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RESEARCH ARTICLE

Curcumin loaded Solid lipid nanoparticles enhanced efficacy in vascular dementia against Homocysteine induced toxicity

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Abstract

Vascular dementia (VaD), a diverse group of brain disorders with cognitive decline is attributable to cerebrovascular pathologies. Recent studies have shown that mitochondrial dysfunctions and oxidative stress are involved in cognitive decline. The aim of the present study was designed to evaluate the effects of Curcumin loaded solid lipid nanoparticles (Cur-SLNP) in vascular dementia (VaD) against homocystiene (HCY) induced toxicity by behavioral and biochemical assessment in different regions of the brain. The sign of VaD i.e., learning and memory levels were evaluated with different behavioural assessment like plus maze test. Neurobehavioral analyses revealed that Cur-SLNP administration successfully ameliorated cognitive decline observed in HCY rats. Compared to HCY administration, Cur-SLNP 10 mg/kg and 25 mg/kg administration showed ameliorative effect in behavioural and biochemical assessment induced by HCY. We found that lipid peroxidation levels decreased significantly in striatum ($p<0.01$), cortex ($p<0.001$), and hippocampus ($p<0.001$) after oral administration of Cur-SLNP with HCY animals. Hence, the present findings suggest that the neuroprotective role of Cur-SLNP against HCY induced toxicity could be a novel and promising therapeutic strategy in VaD as well in other age-related neurodegenerative disorders.

Keywords: Vascular dementia; Curcumin; Solid lipid nanoparticles; Homocysteine; Cognitive decline

Introduction

The impairment of cerebral blood flow is one of the primary pathophysiological events for the development of vascular dementia (VaD) [1]. VaD occurs as a result of blocked or diseased vascular system with reduced blood supply to the brain [2, 3]. Impairments in executive frontal lobe functions, such as attention, planning, and speed of mental processing, have observed in VaD [4]. The global incidence of VaD is higher than prevalence rate. Worldwide it is estimated around 6 to 12 cases per every 1000 population and it occurs above the 70 years of age [5]. The average duration of the development of VaD pathogenesis is around 5 years and the survival rate are lower than Alzheimer's disease (AD). In India, VaD is preventable with some alternative therapy like herbal approach, aroma therapy, Ayurvedic treatments and other lifestyle management [6].

Polyphenols are natural substances that are present in plants, fruits, and vegetables, including olive oil, red wine, and tea [7]. The yellow pigment extracted from the rhizome of Curcuma longa, curcumin, a polyphenolic non-flavonone compound, is the pharmacologically active substance of turmeric [8]. Curcumin is nontoxic and has antioxidant, anti-inflammatory, and antiproliferative activities. Curcumin shows antioxidant activity equivalent to vitamins C and E [9]. Homocysteine (HCY); a sulfur containing amino acid derived from the metabolism of methionine, is an independent risk factor for cardiovascular disease [10]. The thiol group of HCY is readily oxidized in plasma and culture medium, resulting in the generation of reactive oxygen species (ROS). HCY is an excitatory amino acid,

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which markedly enhances the vulnerability of neuronal cells to excitotoxic and oxidative injury [11]. An elevated plasma level of HCY (more than 14 μM) is termed Hyperhomocysteinemia (HHCY) [12]. HCY is recognized as an independent risk factor for myocardial infarction, coronary artery disease, strokes, genetic disorders, Alzheimer's diseases (AD) and cognitive impairment [13].

Nano-antioxidants constitute a new wave in the delivery of antioxidants for prevention and treatment of neurodegenerative diseases involving oxidative stress [14]. During the past few years, solid lipid nanoparticles (SLNP) have attracted much attention in the field of drug delivery. SLNP present some excellent material properties, such as small particle size, biocompatibility, chemical and mechanical stability, and easy functionalization ability [15]. There are several methods of preparation of SLNP. In the current study, we primarily aimed to prepare curcumin-loaded solid lipid nanoparticles (Cur-SLNP) against HCY induced oxidative stress using behavioral studies, as well as biochemical parameters.

Materials and methods

Drugs and Chemicals

D-L-Homocysteine (HCY), Curcumin (95% curcumin and 5% of methoxycurcumin and bismethoxycurcumin as the other two curcuminoids) was purchased from National Scientific Products. Glyceryl monostearate (Gattefosse, India), Stearic acid, PVA, PEG 4000 (Loba chemie Pvt. Ltd. India), 2-Thiobarbituric acid (TBA) were also used in the study.

All other chemicals and reagents were of analytical grade and were used without further purification.

Animals

Male Sprague dawley rats (250 $\pm$ 20g) were purchased from Sri Raghavendra Enterprises, Bangalore, Karnataka and maintained in the animal house facility provided by Acharya Nagarjuna University with standard humidity (44 %–60 %) and temperature (22 $\pm$ 5 °C) with a 12 h light and dark cycle. Animals allowed to have free access to food and water ad libitum. The Institutional Animal Ethical Committee approved the experimental protocol (ANUCPS/IAEC/AH/P/14/2019), of Acharya Nagarjuna University College of Pharmaceutical Sciences, and all the experiments were conducted as per the committee for the purpose of control and supervision of experiments on animals (CPCSEA) guidelines, Govt. of India.

Dose selection

The doses of HCY, Cur, and Cur-SLNP were selected from previous literature [16].

Treatment Schedule

The entire treatment protocol was for 14 days. In this experimental design, rats were randomly divided into four groups, consists of 6 animals each and treatment schedule was summarized as follows. Group - I : normal control with saline i.v. ; Group - II : HCY (400 μg/kg, i.v) ;Group – III : Cur (25mg/kg, p.o.) + HCY (400 μg/kg, i.v);Group- IV : Cur (50mg/kg, p.o.) + HCY (400 μg/kg, i.v);Group - V : Cur-SLNP (10 mg/kg, p.o.) + HCY (400 μg/kg, i.v);Group-VI : Cur-SLNP (25 mg/kg, p.o.) + HCY (400 μg/kg, i.v)

All the treatments were continued for 14 days. DL-Homocystiene was dissolved in normal saline (0.9% NaCl) administered through caudal vein. On the 0, 7 &14^{th} days after 24 hr of treatment the animals were assessed for various physical and behavioural parameters and on 15^{th} day animals were euthanized under diethyl ether to isolate the brain samples and they were used for biochemical estimations on different parts of the brain i.e in striatum, cortex and hippocampus.

Preparation of Cur-SLNP

The Curcumin loaded SLNP (Cur-SLNP) were prepared according to a modified emulsion/solvent evaporation method **[17]** (Fig. 1). In brief, 17.5 mg Curcumin, 42.5 mg phospholipid, and 100 mg GMS were dissolved in ethanol (organic phase) at 60 ° C. The aqueous phase (1% PVA containing 1% PEG 400, w/v) was heated to the same temperature of the organic phase. Then the organic phase was dropped into the hot aqueous phase under rapid stirring at 1200 rpm for dispersion. After that the homogeneous suspension was poured into the dispersed phase (1% Tween-60 containing 1% PEG 400, w/v) under stirring at 1000 rpm for 4 h at 2 ° C in an ice bath to allow for the hardening of the SLNP.

Characterization of Cur-SLNP

Particle size analysis

Mean diameter (Z-average) and polydispersity index (PDI) of the SLNP in the dispersion were measured by dynamic light scattering using Zetasizer (Mastersizer 2000, Malvern Instruments, UK) at 25°C. PDI was used as measurement of size distribution i.e. heterogeneity in the size of molecules present in a sample. Before analysis, the formulation was suitably diluted with double distilled water and gently agitated for 10 minutes. The measurements were performed in triplicates.

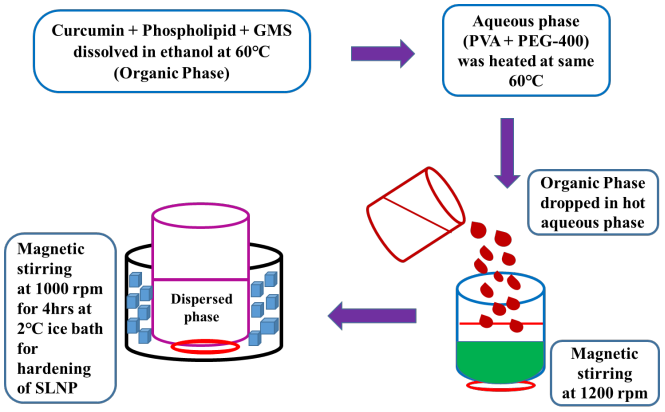

Fig.1 Method of Preparation of Cur-SLNP by Solvent evaporation method

Figure 1 Method of preparation of solid lipid nanoparticles

Zeta potential

Zeta potential of prepared SLNP was measured to assess the surface charge and stability. Zeta potential of SLNP was assessed by determining the particle electrophoretic mobility using Zetasizer (Mastersizer 2000, Malvern Instruments, UK) at 25°C. Samples were prepared by re-dispersing the lyophilized nanoparticles in double distilled water as dispersing medium. The measurements were repeated thrice for each sample.

Field Emission Scanning Electron Microscopy (FESEM)

Shape and surface morphology of Cur-SLNP were observed using FESEM at an excitation voltage of 10 kV (SU8010 Hitachi). A few drops of Cur-SLNP allowed to dry on a coverslip. The samples were coated with platinum using an Ion Sputter (MC-1000). Photo-micrographs of Cur-SLNP were taken at suitable magnification.

Behavioral assessment

Assessment of cognitive performance

Elevated plus maze test The cognitive performance was measured by elevated plus maze test. The plus maze having four arms (50 cm×10 cm) with two open arms and two closed arms, closed with 40 cm height wall connected with a central platform of 10×10 cm dimensions. Memory acquisition was measured on the 0th, 7th and 14th day of the experiment by placing the animals individually on the open arm facing opposite to the central platform; the time taken by the animal to move from an open arm to the closed arm was measured as transfer latency (TL) [18].

Preparation of brain homogenate for biochemical estimations

On the 15th day, animals were euthanized under deep anesthesia by using ketamine hydrochloride (50 mg/kg, i.p) and xylazine (10 mg/kg, i.m). The brains were isolated and washed with the ice-cold isotonic saline solution by removing the cerebellum. Half of the brain hemispheres were separated and sectioned into the striatum, cortex, hippocampus, and were homogenized in ice-cold extraction buffer solution (10mM Tris-HCl, pH 7.4, 0.44M sucrose, 10mM EDTA and 0.1% BSA). The homogenates were centrifuged at 3354 ×g (Thermo scientific, sorvall-ST8R) with 4 °C for 30 min of time to get the supernatants. The supernatants once again were centrifuged at 3354 ×g at 4 °C for 45 min. The mitochondrial pellets were collected, washed with extraction buffer and the solutions were centrifuged at 3354 ×g at 4 °C for 45 min. The pellets were re-suspended in suspension buffer (0.44M sucrose in 10mM Tris-HCl, pH 7.4) and suspensions were used for the estimations. Before re-centrifugation, some portions of the supernatants were used for the estimations of oxidative stress parameters [19].

Measurement of Biochemical assessment

Lipid peroxidation

Lipid peroxidation is the measure of cellular injury by producing malonaldehyde (MDA). In this present assay, we measured cellular injury by measuring MDA levels in the tissue homogenate. As described by Ohkawa et al. to the 0.1 ml of tissue sample added 2 ml of the thiobarbituric acid reaction mixture (TBAR) consists of trichloroacetic acid (1 ml of 10% solution) and thiobarbituric acid (1 ml of 0.67% solution). The reaction mixture allowed to boil in a water bath for 30 min and cooled on an

ice bath for 10 min. After cooling the reaction mixture was centrifuged for 10 min at 4830 $\times$g to collect the supernatant and was observed for absorbance at 532 nm (Shimadzu UV-1800, UV–vis spectrophotometer). The values were expressed in the nmol MDA/g wet weight of tissue, and the calculation was done by using an extinction coefficient of 1.56×105 $M-1$ cm-1 [20, 21].

Statistical analysis

Each group of 6 animals (n=6) animals was assigned to a specific drug treatment. All the values are expressed as means $\pm$ S.E.M and were statistically analyzed by using Graph Pad Prism software (Version 5.01). One-way analysis of variance (ANOVA) followed by Tukey's post hoc test for behavioral assessments and oxidative stress parameters in striatum, cortex and hippocampus. In all the tests a P value < 0.05 was statistically significant.

Results and discussion

Particle size (Z-average) analyses revealed that Cur-SLNP were of homogenous size. Average particle size of Cur-SLN was found to be 154.8 nm as measured by dynamic light scattering (DLS). The actual particle size of SLNP produced was found to be < 200 nm. Polydispersity index (PDI), a measurement of size distribution, of Cur-SLNP formulation was found to be 0.928 **(Fig. 2).** The PDI below 0.3 indicates that the lipid nanoparticles are homogeneous in size and were in a state of acceptable monodispersity distribution, with low variability and no aggregation [22] [23] . FE-SEM analysis further confirmed that the Cur-SLNP preparation consisted of spherical and smooth surface morphology **(Fig. 3).** These results clearly showed that Cur-SLNP prepared were of uniform size and shape.

Zeta potential was evaluated to determine particle surface charge, which could be used to stabilize colloidal suspension. Nanoparticles have a natural tendency to aggregate, however, Nanoparticles with high negative or positive zeta potential would repel each other. It has been shown that particles could stably disperse when the absolute value of zeta potential is above 30 mV due to electrostatic repulsions between the particles, while potentials of 5-15 mV resulted in limited flocculation in solution. Our results showed that the zeta potential of Cur-SLNP formulation was -10.9 mV **(Fig. 3).** The observed zeta potential suggests that Cur-SLNP prepared may be stable for longer periods.

During the treatment schedule, Plus-maze was used to measure the memory by transfer latency time on 7^{th} day and 14^{th} day was represented in **Table. 1 and Fig. 5**. On 0^{th} day, there was no significant change in the groups. On the 7^{th} day, HCY treated group showed decreased transfer latency time with the significance (p<0.001) when compared with control group and HCY group showed ameliorative effect with the significant (p<0.001) change by decreased transfer latency time when compared with (Cur – 50mg + HCY, Cur-SLNP (10 mg/kg/day and 25 mg/kg/day)) + HCY. Cur-25mg + HCY treated group showed significant improvement (p<0.05, p<0.01, and p<0.001) when compared with (Cur – 50mg + HCY, Cur-SLNP (10 mg/kg/day and 25 mg/kg/day)) + HCY. On the 14^{th} day, there was a decreased loss of memory in HCY treated group when compared with control group and HCY treated group showed decreased transfer latency time with significance (p<0.05, p<0.001, and p<0.001) when compared with (Cur – 50mg + HCY, Cur-SLNP (10 mg/kg/day and 25 mg/kg/day)) + HCY. Cur-25mg + HCY treated group showed significant improvement (p<0.01) when compared with Cur-SLNP (10mg/kg/day) + HCY. Cur-50mg + HCY group showed increased significance in the loss of memory (p<0.01) compared with Cur-SLNP (25 mg/kg) + HCY.

In lipid peroxidation Table. 2 and Fig. 6 represents the results observed in striatum, cortex, and hippocampus. Homocysteine (HCY) treatment group significantly increased (p<0.001) the MDA level in striatum, cortex and hippocampus regions of the brain as compared to control group.

In striatum, HCY group showed significant change when compared to (p<0.01) with Cur (50 mg) + HCY, (p<0.001) with Cur – SLNP (10mg/kg/day and 25 mg/kg/day) + HCY. Cur (25 mg) + HCY group showed significant change (p<0.01 and p<0.001) when compared with Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY. Cur (50 mg) + HCY group showed significant change (p<0.01) when compared with Cur-SLNP (25 mg/kg/day) + HCY.

In cortex, HCY group showed significant change when compared to (p<0.05) with Cur (25 mg) + HCY, (p<0.01) with Cur (50 mg) + HCY, (p<0.001) with Cur – SLNP (10mg/kg/day and 25 mg/kg/day) + HCY. Cur (25 mg) + HCY group showed significant change (p<0.01 and p<0.001) when compared with Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY. Cur (50 mg) + HCY group showed significant change (p<0.05 and p<0.001) when compared with Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY.

In hippocampus, HCY group showed significant change when compared to (p<0.05) with Cur (50 mg) + HCY, (p<0.001) with Cur – SLNP (10mg/kg/day and 25 mg/kg/day) + HCY. Cur (25 mg) + HCY group showed significant change (p<0.05 and p<0.001) when compared with Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY. Cur (50 mg) + HCY group showed significant change (p<0.05 and p<0.001) when compared with Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY.

Oxidative neurotoxicity plays an important role in the pathogenesis. Literature reports revealed that increasing HCY concen-

Measurement Results

Date	:	31 August 2019 12:16:44
Measurement Type	:	Particle Size
Sample Name	:	C-SLN-Size
Scattering Angle	:	90
Temperature of the holder	:	25.0 deg. C
T% before meas.	:	29168
Viscosity of the dispersion medium	:	0.895 mPa.s
Form Of Distribution	:	|Standard|
Representation of result	:	Scattering Light Intensity
Count rate	:	4281 kCPS

Calculation Results

Peak No.	S.P.Area Ratio	Mean	S. D.	Mode
1	1.00	191.7 nm	11.6 nm	189.8 nm
2	---	--- nm	--- nm	--- nm
3	---	--- nm	--- nm	--- nm
Total	1.00	191.7 nm	11.6 nm	189.8 nm

Histogram Operations

Size (Median)	:	189.8 nm
Mode	:	189.8 nm
% Cumulative (1)	:	10.0 (%) - 174.8 (nm)
% Cumulative (2)	:	50.0 (%) - 189.8 (nm)
% Cumulative (3)	:	90.0 (%) - 212.2 (nm)
% Cumulative (4)	:	30.0 (%) - 182.2 (nm)
% Cumulative (5)	:	40.0 (%) - 186.0 (nm)
% Cumulative (6)	:	50.0 (%) - 189.8 (nm)
% Cumulative (7)	:	20.0 (%) - 178.5 (nm)
% Cumulative (8)	:	70.0 (%) - 199.8 (nm)
% Cumulative (9)	:	95.0 (%) - 215.4 (nm)
% Cumulative (10)	:	100.0 (%) - 8510.6 (nm)

Cumulant Operations

Z-Average	:	154.8 nm
PI	:	0.928

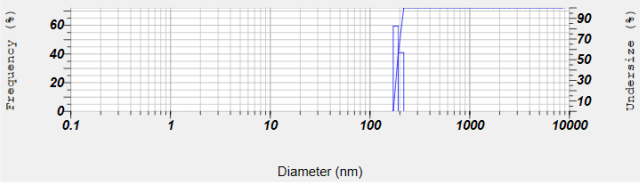

Figure 2 Particle size

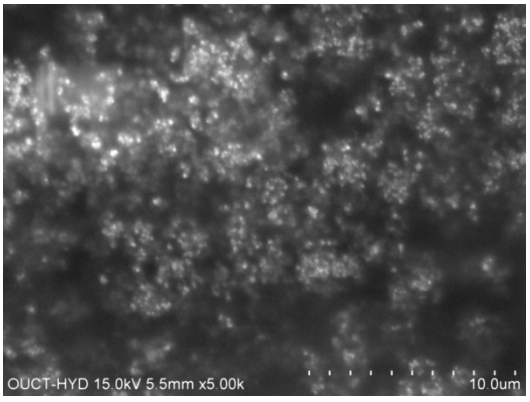

Figure 3 FESEM of Cur-SLNP

trations enhanced the TBARS formation in brain synaptosomes in a concentration-dependent manner [24]. In this study, results were also in line with these findings.

When the production of oxidative free radicals increases or the protection mechanism is decreased, they cause cellular damage leading to the release of MDA. It is a product of tissue lipid peroxidation indicates the level of tissue damage by its quantity. Several studies on Curcumin proved that antioxidant activity of solid lipid nanoparticles improved learning and memory deficits by protecting the nervous system against HCY toxicity [25, 26].

The end product of lipid peroxidation MDA might be used as a biomarker for the assessment of treatment efficacy of the various drugs used in VaD. Furthermore, maintaining of the antioxidant defense system of all the three regions of the brain is essential for reversing the oxidative stress induced by HCY. Treatment with the Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) +

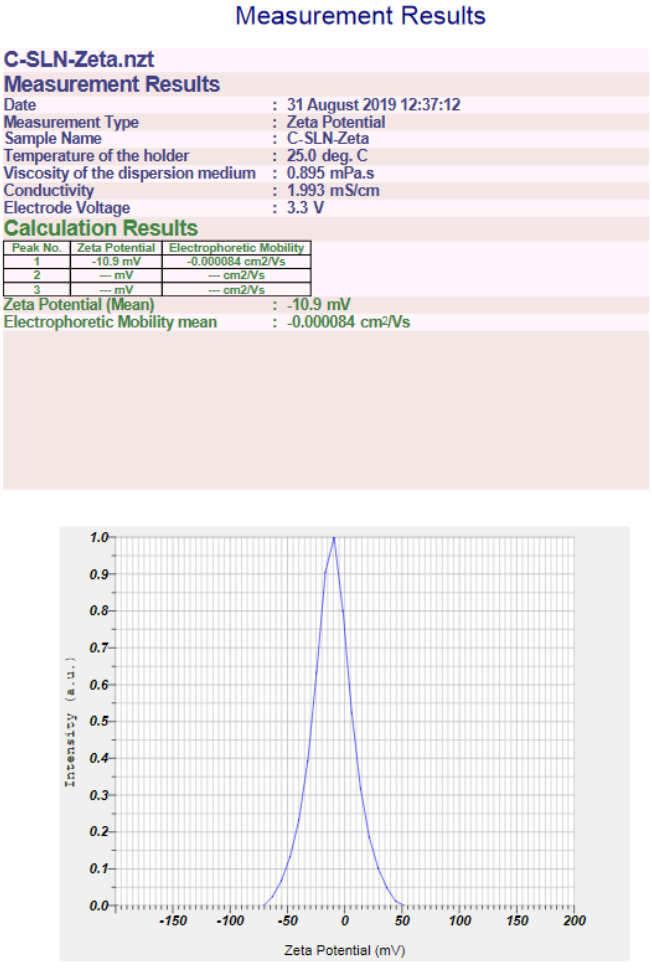

Figure 4 Zeta potential of Cur-SLNP

Table 1 Effect of Cur-SLNP on elevated plus maze

S.No	Treatment Groups	Transfer Latency time (Mean ± SEM)		
		0^{th} day	7^{th} day	14^{th} day
1	Normal	20.2 ± 2.40	29.0 ± 3.61	27.0 ± 4.36
2	HCY (400 μg/kg/day)	21.0 ± 3.15	92.0 ± 10.8 A***	83.3 ± 6.94 A***
3	Cur (25 mg) + HCY	22.8 ± 4.18	76.3 ± 3.84	65.0 ± 4.58
4	Cur (50 mg) + HCY	22.8 ± 4.99	41.7 ± 7.13 B*** C*	52.7 ± 5.90 B*
5	Cur-SLNP (10 mg) + HCY	18.8 ± 2.92	28.0 ± 5.69 B*** C**	29.3 ± 5.24 B*** C**
6	Cur-SLNP (25 mg) + HCY	22.6 ± 3.17	21.0 ± 3.79 B*** C***	17.0 ± 3.61 B*** C*** D**

Values are expressed as mean ± SEM (n=6). One-Way ANOVA followed by Tukey's post hoc test. ***p<0.001, ** p<0.01, *p<0.05 were considered as statistically significant. A-HCY compared with control group; B-HCY compared with Cur – 25mg + HCY, Cur – 50mg + HCY, Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY; C-Cur – 25mg + HCY compared with Cur – 50mg + HCY, Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY; D-Cur – 50mg + HCY compared with Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY; E-Cur-SLNP (10mg/kg) + HCY compared with Cur-SLNP (25mg/kg) + HCY

Table 2 Effect of Cur-SLNP on MDA in striatum, cortex, and hippocampus.

S.No	Treatment Groups	MDA (nm/gm wet tissue) Striatum	Cortex	Hippocampus
1	Normal	3.83 ± 0.581	2.84±1.04	4.61 ± 0.849
2	HCY (400 μg/kg/day)	15.5 ± 1.26 A***	13.5 ± 0.681 A***	11.6 ± 0.731 A***
3	Cur (25 mg) + HCY	11.2 ± 1.11	9.40 ± 0.483 B*	8.83 ± 0.510
4	Cur (50 mg) + HCY	8.33 ±1.25 B**	8.17 ± 0.383 B**	8.01 ± 0.599 B*
5	Cur-SLNP (10 mg) + HCY	5.00 ± 0.794 B*** C**	4.82 ± 0.677 B*** C** D*	4.75 ± 0.664 B*** C* D*
6	Cur-SLNP (25 mg) + HCY	2.42 ± 0.510 B*** C*** D**	2.48 ± 0.662 B*** C*** D***	2.37 ± 0.642 B*** C*** D***

Values are expressed as mean ± SEM (n=6). One-Way ANOVA followed by Tukey's post hoc test. ***p<0.001, ** p<0.01, *p<0.05 were considered as statistically significant. A-HCY compared with control group; B-HCY compared with Cur – 25mg + HCY, Cur – 50mg + HCY, Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY; C-Cur – 25mg + HCY compared with Cur – 50mg + HCY, Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY; D-Cur – 50mg + HCY compared with Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY; E-Cur-SLNP (10mg/kg) + HCY compared with Cur-SLNP (25mg/kg) + HCY

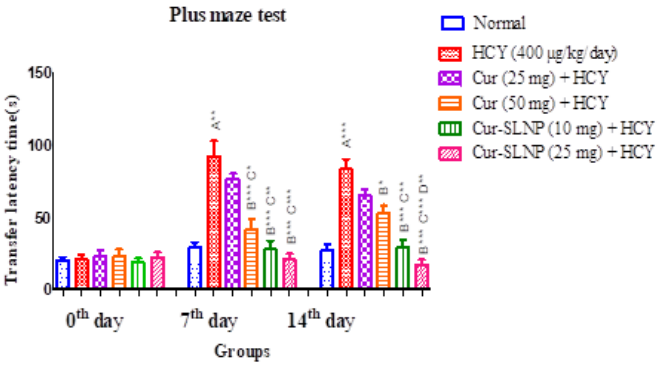

Figure 5 Effect of Cur-SLNP on Plus – maze. Values are expressed as mean ± SEM (n=6). One-Way ANOVA followed by Tukey's post hoc test. ***p<0.001, ** p<0.01, *p<0.05 were considered as statistically significant. A-HCY compared with control group; B-HCY compared with Cur – 25mg + HCY, Cur – 50mg + HCY, Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY; C-Cur – 25mg + HCY compared with Cur – 50mg + HCY, Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY; D-Cur – 50mg + HCY compared with Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY; E-Cur-SLNP (10mg/kg) + HCY compared with Cur-SLNP (25mg/kg) + HCY

HCY significantly reduced the MDA by inhibiting the lipid peroxidation, at all the three regions of the brain. Most of these studies relate the ameliorative effect of naturally available active constituents to their reversing the oxidative stress by scavenging the reactive oxygen species. In the present study, Curcumin solid lipid nanoparticles exhibited neuroprotection by decreasing the elevated levels of lipid peroxidation, indicating its neuroprotection against HCY neurotoxicity might be due to its antioxidant potential.

In conclusion, the prepared Cur-SLNP showed mean parti-cle size, PDI and zeta potential for uniform size, shape, and stability for longer duration. The study demonstrates that Cur-SLNP ameliorate the VaD induced oxidative stress and cognitive deficits. The efficacy of Curcumin was enhanced by using SLNP. This study summarizes that Cur-SLNP improved bioavailability (BA) to cross BBB and would be a great success in reduction of dose. Therefore, Cur-SLNP may be potential therapeutic strategy in ameliorating HCY induced VaD by behavioural assessment and attenuated the biochemical assessment in different regions of the brain. Treatment with Cur-SLNP ameliorated the special memory impairment by decreasing transfer latency in plus maze and reduced MDA levels. Hence, Cur-SLNP could be considered as a promising therapeutic agent in the management of VaD, but further studies needed for the understating of molecular mechanisms involved in the neuroprotection of Cur-SLNP against HCY induced neurotoxicity.

Conflict of interest

There is no conflict of interest.

Acknowledgments

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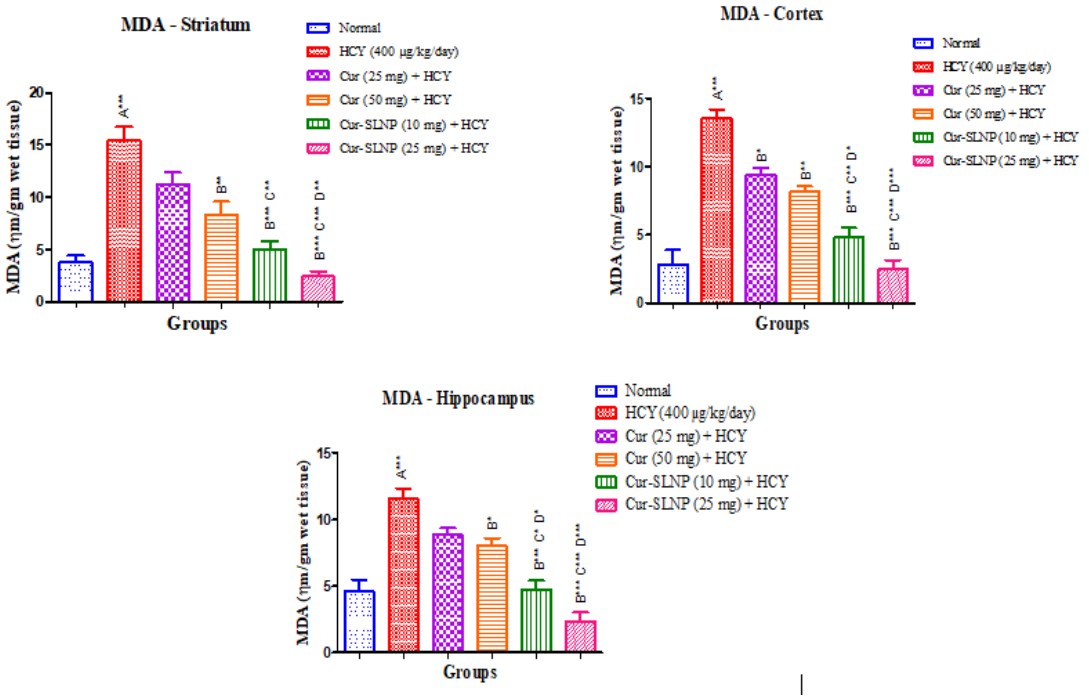

Figure 6 Effect of Cur-SLNP on MDA in striatum, cortex, and hippocampus . Values are expressed as mean ± SEM (n=6). One-Way ANOVA followed by Tukey's post hoc test. ***p<0.001, ** p<0.01, *p<0.05 were considered as statistically significant. A-HCY compared with control group; B-HCY compared with Cur – 25mg + HCY, Cur – 50mg + HCY, Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY; C-Cur – 25mg + HCY compared with Cur – 50mg + HCY, Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY; D-Cur – 50mg + HCY compared with Cur-SLNP (10 mg/kg/day and 25 mg/kg/day) + HCY; E-Cur-SLNP (10mg/kg) + HCY compared with Cur-SLNP (25mg/kg) + HCY

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