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Neuroprotective effect of curcumin on okadaic acid induced memory impairment in mice

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ABSTRACT

Okadaic acid (OKA) has been observed to cause memory impairment in human subjects having seafood contaminated with dinoflagellate (*Helicondria okadai*). OKA induces tau hyperphosphorylation and oxidative stress leading to memory impairment as our previous study has shown. Curcumin a natural antioxidant has demonstrated neuroprotection in various models of neurodegeneration. However, the effect of curcumin has not been explored in OKA induced memory impairment. Therefore, present study evaluated the effect of curcumin on OKA (100 ng, intracerebrally) induced memory impairment in male Swiss albino mice as evaluated in Morris water maze (MWM) and passive avoidance tests (PAT). OKA administration resulted in memory impairment with a decreased cerebral blood flow (CBF) (measured by laser doppler flowmetry), ATP level and increased mitochondrial $(Ca^{2+})i$, neuroinflammation (increased TNF-α, IL-1β, COX-2 and GFAP), oxidative-nitrosative stress, increased Caspase-9 and cholinergic dysfunction (decreased AChE activity/expression and α7 nicotinic acetylcholine receptor expression) in cerebral cortex and hippocampus of mice brain. Oral administration of curcumin (50 mg/kg) for 13 days significantly improved memory function in both MWM and PAT along with brain energy metabolism, CBF and cholinergic function. It decreased mitochondrial $(Ca^{2+})i$, and ameliorated neuroinflammation and oxidative-nitrostative stress in different brain regions of OKA treated mice. Curcumin also inhibited astrocyte activation as evidenced by decreased GFAP expression. This neuroprotective effect of curcumin is due to its potent anti-oxidant action thus confirming previous studies. Therefore, use of curcumin should be encouraged in people consuming sea food (contaminated with dinoflagellates) to prevent cognitive impairment.

1. Introduction

There are several reports which demonstrate that the population consuming sea food containing dinoflagellates (*Helicondria okadai*) suffer from memory impairment due to presence of okadaic acid (OKA), a selective and potent inhibitor of serine/threonine phosphatases 1 (PP1) and 2A (PP2A) (www.aristatek.com/Newsletter/DEC07/DEC07ts.aspx; Cohen et al., 1990). This observation was also confirmed by our study which demonstrated that OKA (ICV) induced memory impairment in rats due to oxidative stress and increased mitochondrial calcium ion $(Ca^{2+})i$ (Kamat et al., 2010). The OKA increased levels of $(Ca^{2+})i$ which lead to impairment of mitochondrial electron transport system resulting in mitochondrial dysfunction and generation of intracellular reactive nitrogen and oxygen species. This initiated apoptotic cell death as evident by increased Caspase-3 and Caspase-9 in rat brain areas (Kamat et al., 2011). Reactive nitrogen and oxygen species also contribute in activation of glial cells, which initiates neuroinflammatory processes by releasing pro-inflammatory cytokines such as TNF-α and IL-1β (Tanaka et al., 2006; Kamat et al., 2012a). Further TNF-α stimulates COX-2 in neurons, astrocytes and microglia leading to neuronal and synaptic loss that is associated with neurodegeneration (Kukreja et al., 1986; Cakala et al., 2007; Niranjan et al., 2010).

OKA induces neurodegeneration along with Tau hyperphosphorylation; GSK3β activation which is a characteristic feature of Alzheimer's disease pathology (Kamat et al., 2013). Alzheimer's disease and other types of dementia are associated with a reduction in cerebral blood flow (CBF) due to oxidative stress and impaired endothelial function (Prohovnik et al., 1988; O'brien et al., 1992). Decreased CBF impairs glucose and energy metabolism which leads to increased cellular dysfunction (Olanow, 1993). Disturbed glucose and energy metabolism impairs cholinergic system which accounts nearly 50% of dementias (Areosa and Sherriff, 2003). This results in decreased level of choline acetyl

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transferase (ChAT) activity and acetylcholine (Blokland and Jolles, 1993). Functions of cholinergic system are also affected by α7 nicotinic acetylcholine receptor (α7 nAChR), present in basal forebrain cholinergic projection neurons and hippocampus (Nordberg and Winblad, 1986). There is loss or down regulation of the α7 nicotinic acetylcholine receptor in various forms of dementia as well as in OKA induced impairment of memory (Court et al., 2000; Kamat et al., 2012b).

Curcumin a polyphenolic flavonoid found in turmeric (*Curcuma longa*), is a yellow curry spice with a long history of use in Indian cuisine and traditional herbal medicines (Ammon and Wahl, 1991). It shows anti-oxidant, free radical scavenger (Molina-Jijon et al., 2011), anti-inflammatory (Buhrmann et al., 2011), anti-amyloid (Wang et al., 2010) and anti-ischemic properties (Shukla et al., 2008). It crosses blood brain barrier (Mishra and Palanivelu, 2008) and gives, as we have reported, neuroprotection in streptozotocin induced memory impaired rats (Agrawal et al., 2010; Awasthi et al., 2010). In this study, we are investigating the neuroprotective effect of curcumin in OKA induced memory impairment model which causes tau hyperphosphorylation thus mimicking certain aspects of Alzheimer's disease. Further, we explored the effect of curcumin on mitochondrial $(Ca^{2+})i$, neuroinflammation, brain energy metabolism, cerebral microcirculation, neuronal apoptosis, brain atrophy and cholinergic dysfunction.

2. Materials and methods

2.1. Animals

All experiments were carried out with male Swiss albino mice (25–30 g). Mice were procured from the Division of Laboratory Animal Services, Central Drug Research Institute, Lucknow, India. Experiments were approved by Institutional Animal Ethics Committee (IAEC), and performed according to internationally followed ethical standards. Mice were kept in a polyacrylic cages (22.5 × 37.5 cm) and maintained under standard housing conditions (room temperature 23–25 °C and humidity 60–65%) with a 12 h light and dark cycle. Food and water were available ad libitum but food was not allowed from 1 h prior to the behavioural study.

2.2. Materials

Curcumin was purchased from Kancor, India. Okadaic acid, acetylthiocholine iodide (AChI), chloral hydrate, 2,7-dichlorofluorescin-diacetate (DCF-DA), Fura-2AM and other biochemical's were purchased from Sigma-Aldrich, USA. ATP colorimetric/fluorometric assay kit was purchased from Biovision, USA (Catalog ♯K354-100). Primer for AChE, α7 nicotinic acetylcholine receptor, GFAP, TNF-α, IL-1β, COX-2, Caspase-9 and β-actin was purchased from Integrated DNA technology (IDT), USA. Taq PCR kit (Fermentas, USA) and cDNA synthesis kit (Qiagen, USA).

2.3. Drug administration

2.3.1. Standardization of intracerebral (IC) okadaic acid induced memory impairment in mice

The mice were anesthetized with chloral hydrate (300 mg/kg, qi.p.). A midline sagittal incision was made in the scalp. A 27 gauge hypodermic needle attached to a 100 μl Hamilton syringe was inserted (2.5 mm depth) perpendicularly through the skull into the brain. Okadaic acid, dissolved in freshly prepared sterile artificial cerebrospinal fluid (aCSF-147 mM NaCl, 2.9 mM KCl, 1.6 mM $MgCl_2$, 1.7 mM $CaCl_2$ and 2.2 mM dextrose) was infused once (50 and 100 ng/10 μl) by IC route (Haley and McCormick, 1957) but only dose of 100 ng OKA (IC) showed a significant memory impairment in Morris water maze and passive avoidance tests as evaluated after 13 days (Kamat et al., 2010) (Fig. 1).

2.3.2. Experimental protocol and administration of curcumin

To study the preventive effect of curcumin, a dose of (50 mg/kg, p.o.) was selected on the basis of our earlier lab report in which significant memory improving ability and anti-oxidant property of curcumin has been demonstrated (Awasthi et al., 2010). Curcumin was suspended in gum acacia (1.0% w/v) and administered for 13

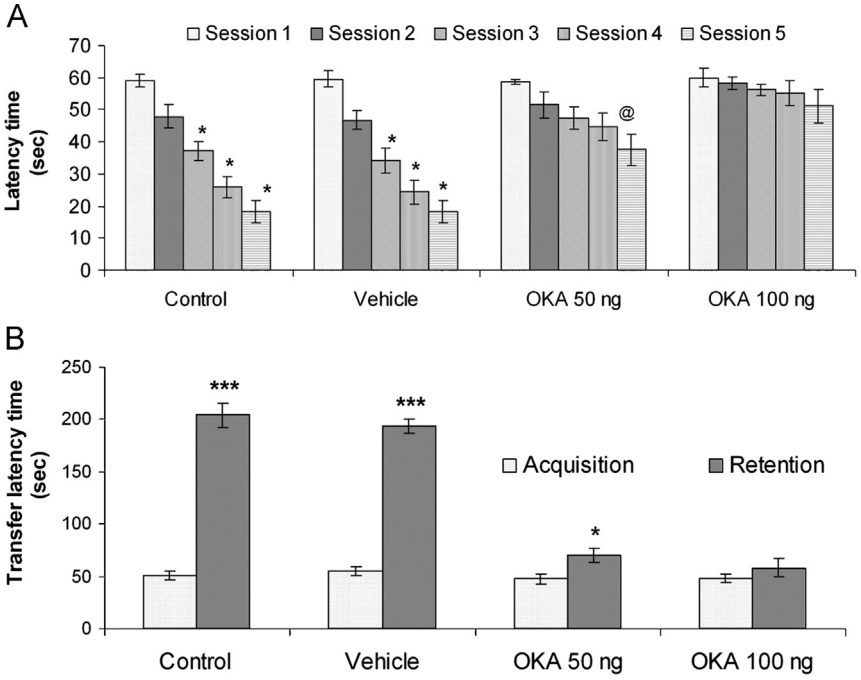

Fig. 1. Standardization of okadaic acid induced memory impairment in mice. (A) Morris water maze test. Data values are expressed as mean latency time (s) ± S.E.M. ($n=6$). (* and @) significant decrease in latency time ($^{@}P<0.01$ and $^{*}P<0.001$) vs acquisition trial. (B) Passive avoidance test. Data values are expressed as mean transfer latency time (s) ± S.E.M. ($n=6$). *Significant difference ($^{*}P<0.05$, $^{***}P<0.001$) in comparison to acquisition trial.

days, starting from single OKA (IC) 100 ng injection. It was administered orally 1 h before OKA administration on day 1.

Animals were randomly divided into five groups of 6–8 animals each.

Group 1 Control mice treated with vehicle of curcumin [1.0% (w/v) gum acacia] for 13 days.
Group 2 Mice injected intra cerebrally (IC) with aCSF, (vehicle of OKA) once and treated with vehicle of curcumin for 13 days.
Group 3 Mice injected intra cerebrally (IC) OKA once and treated with vehicle of curcumin for 13 days.
Group 4 Mice injected intra cerebrally (IC) OKA once and treated with curcumin for 13 days.
Group 5 Mice treated with curcumin for 13 days in per se.

2.3.3. Evaluation of memory function

2.3.3.1. The acquisition and retention of a spatial navigation task was examined by Morris water maze test. Memory function was assessed on 13th day of post OKA administration in Morris water maze followed by Morris (1984). The Morris water maze (MWM) consists of a large circular black pool of 120 cm diameter, 50 cm height, filled to a depth of 30 cm with water temperature 25 ± 1 °C, and was kept in a dark room. Within the pool, a submerged black colored round platform of 8 cm diameter was placed 1–1.5 cm below water surface. Data were acquired through a video camera connected to the computerized tracking system (Columbus Instruments, USA) fixed above the center of the pool. During the experiment each animal was placed in the water to find out the hidden platform to escape from the swimming. Same starting position was used on each trial. The trial was given to mice for a maximum time of 60 s (cut off time) to find the hidden platform and allowed to stay on platform for 20 s. Mice failing to find the platform were gently guided to find platform only in 1st trial (acquisition trial). The animals were given a daily session of 3 trials per day for 5 days. During a session, latency time to reach the platform and path length was recorded for individual animal in each trial and mean latency time was calculated. Significant decrease in latency time in subsequent sessions (retention) from that of 1st session (acquisition) was considered as successful learning.

In order to assess the possibility of drug interference with animal sensory and motor coordination or the animal motivation, the capability of mice to escape to a visible platform was tested in this study. For the test, platform was placed on a new location inside the pool 1 cm above the water line. Mice were allowed to swim for 60 s. Time to reach the platform was recorded as escape latency (Kamal et al., 2000).

A probe trial was performed 1 h after the last water maze session to access the extent of memory consolidation. The time spent in the target quadrant indicates the degree of memory consolidation that has taken place after learning. The individual mice were placed into the pool as in the training trial, except that the hidden platform was removed from the pool (Kamal et al., 2000). The time spent in target quadrant was measured for 60 s. In probe trial, each mouse was placed at a start position directly opposite to platform quadrant. Further, the path length in target quadrant/average path length in other three quadrants, Time spent in target quadrant/average time spent in other three quadrants and number of times crossing over the platform site of each mouse was also measured and calculated.

2.3.3.2. Assessment of learning and memory by passive avoidance test. The mice were subjected to the passive avoidance test (PAT) by keeping them in a light compartment with an intensity of 8 [scale from 0 to 10 (brightest)] in a computerized shuttle box, operated with a software programme PACS 30 (Columbus Instruments, Ohio, USA). There is automated guillotine door between light compartment and dark compartment. The mice were acclimatizing for 30 s period; the guillotine door was automatically opened and closed after each entry of the mouse into the dark compartment. The mouse received a low-intensity foot shock (0.5 mA; 10 s) in the dark compartment. Infrared sensors monitored the transfer of the animal from one compartment to another, which was recorded as transfer latency time (TLT) in seconds. The 1st trial was acquisition trial and retention was tested in a 2nd trial (retention) given 24 h after the 1st trial. The duration of a trial was 270 s. The shock was not delivered in the retention trials to avoid reacquisition. The criterion for learning was taken as an increase in the TLT on retention trials as compared to acquisition trial (Awasthi et al., 2010).

2.3.3.3. Spontaneous locomotor activity. Spontaneous locomotor activity (SLA) was assessed in Optovarimex activity meter (Columbus Inc, USA) prior to trial on water maze test to check any change in SLA and abnormal locomotor activity may affect the memory tests. Every animal were observed for 5 min after a period of 10 min for acclimatization.

2.4. Measurement of cerebral blood flow

CBF was measured by laser Doppler flowmetry (LDF 100, BIOPAC, USA) on 13th day (1 h after administration of vehicle or curcumin) in a separate set of all animal groups. LDF qualitatively measures CBF in arbitrary blood perfusion units (BPU) (Tonnesen et al., 2005; Tota et al., 2010). The mice were anesthetized with chloral hydrate (300 mg/kg, i.p.) and a 0.5 mm diameter micro-fiber laser doppler probe was placed on the skull (6 mm lateral and 1 mm posterior of bregma) and CBF was monitored in brain cortical region (Stenman et al., 2007). CBF was measured for 10 min time period and recording after each 30 s continuously at above-given co-ordinates and average values of blood flow were calculated for given time period.

2.5. Biochemical and molecular parameters

Biochemical and molecular parameters estimations were performed in treatment group. For biochemical assays, mice were sacrificed with excess dose of ether anesthesia after completion of the behavioral studies. Brain was removed quickly and kept on ice-cold plate and then dissected into cerebral cortex and hippocampus according to the Glowinski and Iversen (1966) and homogenized in sodium phosphate buffer (0.03 M, pH 7, 10% w/v) by using an Ultra-Turrax T25 (USA) homogenizer at 9500 rpm.

2.5.1. Estimation of lipid peroxidation

MDA, an end product of lipid peroxidation was measured spectrophotometrically using 1,1,3,3 tetra ethoxy propane as standard at 532 nm wavelength as described previously (Colado et al., 1997; Kamat et al., 2010).

2.5.2. Estimation of glutathione

GSH, an endogenous antioxidant, its level was determined by reaction with 5,5′-dithiobis 2-nitrobenzoic acid (DTNB) (Ellman, 1959; Kamat et al., 2010) using reduced glutathione as standard, at 412 nm wavelength.

2.5.3. Estimation of nitrite level

Nitrite level was estimated by Griess reagent according to previous reports (Green et al., 1982; Kamat et al., 2010).

2.5.4. Estimation of reactive oxygen species

Intracellular reactive oxygen species was estimated by CARY Eclipse, fluorescence spectrometer with excitation wavelength of 488 nm and emission wavelength of 530 nm using the oxidation sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (DCF-DA) (Oyama et al., 1992; Kamat et al., 2010; Tota et al., 2012).

2.5.5. Acetylcholinesterase (AChE) assay

The brain homogenate in volume of 500 μl was mixed with 1% Triton X-100 (1% w/v in 0.03 M sodium phosphate buffer, pH-7) and samples were centrifuged at 100,000*g* at 4 °C in a Beckman Ultracentrifuge (LE 80, USA), using a fixed angle rotor (80 ti) for 60 min. Supernatant was collected and acetylcholinesterase (AChE) activity estimated as described previously (Ellman et al., 1961; Tota et al., 2012).

2.5.6. Estimation of ATP

ATP was estimated in mice brain as described by Tota et al. (2010) using ATP colorimetric/fluorometric assay kit (Biovision, Catalog #K354-100) and performed according to manufacturer instructions.

2.5.7. Mitochondrial calcium ion [Ca^{2+}]i estimation

Mice brain mitochondria were prepared as described previously (Partridge et al., 1994; Kamat et al., 2010) and calcium levels were measured by using the radiometric calcium indicator fura-2AM. The mitochondrial suspension were loaded with 5 μM fura-2AM in HEPES buffer for 30 min at 37 °C in water bath, protected from light. After completion of reaction, mitochondrial suspension was centrifuged and the mitochondrial pellet was resuspended in fresh, dye-free HEPES buffer (145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose, 5 mM HEPES, pH 7.4) and kept on ice, protected from light, until experiments were performed. Mitochondrial [Ca^{2+}]i concentration were assayed fluorometrically measured by the dual wavelength method described by Grynkiewicz et al. (1985) with excitation wavelengths alternating between 340 and 380 nm (slit widths 5 nm), and an emission wavelength of 510 nm (slit width 10 nm). The assay was performed in a thermo stated cuvette at 37 °C. Mitochondrial Ca^{2+} was expressed as percentage of vehicle control.

2.5.8. Transcriptional analysis of AChE, α7 nicotinic acetylcholine receptor, GFAP, TNF-α, IL-1β, COX-2, and Caspase-9 genes by RT-PCR

Expression of various genes was studied in cerebral cortex and hippocampus of mice brain by reverse transcription polymerase chain reaction (RT-PCR). RNA was isolated from brain region using TRIzol reagent (Sigma) as directed by the manufacturer. Concentration and purity of RNA were determined spectrophotometrically using Gene Quant. Approximately 2 μg of total RNA was reverse transcribed using reverse transcriptase (RT) in a 20 μl mixture containing oligo-(dT)-primer, RNase Inhibitor, dNTP mix and 5 × reaction buffer (Omniscript RT kit). The resultant cDNA was amplified separately with specific primer for AChE, a7 nicotinic acetylcholine receptor, GFAP, TNF-α, IL-1β, COX-2, Caspase-9 and β-actin using Taq PCR core kit (Qiagen, USA). Briefly, cDNA (2 μl) was amplified in a 20 μl reaction volume containing 1 U Taq polymerase, 200 μM (each) dNTP mix and 2 μl 10 × Taq buffer with specific primers. The polymerase chain reaction mixture was amplified in a DNA thermal cycler (Bioer XP cycler) through 35 cycles at the specifications described in Table 1. The PCR products were detected by electrophoresis on a 1.2% agarose gel containing ethidium bromide. Band intensities were quantified by computerized densitometry (Alpha Imager gel documentation system) and normalized with respect to actin mRNA (Kamat et al., 2011).

2.5.9. Protein estimation

Protein was measured in all brain samples by the method of Lowry et al. (1951) for the biochemical estimation unless otherwise specified. Protein has estimated by the method of Wang and Smith (1975) only in the samples prepared for acetylcholine esterase activity. Bovine serum albumin (BSA) (0.1–1 mg/ml) was used as a standard.

2.6. Statistical analysis

Statistical analyses were performed (GraphPad Prism) using Student's *t* test when 2 conditions were compared and 1-way ANOVA followed by *Tukey test* for multiple comparisons. Results are represented as mean ± S.E.M. and *P* values, are specified in each figure legend. *P* values less than 0.05 were considered significant.

3. Results

3.1. Effects of curcumin on OKA induced memory deficit in Morris water maze test

3.1.1. Analysis of latency time

The mean escape latency time of acquisition trial did not differ between any of the groups in Morris water maze test. But the control ($P < 0.001$), vehicle ($P < 0.001$) and OKA (50 ng) ($P < 0.01$) groups showed learning during retention trials as shown by a significant decrease in latency time as compared to acquisition trials. However, no significant improvement in learning ($P > 0.05$) was found in OKA

Table 1
Primer sequences and RT-PCR conditions for different genes.

S. No.	Gene name	Primer sequence	Product size (bp)	Annealing temperature (°C)
1	AChE	Forward 5′-GAT CCC TCG CTG AAC TAC ACC-3′ Reverse 5′-GGT TCT TCC AGT GCA CCA TGT AGG AG-3′	331	60
2	a7 nAChR	Forward 5′-GTG GAA CAT GTC TGA GTA CCC CGG AGT GAA-3′ Reverse 5′-GAG TCT GCA GGC AGC AAG AAT ACC AGC A-3′	510	60
3	COX-2	Forward 5′-GAA GTG GGG GTT TAG GAT CAT C-3′ Reverse 5′-CCT TTC ACT TTC GGA TAA CCA-3′	381	60
4	TNF-α	Forward 5′-TAC TGA ACT TCG GGG TGA TTG GTC C-3′ Reverse 5′-CAG CCT TGT CCC TTG AAG AGA ACC-3′	295	70
5	IL-1β	Forward 5′-GCT ACC TAT GTC TTG CCC GT-3′ Reverse 5′-GAC CAT TGC TGT TTC CTA GG-3′	543	65
6	GFAP	Forward 5′-TCCTGGAACAGCAAAACAAG -3′ Reverse 5′-CAGCCTCAGGTTGGTTCAT-3′	256	58
7	Caspase-9	Forward 5′-AGC CAG ATG CTG TCC CAT AC-3′ Reverse 5′-CAG GAG ACA AAA CCT GGG AA-3′	132	60
8	β-actin	Forward 5′-GCC ATG TAC GTA GCC ATC CA-3′ Reverse 5′-GAA CCG CTC ATT GCC GAT AG-3′	352	55.7

(100 ng) treated mice (Fig. 1A). As shown in Fig. 2A, pre-treatment with curcumin significantly ($P<0.001$) decreased latency time in OKA treated animals suggesting improvement in spatial memory in mice. Further, per se curcumin had no significant ($P>0.05$) effect during any session as compared to control and vehicle groups.

3.1.2. Analysis of path length

The average path length in acquisition trial did not differ between any of the groups in Morris water maze test (Fig. 2B). But the control ($P<0.001$) and vehicle ($P<0.001$) groups showed learning during the retention trials as shown by a significant decrease in path length as compared to acquisition trials. However, no significant improvement in learning ($P>0.05$) was found in OKA treated mice as is evident by no decrease in the path length. Pre-treatment with curcumin significantly ($P<0.001$) decreased path length in OKA treated animals suggesting improvement in spatial memory in mice. The representative swim pattern of different groups of animals during last trial of session 5 was shown in Fig. S1.

3.1.3. Visible platform test

All the groups showed equivalent efficacy in locating a visible platform ($P>0.05$) implying absence of motor sensory differences between the experimental groups (Fig. S2).

3.1.4. Probe trial

The probe trial data is depicted in Fig. 3A–C, which provides four representations of selective performance in the retention test. Fig. 3A is a standard measure and compares time spent in the target quadrant against the average time spent in other three quadrants. The time spent in target quadrant was significantly high in control and vehicle groups ($P<0.01$) in comparison to the OKA group. It was further observed that the target quadrant preference was completely lost in OKA injected animals ($P>0.05$). The treatment with curcumin prevented the memory impairment as indicated by the significant ($P<0.01$) increase in the time spent in target quadrant in comparison to average time spent in other three quadrants. Further, inter group analysis showed a significant increase ($P<0.05$) in the time spent in target quadrant of curcumin treated animals as compared to OKA group. However there was no significant ($P>0.05$) difference in the time spent in target quadrant of control, vehicle and per se curcumin groups. The total path length in target quadrant and average path length in other three quadrants is shown in Fig. 3B. Except the OKA injected group, all other animals showed a significantly high ($P<0.05$) path length in target quadrant in comparison to average path length in other three quadrants. Further, in the OKA group, the path length in target quadrant was significantly less ($P<0.05$) as compared to control and vehicle groups. Curcumin group exhibited significantly ($P<0.05$) higher path length in target quadrant in comparison to that of OKA group. However there was no significant ($P>0.05$) difference in path length in target quadrant of control, vehicle per se curcumin groups.

Probe trial study also revealed that OKA treated animals showed significantly less ($P<0.05$) platform crossings when compared with control and vehicle groups, indicating their inferior search accuracy for the hidden platform. Curcumin administration in OKA injected mice improved search accuracy as indicated by significantly higher ($P<0.05$) platform crossings in comparison to OKA group (Fig. 2C). The representative swim pattern of different groups of animals during probe trial was shown in Fig. S3.

3.2. Effects of curcumin on OKA induced memory deficit in the passive avoidance test

The transfer latency time (TLT) significantly increased in the retention trials as compared to the acquisition trial in control ($P<0.001$), vehicle (aCSF) ($P<0.001$) and OKA (50 ng) ($P<0.01$) treated mice. No significant increase in the TLT of the retention trials as compared to the acquisition trial was observed in the OKA (100 ng) treated mice ($P>0.05$) (Figs. 1B, 4). However, curcumin

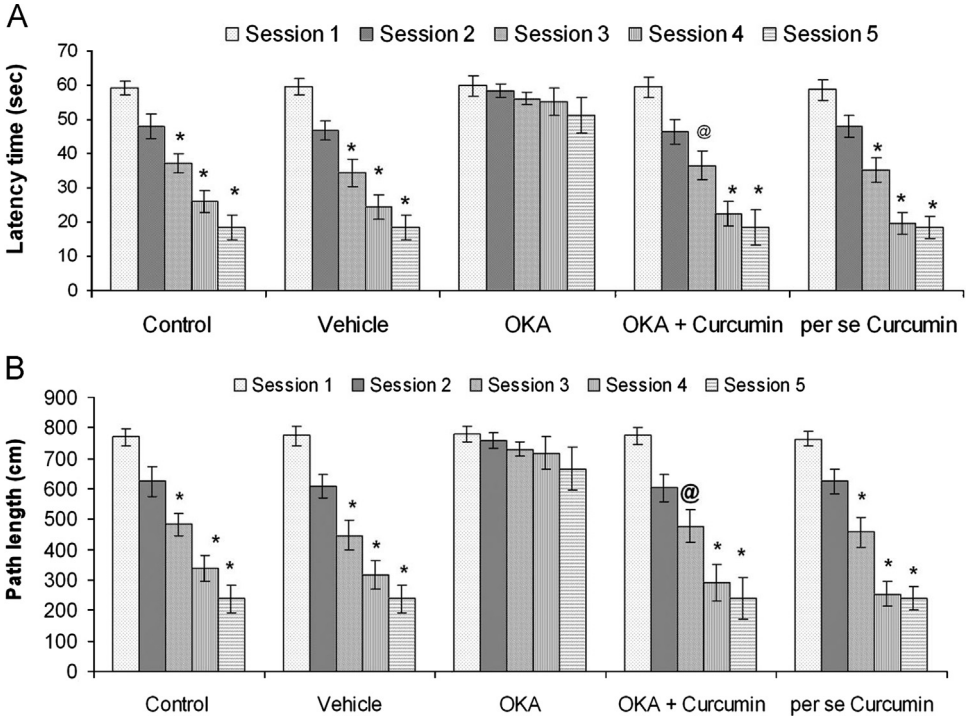

Fig. 2. Effect of curcumin pretreatment on okadaic acid (IC) induced memory impairment in mice. (A) Comparison of latency time. Data values are expressed as mean latency time (s) ± S.E.M. ($n=6$). (* and @) significant decrease in latency time ($^{@}P<0.01$ and $^{*}P<0.001$) vs acquisition trial. (B) Comparison of path length. Data values are expressed as mean path length (cm) ± S.E.M. ($n=6$). (* and @) significant decrease in path length ($^{@}P<0.01$ and $^{*}P<0.001$) vs acquisition trial.

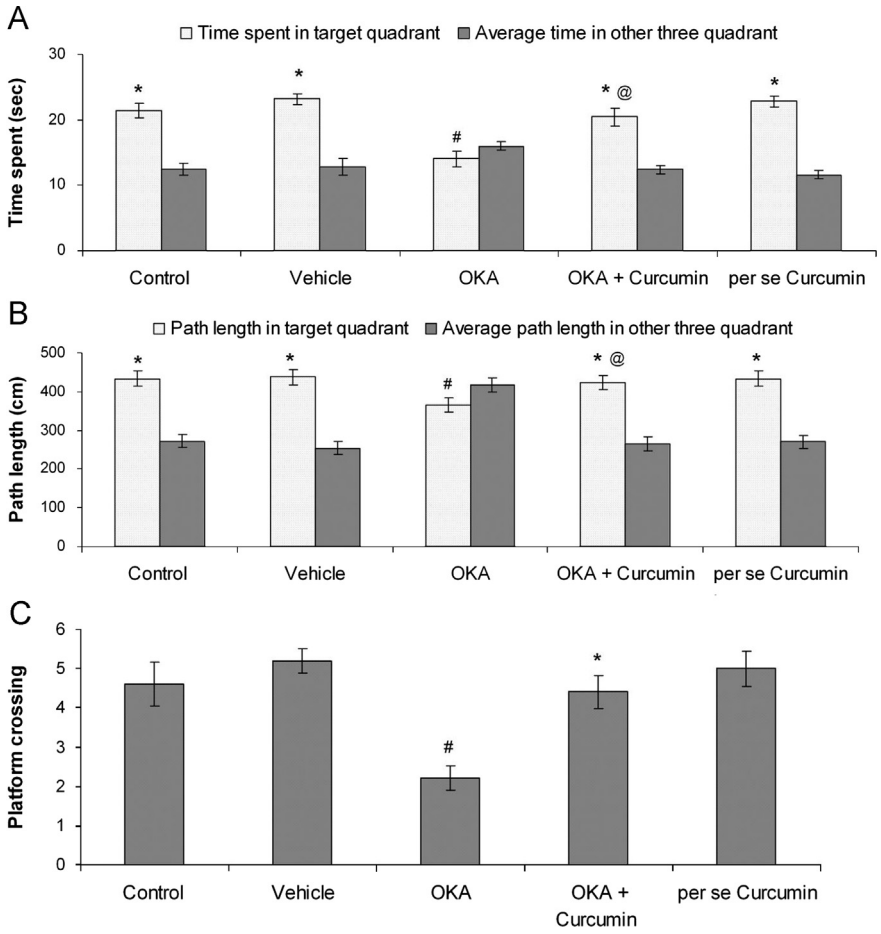

Fig. 3. Effect of curcumin on probe trial performance in Morris water maze. (A) Probe trial performance as measured by comparing time spent in the target quadrant with an average time spent in all three non-target quadrants. Data values are expressed as mean time spent (s) ± S.E.M. ($n=6$). (*) Significant difference in time spent ($^{*}P<0.01$) vs average time spent in other three non-target quadrants of respective group, (♯) significant difference in time spent in target quadrant ($^{\sharp}P<0.05$) vs control and vehicle group and (@) significant difference in time spent in target quadrant ($^{@}P<0.05$) vs okadaic acid group. (B) Probe trial performance as measured by comparing path length in the target quadrant with an average path length in all three non-target quadrants. Data values are expressed as mean path length (cm) ± S.E.M. ($n=6$). (*) Significant difference in path length ($^{*}P<0.01$) vs average path length in other three non-target quadrant of respective groups, (♯) significant difference in path length in target quadrant ($^{\sharp}P<0.05$) vs control and vehicle group and (@) significant difference in path length in target quadrant ($^{@}P<0.05$) vs okadaic acid group. (C) Number of crossings of training site (± S.E.M.) during the probe trial. (♯) Significant decrease ($^{\sharp}P<0.01$) vs control and vehicle group and (*) significant increase ($^{*}P<0.01$) vs okadaic acid group. Q1—quadrant 1, Q2—quadrant 2, Q3—quadrant 3 and TQ—target quadrant. In probe trial, each mouse was placed at a start position in the quadrant 2 and allowed to swim for 60 s.

treatment significantly reversed memory deficit and increased transfer latency in OKA injected animals ($P<0.001$). There was no significant difference ($P>0.05$) in TLT of the 1st trial among the different groups (Fig. 4).

3.3. Effects of curcumin on locomotor activity

No significant change in locomotor activity was observed in any group [ambulatory: $P>0.05$; vertical: $P>0.05$; and total: $P>0.05$].

3.4. Effect of curcumin on cerebral blood flow

Administration of OKA significantly ($P<0.001$) reduced CBF in comparison to control and vehicle groups. Curcumin significantly ($P<0.001$) restored CBF as compared to the OKA treated mice. However there was no significant difference in CBF between curcumin per se, control and vehicle groups ($P>0.05$) (Fig. 5).

3.5. Estimation of biochemical parameters

3.5.1. Measurement of mitochondrial calcium ion [Ca^{2+}]i

The level of mitochondrial [Ca^{2+}]i in the brain region was measured after finishing the behavioural studies. There was a significant increase in [Ca^{2+}]i in hippocampus ($P<0.01$) and cerebral cortex ($P<0.01$) of OKA treated mice as compared to vehicle control mice. As shown in Fig. 6, treatment with curcumin significantly ($P<0.01$) reduced amount of [Ca^{2+}]i as compared to OKA group. Per se curcumin treatment has no significant effect ($P>0.05$) on [Ca^{2+}]i as compared to vehicle control mice.

3.5.2. Measurement of malondialdehyde (MDA) and nitrite level

The MDA (nmol/mg protein) and nitrite (μg/mg protein) in the mice brain regions were estimated after completion of behavioral studies. The MDA (Fig. 7A) and nitrite (Fig. 7C) were significantly increased in cerebral cortex ($P<0.001$) and hippocampus ($P<0.001$) of OKA treated mice in comparison to control and vehicle groups. Treatment with curcumin significantly ($P<0.001$) decreased MDA and nitrite level in both the brain regions in

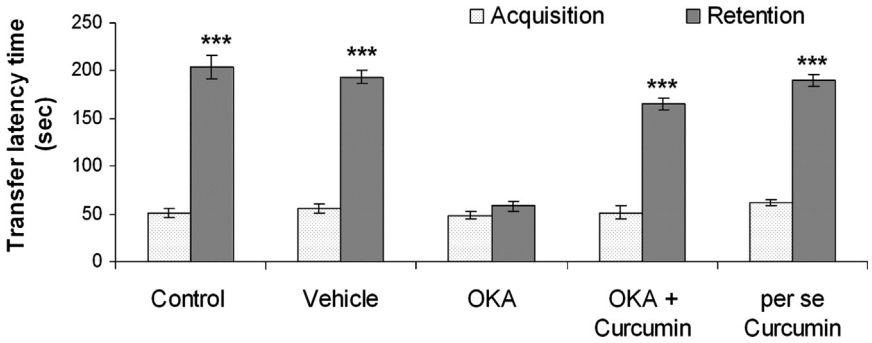

Fig. 4. Effect of curcumin on OKA induced memory impairment in passive avoidance test in mice. Data values are expressed as mean transfer latency time (s) ± S.E.M. *Significant difference ($^{*}P < 0.001$) in comparison to acquisition trial.

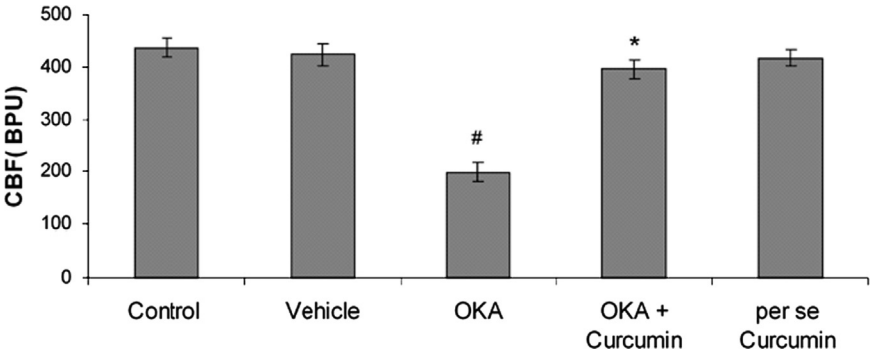

Fig. 5. Effect of curcumin on cerebral blood flow (CBF) in OKA (IC) induced memory deficit mice. CBF was measured in arbitrary blood perfusion units and expressed as blood perfusion units (BPU) ± S.E.M. ($n=6$). #Significant difference ($^{\#}P < 0.001$) in CBF as compared to control and vehicle treated group and ⁎significant difference ($^{*}P < 0.001$) in CBF as compared to the OKA group.

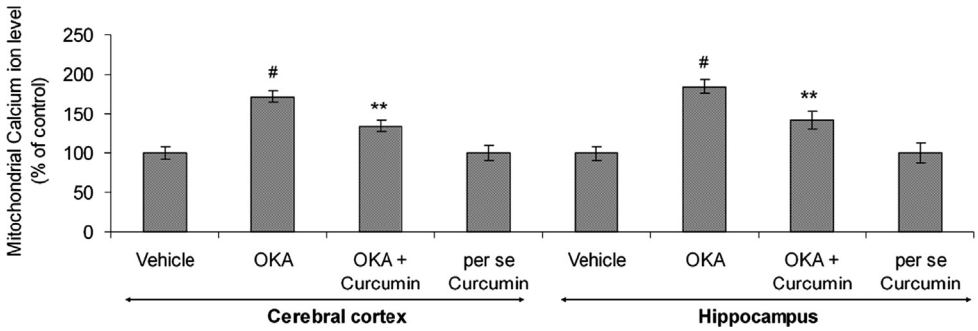

Fig. 6. Effect of curcumin on mitochondrial [Ca^{2+}]i. level in OKA (IC) induced memory deficit mice brain. Graph shows mitochondrial [Ca^{2+}]i level calculated in nmol/mg protein and expressed as % of control ($n=6$). #Significant difference ($^{\#}P < 0.01$) vs vehicle group; *significant difference ($^{**}P < 0.01$) vs OKA group.

comparison to OKA. However, per se treatment of curcumin did not change MDA and nitrite in both hippocampus ($P > 0.05$) and cerebral cortex ($P > 0.05$) vs. control and vehicle group.

3.5.3. Glutathione (GSH) level

The vehicle (aCSF) (IC) administration had no significant ($P > 0.05$) effect on GSH (μg/mg protein) level in comparison with control group. While, there was a significant reduction in the GSH levels, as compared to control and vehicle, in cerebral cortex ($P < 0.001$) and hippocampus ($P < 0.001$) of OKA treated group. The GSH level was significantly higher ($P < 0.001$) in cerebral cortex and hippocampus of curcumin treated group in comparison to OKA group. Per se curcumin did not significantly affect GSH levels in hippocampus ($P > 0.05$) and cerebral cortex ($P > 0.05$) in mice brain regions as compared to control and vehicle groups (Fig. 7B).

3.5.4. Measurement of reactive oxygen species

Production of reactive oxygen species was measured relative to control. Treatment with OKA significantly increased ($P < 0.001$) reactive oxygen species generation in cerebral cortex and hippocampus region of mice brain. Administration of curcumin significantly ($P < 0.001$) reduced the amount of reactive oxygen species in both brain region vs OKA group. Per se curcumin had no significant effect on reactive oxygen species level in cerebral cortex ($P > 0.05$) and hippocampus ($P > 0.05$) of mice brain vs. vehicle control group (Fig. 7D).

3.5.5. ATP level

ATP level was measured relative to control. A significant reduction in the ATP as compared to vehicle control was observed

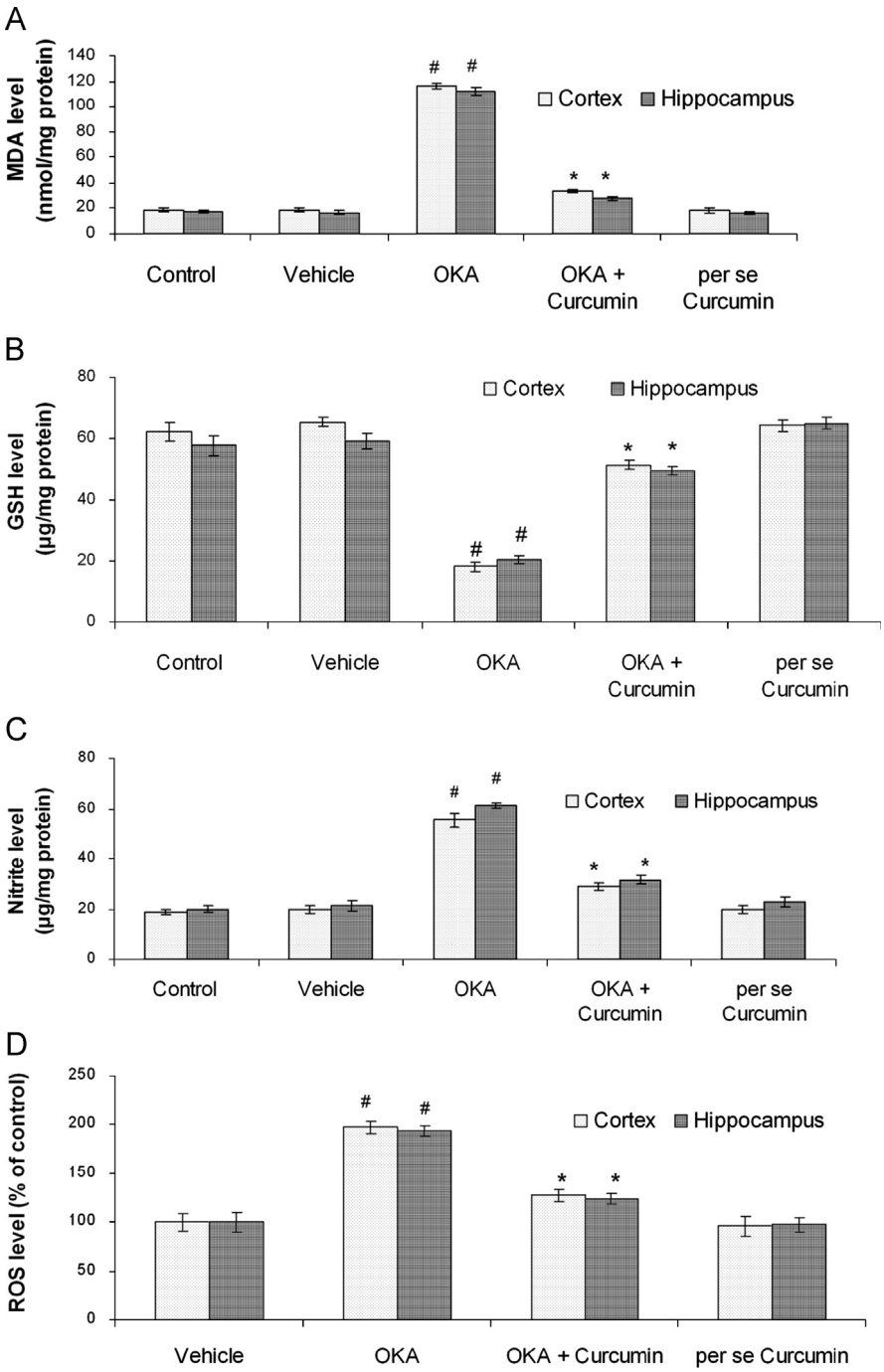

Fig. 7. (A) Effect of curcumin on MDA level in OKA (IC) induced memory deficit mice brain. Data values are expressed as mean MDA level (nmol/mg protein) ± S.E.M. ($n=6$) #significant difference (#$P<0.001$) in MDA level as compared to control and vehicle treated group and *significant difference (*$P<0.001$) in MDA level as compared to the OKA group. (B) Effect of curcumin on GSH level in OKA (IC) induced memory deficit mice brain. Data values are expressed as mean GSH level (µg/mg protein) ± S.E.M. ($n=6$) #significant difference (#$P<0.001$) in GSH level as compared to control and vehicle treated group and *significant difference (*$P<0.001$) in GSH level as compared to the OKA group. (C) Effect of curcumin on nitrite level in OKA (IC) induced memory deficit mice brain. Data values are expressed as mean nitrite level (µg/mg protein) ± S.E.M. ($n=6$) #significant difference (#$P<0.001$) in nitrite level as compared to control and vehicle treated group and *significant difference (*$P<0.001$) in nitrite level as compared to the OKA group. (D) Effect of curcumin on Reactive oxygen species generation in OKA (IC) induced memory deficit mice brain. Graph shows mean florescence intensity expressed as % of control ($n=6$). #Significant difference (#$P<0.001$) as compared to control group and *significant difference (*$P<0.001$) as compared to the OKA group.

in cortex ($P<0.001$) and hippocampus ($P<0.001$) of OKA treated group. This reduction in ATP level was reversed in cerebral cortex and hippocampus by preventive administration of curcumin ($P<0.001$) in comparison to OKA treated group. Per se curcumin did not significantly ($P>0.05$) affect ATP level in as compared to vehicle control group (Fig. 8).

3.5.6. AChE activity

The vehicle (aCSF) (IC) administration had no significant ($P>0.05$) effect on AChE activity (µmol/min/mg protein) in both brain region in comparison with control group. While, there was a significant reduction in the AChE activity in cerebral cortex ($P<0.01$) and hippocampus ($P<0.01$) of OKA treated group as

compared to control and vehicle groups. This reduction in AChE activity was restored in cerebral cortex and hippocampus by preventive administration of curcumin ($P<0.01$) in comparison to OKA group. Per se curcumin did not significantly ($P>0.05$) affect AChE activity in both mice brain regions as compared to control and vehicle groups (Fig. 9).

3.5.7. Brain weight

As shown in Fig. S4 the brain weight was significantly decreased in OKA treated mice as compared to control and vehicle groups ($P<0.01$). Preventive treatment with curcumin in OKA treated mice significantly ($P<0.05$) increased brain weight. However, per se curcumin treatment has no significant effect on brain weight ($P>0.05$) as compared to control and vehicle groups.

3.6. Transcriptional analysis

3.6.1. Effect of curcumin on GFAP and COX-2 mRNA expression in brain of OKA induced memory impaired mice

As shown in Fig. 10B and C, the GFAP and COX-2 mRNA expression was significantly increased in cerebral cortex ($P<0.01$) and hippocampus ($P<0.01$) of OKA treated mice as compared to vehicle control group. Preventive treatment with curcumin in OKA treated mice significantly ($P<0.05$) decreased GFAP and COX-2 mRNA level in cerebral cortex and hippocampus vs OKA group. However, per se curcumin treatment has no significant effect on GFAP and COX-2 mRNA expression in cerebral cortex ($P>0.05$) and hippocampus ($P>0.05$) as compared to vehicle control group.

3.6.2. Effect of curcumin on TNF-α and IL-1β mRNA expression in OKA induced memory impaired mice

As shown in Fig. 11B and C, the TNF-α and IL-1β mRNA expression was significantly increased in cerebral cortex ($P<0.01$) and hippocampus ($P<0.01$) of OKA treated mice as compared to vehicle control group. Preventive treatment with curcumin in OKA treated mice significantly ($P<0.01$) decreased TNF-α and IL-1β mRNA level in cerebral cortex and hippocampus vs OKA group. However, per se curcumin treatment has no significant effect on TNF-α and IL-1β mRNA expression in cerebral cortex ($P>0.05$) and hippocampus ($P>0.05$) as compared to vehicle control group.

3.6.3. Effect of curcumin on AChE and α7 nicotinic acetylcholine receptor mRNA expression in OKA induced memory deficit mice brain

As shown in Fig. 12B and C, the AChE and α7 nicotinic acetylcholine receptor mRNA expression was significantly decreased in cerebral cortex ($P<0.01$) and hippocampus ($P<0.01$) of OKA treated mice as compared to vehicle control group. Preventive treatment with curcumin in OKA treated mice significantly ($P<0.05$) increased AChE and α7 nicotinic acetylcholine receptor mRNA level in cerebral cortex and hippocampus vs OKA group. However, per se curcumin treatment has no significant effect on AChE and α7 nicotinic acetylcholine receptor mRNA expression in cerebral cortex ($P>0.05$) and hippocampus ($P>0.05$) as compared to vehicle control group.

3.6.4. Effect of curcumin on Caspase-9 mRNA expression in OKA induced memory deficit mice brain

As shown in Fig. 12D, the Caspase-9 mRNA expression was significantly increased in cerebral cortex ($P<0.01$) and

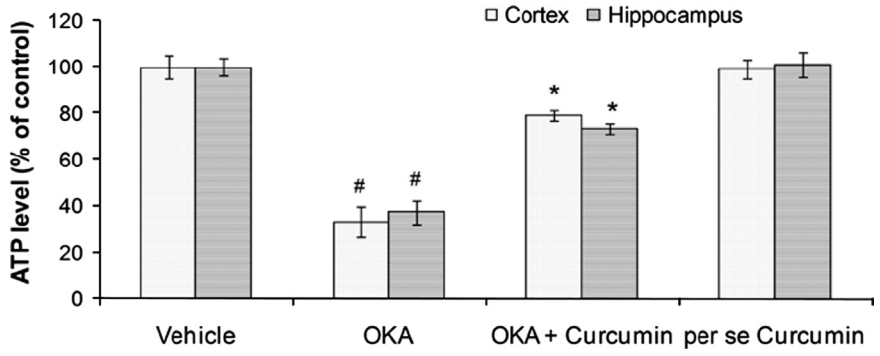

Fig. 8. Effect of curcumin on ATP level in OKA (IC) induced memory deficit mice brain. Data values are expressed as % of control ($n=6$). #Significant difference (# $P<0.001$) in ATP level as compared to vehicle treated group and *significant difference (*$P<0.001$) in ATP level as compared to the OKA group.

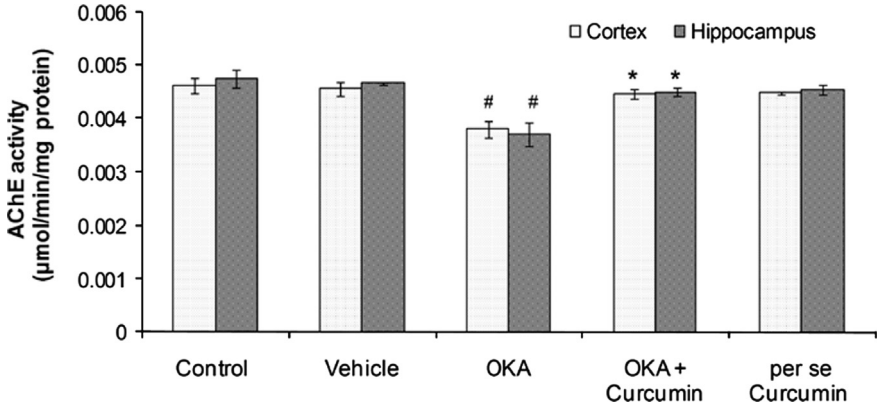

Fig. 9. Effect of curcumin on AChE activity in OKA (IC) induced memory deficit mice brain. Data values are expressed as mean AChE activity (µmol/min/mg protein) ± S.E.M. #Significant difference (#$P<0.01$) in AChE activity as compared to control and vehicle treated group and *significant difference (*$P<0.01$) in AChE activity as compared to the OKA group.

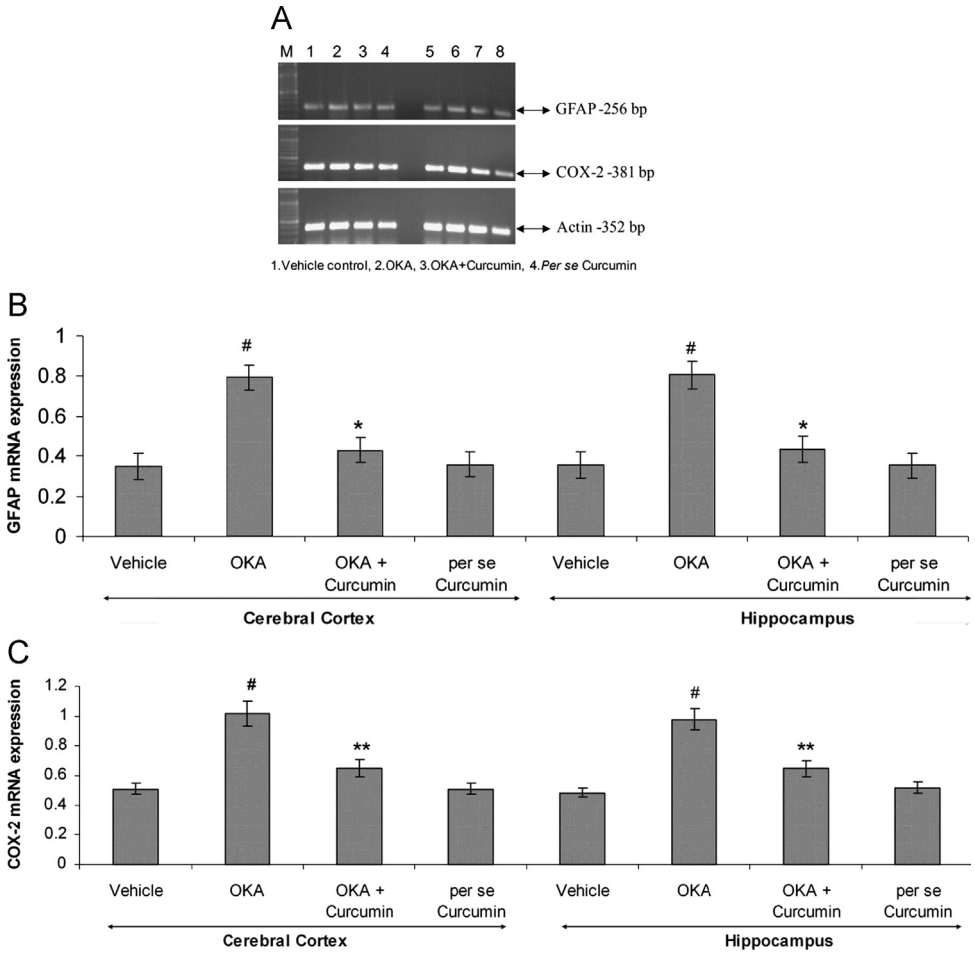

Fig. 10. (A) mRNA expression of GFAP, COX-2 and β-actin. (B) GFAP and (C) COX-2 mRNA levels (normalized to level of β-actin). (♯) Significant difference ($^{\#}P < 0.01$) in comparison to respective region of vehicle control group and (*) significant difference ($^{*}P < 0.05$, $^{**}P < 0.01$) in comparison to OKA group.

hippocampus ($P < 0.01$) of OKA treated mice as compared to vehicle control group. Preventive treatment with curcumin in OKA treated mice significantly ($P < 0.05$) decreased Caspase-9 mRNA levels in cerebral cortex and hippocampus vs OKA group. However, per se curcumin treatment has no significant effect on Caspase-9 mRNA expression in cerebral cortex ($P > 0.05$) and hippocampus ($P > 0.05$) as compared to vehicle control group.

4. Discussion

The present study was planned to examine the effect of curcumin on impairment in memory, mitochondrial calcium ion level, neuroinflammation, brain energy metabolism, cerebral circulation, apoptosis, brain atrophy and cholinergic dysfunction in intracerebral (IC) OKA induced memory impairment in mice.

Recently our lab showed that OKA caused memory impairment in rats, which was prevented by clinically used anti-dementic drug donepezil (5 mg/kg) and memantine (10 mg/kg) (Kamat et al., 2010). In the present study OKA caused memory impairment in mice as shown by no significant change in latency time and path length in water maze test. Further, probe trial also confirmed the memory impairment as OKA injected mice lost the target quadrant preference and showed reduced number of platform crossings. Curcumin improved memory in OKA injected mice as evidenced by significant decrease in latency time and path length. Curcumin treated animals also exhibited improved target quadrant preference as shown by time spent in platform zone and number of platform crossings. Further, per se curcumin has no significant effect on memory function vs. control and vehicle (aCSF). Moreover, all the animals showed similar preference for searching visible platform indicating absence of motor-sensory differences between the experimental groups.

In passive avoidance test (PAT), control and vehicle groups had shown improvement in memory as revealed by significant increase in retention latencies as compared with acquisition trial. OKA caused no significant increase in retention latency as compared to acquisition trial indicating impairment in passive avoidance learning. Curcumin administration prevented the OKA induced memory impairment as shown by improved retention latency in PAT. Therefore, improved memory functions due to curcumin in both test models corroborate other findings as well (Ishrat et al., 2009; Agrawal et al., 2010; Awasthi et al., 2010).

The locomotor activity was tested by optovarimex activity meter and we found no significant difference among different groups thus excluding possibility that alteration in locomotors activity may have contributed in performance of animals in memory tests.

OKA administration lead to oxidative stress as evidenced by a significant increase in MDA level and decrease in endogenous antioxidant GSH level. Additionally, there was a significant increase in reactive oxygen species and nitrite levels in brain of OKA treated mice thus confirming our previous finding (Kamat et al., 2010). Reactive oxygen species, which were predominantly

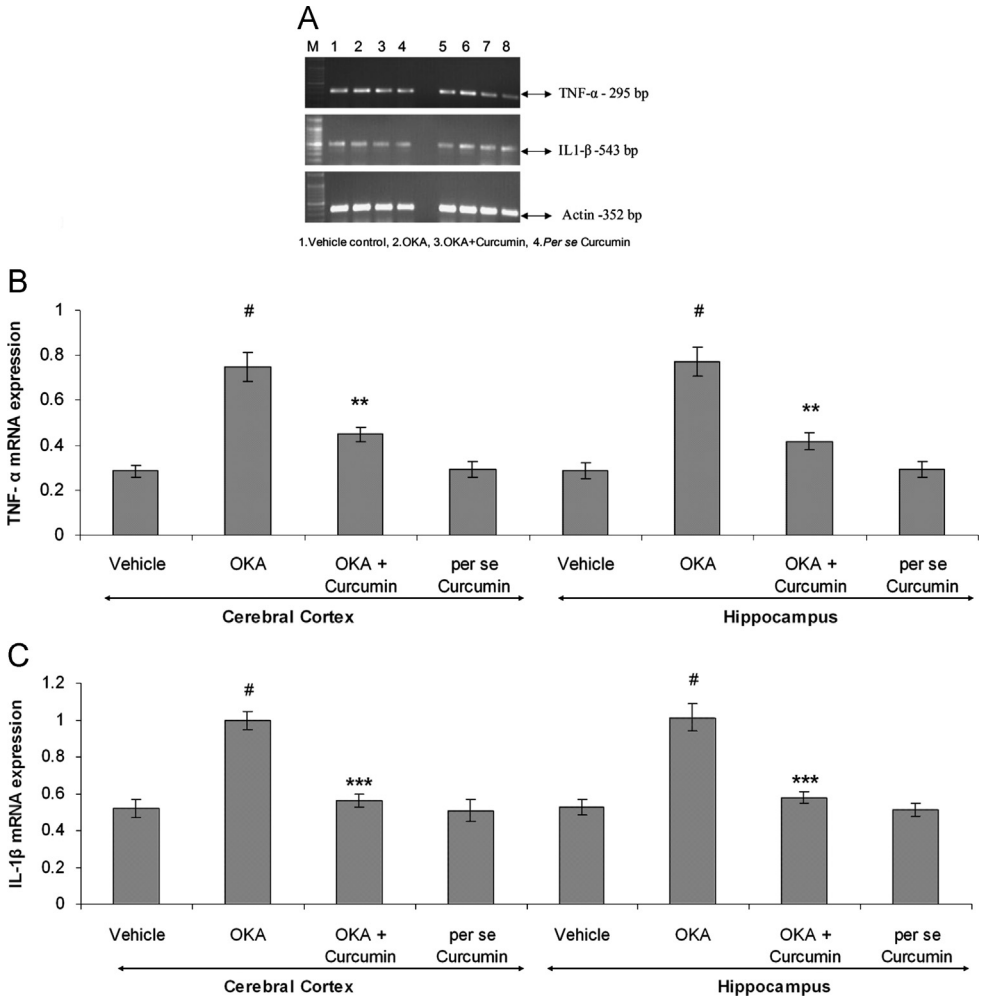

Fig. 11. (A) mRNA expression of TNF-α, IL-1β and β-actin. (B) TNF-α and (C) IL-1β mRNA levels (normalized to level of β-actin). (♯) Significant difference ($^{\#}P < 0.01$) in comparison to respective region of vehicle control group and (*) significant difference ($^{**}P < 0.01$, $^{***}P < 0.001$) in comparison to OKA group.

produced in the mitochondria, if excessive, may lead to the free radical attack of membrane phospholipids and loss of mitochondrial membrane potential, which causes the release of apoptosis-inducing factors (AIF) that activate caspase cascades, cause nuclear condensation, and generate secondary reactive oxygen species (Zamzami et al., 1995). We found significant reduction in oxidative and nitrosative stress in cerebral cortex and hippocampus of curcumin treated mice as shown by decrease in the deleterious oxidants like reactive oxygen species, nitrite, MDA and increase GSH further attributed its potent antioxidant and free radical scavenging activity (Molina-Jijon et al., 2011). Moreover, curcumin is lipophilic and has strong anti-oxidant potential. Presence of phenolic, methoxy and di ketonic groups in its structure has the capability to scavenge reactive nitrogen and oxygen species (Kapoor and Priyadarsini, 2001; Aggarwal and Sung, 2009).

In the present study, we found that OKA significantly elevated mitochondrial $[Ca^{2+}]i$ in cerebral cortex and hippocampus region which was reversed by curcumin. Excessive reactive oxygen species production could cause membrane depolarisation, which leads to the increase of Ca^{2+} influx (Trump and Berezesky, 1995). Rise in levels of intracellular Ca^{2+} through NMDA receptor and voltage-gated calcium channels leads to the impairment in the mitochondrial electron transport system which consequently results into generation of intracellular reactive nitrogen and oxygen species leading to tissue damage (Bonfoco et al., 1995; Kamat et al., 2011). A recent study also support this observation in which curcumin moderated H_2O_2-induced mitochondrial membrane potential loss and increase Ca^{2+} influx by inhibiting reactive oxygen species generation and up-regulating the anti-oxidative proteins including thioredoxin, haem oxygenase-1 and peroxiredoxin-1 in Neuro-2A cells (Zhao et al., 2011).

Activated astrocytes are found in neurofibrillary tangle in Alzheimer's disease (Thangavel et al., 2011). In addition, there is also evidence that GFAP (marker for astrocyte activation) correlates inversely with cognitive function (Kashon et al., 2004). Thus, to confirm the involvement of the astrogliosis in memory deficit, we have measured GFAP mRNA level in cerebral cortex and hippocampus of OKA administered mice. OKA significantly enhanced in GFAP mRNA level suggesting the role of the astrocyte activation in OKA induced cognitive deficits. These finding are accordance to Costa et al. (2012). In previous study also, we found that Lipopolysaccharide (LPS) has significantly up regulated GFAP protein expression in astroglial cells mimicking the reactive gliosis during in vivo patho-physiological conditions of neurodegenerative disease like Alzheimer's disease (Niranjan et al., 2010). Anti-inflammatory drug nimesulide have significantly inhibited LPS induced GFAP expression in astroglial cells in a dose dependent manner (Niranjan et al., 2010). In this study administration of curcumin decreased GFAP mRNA expression. It corroborates the finding of Lim et al. (2001) where curcumin treatment reduced GFAP in Alzheimer transgenic mouse.

In the current study, we observed a significant elevation in the mRNA levels of TNF-α, IL-1β and COX-2 in the cerebral cortex and hippocampus of OKA treated mice which is indicative of enhanced

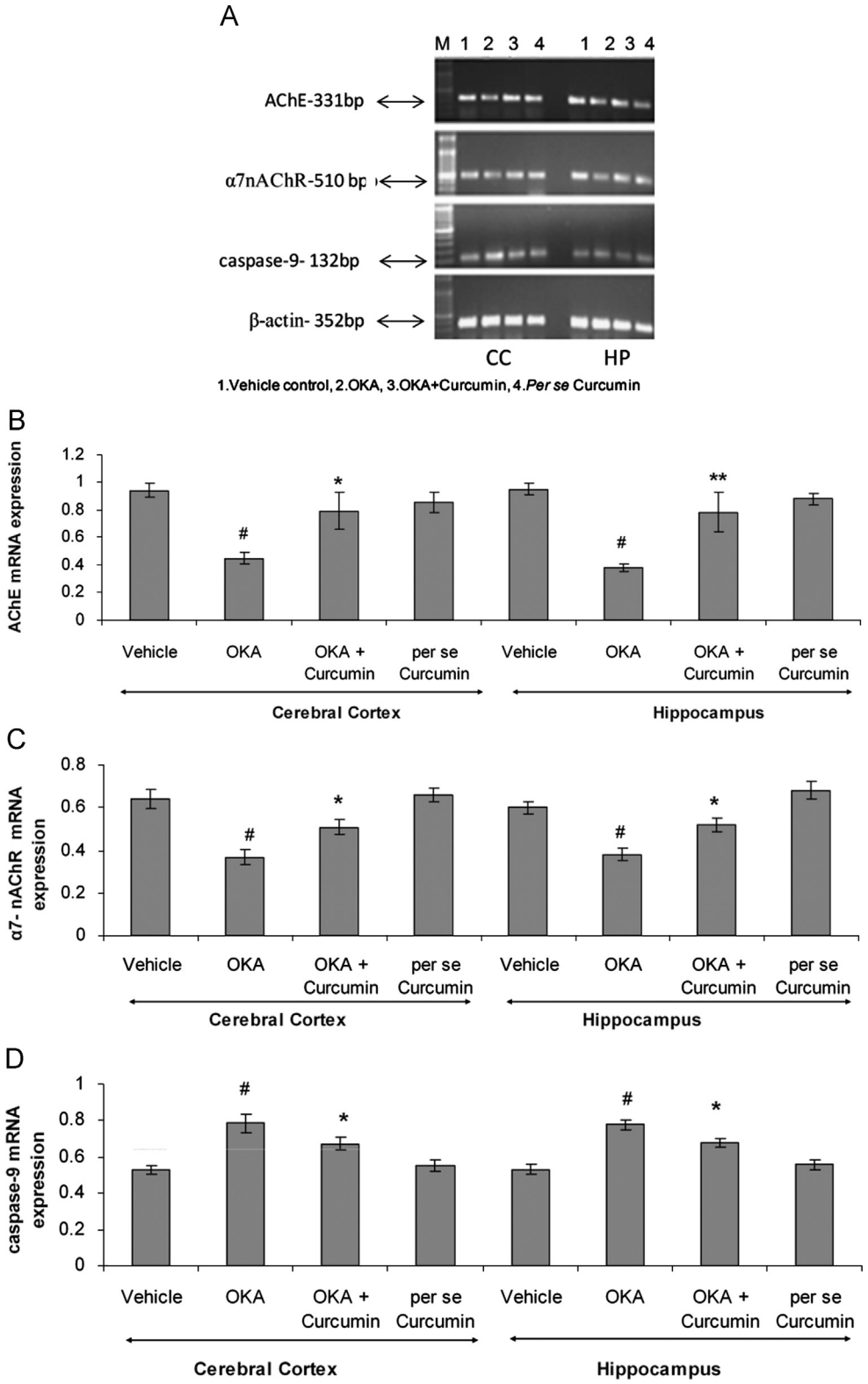

Fig. 12. (A) mRNA expression of AChE, α7 nicotinic acetylcholine receptor, Caspase-9 and β-actin. (B) AChE, (C) α7 nicotinic acetylcholine receptor and (D) Caspase-9 mRNA levels (normalized to level of β-actin). (#) Significant difference ($^{\#}P < 0.01$) in comparison to respective region of vehicle control group and (*) significant difference ($^{*}P < 0.05$, $^{**}P < 0.01$) in comparison to OKA group.

neuroinflammation in the two main regions of brain involved in learning and memory. Activated glial cells releases proinflammatory mediators such as TNF-α and IL-1β which have been suggested important mediators in brain pathology of Alzheimer's disease (Tan et al., 2007; Tanaka et al., 2006). Increased TNF-α level in the brain and plasma, as well as a TNFR1 up regulation in the brain have been detected in Alzheimer's disease patients. Evidence from animal study showed that IL-1β injected directly into the hippocampus leads to memory impairment (Barrientos et al., 2002), probably via the suppression of brain-derived

neurotrophic factor. It is now well recognized that expression of COX-2 is commonly induced during inflammatory processes (Simmons et al., 2004) and experimental evidence revealed that ibuprofen causes a modest decrease in Aβ load, in APP/PS1 mice (Jantzen et al., 2002). Treatment with curcumin significantly reduced the proinflammatory mediators (TNF-α, IL-1β and COX-2) in different brain regions of OKA administered mice. Our results are supported by the findings from Tiwari and Chopra (2012) who found treatment with curcumin significantly reduced proinflammatory mediators (NF-κβ, TNF-α, IL-1β, and TGF-β1) in different brain regions of ethanol-administered animals.

Administration of the OKA leads to a significant reduction in CBF and ATP levels. This finding is in agreement with many clinical studies showing alteration in cerebral microcirculation of Alzheimer's disease patients (Prohovnik et al., 1988; O'Brien et al., 1992; Wyper et al., 1993). Although the exact mechanism for this impairment in the microcirculation is not known, the possible reasons include oxidative stress and endothelial dysfunction leading to restriction of blood flow to the brain (Ajmani et al., 2000). The protection against oxidative damage may avoid the endothelial dysfunction and cerebral circulation as shown by improved CBF in curcumin treated mice. This restored the cerebral circulation along with sustained glucose supply/utilization in brain to meet energy metabolism which leads to increased ATP level in curcumin administered animals.

OKA significantly increased Caspase-9 mRNA expression and atrophy in mice brain, indicating involvement of Caspase mediated cell death in OKA induced neurodegeneration. Caspase-9 is main initiator Caspase which play a pivotal role in neurodegenerative diseases and Caspase mediated apoptotic pathways has dominant role in mediating cell dysfunction and cell death (Yuan and Yankner, 2000). Indeed, reduction in total brain volume (or shrinkage of a brain region known to be involved in cognition) might be expected to be intimately linked to the causal pathway of neurodegeneration (Sabuncu et al., 2011). Curcumin significantly inhibited the activation of Caspase-3 and enhanced the level of Bcl-2 in $A\beta_{25-35}$-treated rat prefrontal cortical neurons (Qin et al., 2009). We also got similar finding that curcumin ameliorated apoptotic cell death and brain atrophy by combating oxidative stress and neuroinflammation. These findings are accordance with Tiwari and Chopra, 2012 who found treatment with curcumin significantly inhibited ethanol-induced apoptotic pathway/cell death cascade.

Loss or down regulation of the neuronal nicotinic acetylcholine receptors (nAChRs) has also been observed in the Alzheimer's disease (Levin, 2002). Further loss of α7 nicotinic acetylcholine receptors leads to enhanced β-amyloid oligomer accumulation in mouse model of Alzheimer's disease (Hernandez et al., 2010). Studies showed a significant reduction in level of ACh and AChE in Alzheimer's disease brain suggesting degeneration of cholinergic neurons (Kristensen, 1990; Perry et al., 1999). In agreement with clinical findings, we found a significant reduction in α7 nicotinic acetylcholine receptor expression and AChE expression/activity in OKA treated mice. The exact mechanism of OKA induced cholinergic dysfunction is not known but neuronal loss due to OKA administration may be behind its cholinergic deficiency and memory deficit as corroborate with our previous finding (Kamat et al., 2012b). Preventive treatment with curcumin attenuated cholinergic dysfunction in OKA treated mice which may attributed its neuroprotective effect. The mechanism underlying the neuroprotective effects of curcumin observed in our study may be due to its anti-oxidant, anti-inflammatory, and anti-apoptotic activities.

5. Conclusion

Curcumin administration significantly improved memory function along with cerebral blood flow and energy metabolism. It also attenuated excitotoxicity, neuroinflammation, oxidative stress, and cholinergic dysfunction in cerebral cortex and hippocampus of OKA treated mice. The results of this study clearly demonstrated beneficial effect of curcumin against OKA induced memory impairment. This study corroborated many preclinical/clinical findings that use of curcumin as a dietary supplement could be neuroprotective. Therefore, use of curcumin should be encouraged to prevent OKA containing seafood related cognitive impairment in human.

Acknowledgments

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ejphar.2013.04.033.

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