroprotective mechanism of statins for AD treatment. In our earlier findings, we have also reported that atorvastatin, simvastatin and pitavastatin reversed memory deficits of rats and mice associated with dementia of AD type.[1, 9] Statins are also known to improve cerebral blood flow,[45] and it has also been reported that agents that improve cerebrovascular function also improve cognition.[46] Therefore noted memory restorative effect of atorvastatin in this study may be due to its beneficial effect on cerebrovascular function exerted via its cholesterol-dependent as well as cholesterol-independent actions.

In the present investigation, donepezil was shown to improve L-methionine-induced endothelial dysfunction, as reflected by endothelium-dependent relaxation; increased serum nitrite levels, decreased serum homocysteine and decrease in oxidative stress levels. Donepzil is known to exert its therapeutic action by inhibiting AChE enzyme and thereby causing an increase in ACh level at the synapse. ACh has been shown to modulate

the level of NO in brain[47] as well as in peripheral tissues.[48] Donepezil is also reported to have antioxidative and neuroprotective actions.[49] Hence donepezil-mediated antioxidative activity with consequent modulation of serum homocysteine and NO levels may be responsible for its protective action in chronic L-methionine-induced endothelial dysfunction.

Donepezil in our study also reversed memory deficit induced by chronic L-methionine (Hhcy). Central cholinergic system plays an important role in the process of learning and memory. Its hypofunction may induce aspects of dementia such as memory loss and disorientation seen in AD. As mentioned above central ACh levels modulate NO concentration in the brain. Many studies in the recent years have implicated a vital role for NO in neurophysiological process of learning and memory.[50] Inhibition of NO system impaired memory in rats.[51, 52] Whereas, stimulation of NO production improved cognitive function in AD patients.[53] NO donors such as molsidomine reversed scopolamine-induced amnesia in rats. NO probably acts as retrograde messenger in the formation of long-term potentiation (LTP) at the molecular level of learning and memory processes.[54] Donepezil is recently approved; drug to be used clinically for memory impairment of AD. Central anti-AChE activity and up-regulation of alpha7-nicotinic receptors are key targets of donepezil-mediated beneficial effects in AD.[55–57] Donepezil as mentioned above also possesses potential neuroprotective and anti-oxidative actions.[49] Therefore donepezil in the present investigation appears to reverse chronic L-methionine-induced memory deficits via multiple actions *viz.*; decrease in brain oxidative stress (decrease TBARS and increase GSH) levels, AChE activity and increase in serum nitrite levels. Furthermore, donepezil mediated beneficial effect in endothelial dysfunction with subsequent improvement of cerebrovascular function may also contribute in reversing chronic L-methionine-induced memory deficits. To the best our knowledge, this is the first report demonstrating that donepezil induces improvement of chronic L-methionine-induced dementia.

On the basis of the above discussion, it may be concluded that chronic L-methionine administration (Hhcy) produces endothelial dysfunction along with impairment of learning and memory (vascular dementia). Our results suggest the potential of donepezil in Hhcy-induced endothelial dysfunction and associated memory deficits.

Acknowledgements The authors would like to thank Dr. A. K. Tiwary for constant support and keen interest in this project. We are also thankful to Ind Swift Ltd. and Wokhardt Ltd. for supplying the free sample of atorvastatin and donepezil respectively.

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