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Mitochondrial dysfunction: A crucial event in okadaic acid (ICV) induced memory impairment and apoptotic cell death in rat brain

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ABSTRACT

Mitochondrial abnormalities have been identified in a large proportion of neurodegenerative diseases. Recently we have reported that intracerebroventricular (ICV) administration of okadaic acid (OKA) causes memory impairment in rat. However involvement of mitochondrial function in OKA induced memory impairment and neuronal damage has not been determined. OKA (200 ng) was administered by ICV route. After 13th day of OKA administration memory function was evaluated by Morris Water Maze test. Following completion of behavioral studies on 16th day, mitochondrial membrane potential, Ca^{2+} and reactive oxygen species were evaluated in mitochondrial preparation of cortex, hippocampus, striatum and cerebellum of rat brain. While ATP, mitochondrial activity, lipid peroxidation and nitrite were investigated in synaptosomal preparation of rat brain areas. The activities and mRNA expression of apoptotic factors, caspase-3 and caspase-9, were studied in rat brain regions. The neuronal damage was also confirmed by histopathological study. OKA treated rats showed memory impairment including increased Ca^{2+} and reactive oxygen species and decreased mitochondrial membrane potential, ATP and mitochondrial activity in mitochondrial preparation. There was a significant increase in lipid peroxidation and nitrite in synaptosomal preparations. Preventive treatment daily for 13 days with antidementic drugs, donepezil (5 mg/kg, p.o) and memantine (10 mg/kg, p.o), significantly attenuated OKA induced mitochondrial dysfunction, apoptotic cell death, memory impairment and histological changes. Mitochondrial dysfunction appeared as a key factor in OKA induced memory impairment and apoptotic cell death. This study indicates that clinically used antidementic drugs are effective against OKA induced adverse changes at behavioral, cellular, and histological levels and mitochondrial dysfunction.

1. Introduction

Okadaic acid (OKA), a selective and potent inhibitor of serine/threonine phosphatases 1 and 2A induces hyperphosphorylation of tau in vitro and in vivo (Cohen et al., 1990; Mudher and Perry, 1998). OKA also increases Ca^{2+} in hippocampal neuronal cell culture through the ionotropic excitatory amino acid receptors resulting in neuronal degeneration (Fernandez et al., 1993). Recently, we reported that intracerebroventricular (ICV) administration of OKA in rats induced spatial memory impairment which was prevented by clinically used antidementic drugs (Kamat et al., 2010).

Recently the pivotal role of mitochondria in excitotoxicity has been highlighted in apoptotic and necrotic cell death (Papadia et al., 2008). Apoptosis plays a significant role in cell loss during neurodegenerative disorders such as Alzheimer's disease (AD) (Loh et al., 2006). A cascade of events like activation of caspases and aspartate-specific cysteine proteases has been proposed to play a key role in apoptosis (Nicholson and Thornberry ,1997). The major apoptotic pathway is characterized by mitochondrial dysfunction with the release of cytochrome c, activation of caspase-9, and subsequently of caspase-3. It has been suggested that caspase-3 is an ultimate effectors caspase whose activation leads to switch on the apoptotic cascade (Lee et al.,

Abbreviations: aCSF, Artificial cerebrospinal fluid; AD, Alzheimer's disease; DCFDA, 2,7 Dichlorofluorescein di-acetate; FURA-2AM, FURA-2 Acetoxy-methyl ester; HE, Hematoxylin and eosin; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; ICV, Intracerebroventricular; LEHD-AFC, Leu-1 = N-Ac-Leu, Asp-4 = Asp-(7-amino-4-trifluoromethylcoumarin; MDA, Malondialdehde; MMP, Mitochondrial membrane potential; MTT, Methylthiazoletetrazolium; NEDA, 1-(Naphthyl) ethylenediamine dihydrochloride; NMDA, N-methyl D-aspartate; OKA, Okadaic acid; R-123, Rhodamine123; ROS, Reactive oxygen species; SDH, Succinate dehydrogenase; TCA, Trichloro acetic acid.

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2005). Evidences of caspase-3 activation were also found in post-mortem study conducted on the brain of AD patient (Engidawork et al., 2001). Biochemical features of apoptosis include loss of plasma membrane phospholipid symmetry and ATP levels (Kroemer and Reed, 2000). In apoptosis alteration in mitochondrial activity occurs i.e. activation of cysteine proteases of caspase family; mitochondrial membrane depolarization, mitochondrial oxyradical production and calcium overload (Keller et al., 1998). The mitochondria are thought to be both generators of reactive oxygen species (ROS) and targets of ROS attack. Indeed, ROS generation is an important mechanism accounting for cellular injury in many neurodegenerative disorders (Valko et al., 2007). The glutamatergic synapses are most likely site for initiation of neurodegenerative process. Activation of glutamate receptors is believed to play a major role in the neuronal cell death (Mattson, 1996). Activation of glutamate receptors causes massive calcium influx through NMDA, voltage-dependent calcium channels (Choi, 1994) and oxyradical production (Mattson, 1996).

In spite of the fact that OKA is known to increases Ca^{2+} influx and ROS generation (Kamat et al., 2010), the involvement of mitochondria in OKA induced neurotoxicity and memory impairments has not been investigated. Therefore, the present study was carried out to explore cellular mechanisms potentially in reference to mitochondrial function in the OKA induced neurotoxicity associated with the memory impairment and also to investigate the effect of antidementic drugs on OKA induced alteration in mitochondrial activity.

2. Methods

2.1. Animal

The adult male Sprague Dawley (SD) rats (225–250 g) were used for experiments. The rats were kept in a polyacrylic cage (22.5×37.5 cm) in group of 6 rats per cage and maintained under standard housing conditions (room temperature 24–27 °C and humidity 60–65%) with a 12 h light and dark cycle. Food and water were available ad libitum but food removed during experimental period from 1 h prior to administration of drug till completion of the trials (approximate 2–3 h). The animals were procured from the Laboratory Animal Division of Central Drug Research Institute, Lucknow. Internationally practiced ethical standards were followed during experiments. The study was approved by Institutional Animals Ethics Committee of Central Drug Research Institute, Lucknow.

2.2. Materials

The chemicals used in the study were purchased from Sigma Aldrich (okadaic acid, methylthiazoletetrazolium, FURA-2AM and 2,7-Dichlorofluorescin-diacetate, R123), Biovision (ATP assay kits, caspase-3 and caspase-9 assay kit) and IDT (caspase-3 and caspase-9 primer). Follin Lowery protein assay reagents and all other chemicals were of analytical grade.

2.3. Intracerebroventricular injection of okadaic acid

Rats were anesthetized with chloral hydrate (300 mg/kg, i.p). The head was positioned in a stereotaxic frame and a midline saggital incision was made in the scalp. Holes were drilled in the skull with dental burr on both the sides over the lateral ventricles using the following coordinates: 0.8 mm posterior to bregma, 1.5 mm lateral to saggital suture and 3.6 mm beneath the surface of brain (Paxinos et al., 1980). Each rat received OKA (200 ng) in volume of 10 µl on each site.

2.4. Experimental design and drug administration

Animals were divided in following groups with six rats in each group:

Control: Rats were treated orally with vehicle (1% aqueous suspension of gum acacia, 1 ml/kg) of memantine and donepezil for 13 days.
aCSF: Rats were injected intracerebroventricularly (ICV) with aCSF and treated with vehicle for 13 days.
OKA 200 ng: Rats were injected ICV with OKA (200 ng) and treated with vehicle for 13 days.
Memantine 10 mg/kg: Memantine (10 mg/kg, p.o.) were administered for 13 days in ICV OKA (200 ng) treated rats.
Donepezil 5 mg/kg: Daily oral administration of donepezil (5 mg/kg) was continued for 13 days in ICV OKA (200 ng) treated rats.

2.5. Assessment of learning and memory by Morris water maze test

Morris water maze is commonly used for test spatial learning and memory in rodents (Morris, 1984). In this test water maze consists a circular pool (120 cm diameter and 50 cm height) colored with black nontoxic dye filled to a depth of 30 cm with water. The temperature in pool was maintained at 26±2 °C. Four equally spaced points around the edge of the pool were designed as N (North), E (East), S (South) and W (West). A black colored round platform of 8 cm diameter was placed 1 cm below the surface of water. The rats were trained to navigate the submerged platform. The rats were given a maximum time of 120 s (cut-off time) to find the hidden platform and were allowed to stay on it for 30 s. Rat that failed to locate the platform within 120 s was put on platform only in the first session. The animals were given a daily session of five trials. Escape latency time (ELT) to reach the platform was recorded in each trial. The mean value of latency time of each session is shown in the Results (Kamat et al., 2010).

2.6. Spontaneous locomotor activity

Spontaneous locomotor activity (SLA) was assessed on Optovarimex activity meter (Columbus Inc USA) 1 h prior to first water maze trial. Each rat was observed for 5 min after a period of 10 min for acclimatization.

2.7. Brain tissue collection

The rats were sacrificed with ether anesthesia at the end of behavioral studies on 16th day. After intracardiac perfusion with chilled normal saline brain was removed quickly and kept on ice-cold plate immediately. Then brain areas cortex, hippocampus, striatum and cerebellum were dissected according to method of Glowinski and Iversen (1966). Mitochondrial, synaptosomal and mRNA preparations of these brain areas were used for estimation of biochemical and molecular parameters.

2.8. Mitochondrial preparation and estimation of various parameters

Mitochondrial preparation was done according to the method described by Partridge et al. (1994). The brain tissue was placed in ice-cold isolation buffer (0.15 M KCl, 20 mM potassium phosphate, pH 7.6). Mitochondrial preparation was obtained by manual homogenization in a glass-Teflon homogenizer and centrifugation (17,000 *g* for 10 min) followed by a Ficoll gradient (10% w/v in isolation buffer) and ultracentrifugation (100,000 *g* for 45 min). The resultant pellet suspended in HEPES (145 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM glucose and 5 mM HEPES, pH 7.4) buffer was used as mitochondrial preparation to estimate ROS, Ca^{2+} and MMP.

2.8.1. Assessment of mitochondrial ROS formation

ROS formation was assessed fluorimetrically using DCFH-DA, a nonfluorescent cell-permeable compound, according to method of Myhre et al. (2003). Mitochondrial preparation was incubated with DCFH-DA (10 mM, 5 µl) in water bath for 30 min at 37 °C in a total reaction mixture of 1 ml. After incubation, the mitochondria were pelleted by centrifugation (1200 *g* for 10 min) and resuspended in fresh HEPES buffer. ROS formation was studied in triplicate for each sample. Fluorescence was recorded at excitation wavelength of 488 nm and emission wavelength of 525 nm. The ROS formation was expressed as percentage of vehicle control.

2.8.2. Mitochondrial calcium ion Ca^{2+} levels

Mitochondrial Ca^{2+} concentration was measured by the dual wavelength method described by Grynkiewicz et al. (1985). Ca^{2+} was calculated according to the following equation: $[Ca^{2+}]i = Kd \times B \times (R - Rmin)/(Rmax - R)$. A Kd of 224 nM was used for the fura-2 complex (Grynkiewicz et al., 1985). B is the ratio of fluorescence intensity of fura-2 to the Ca^{2+} bound fura-2 at 380 nM. Calcium levels were determined using the radiometric calcium indicator fura-2. A stock solution of fura-2AM (1 mM) was made in dimethyl sulfoxide and stored at −20 °C. 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 preparation was centrifuged and the mitochondrial pellet was resuspended in fresh, dye-free HEPES buffer and kept on ice, protected from light, until experiments were performed. Calcium levels were assayed fluorometrically with excitation wavelengths alternating between 340 and 380 nm (slit width 5 nm), and an emission wavelength of 510 nm (slit width 10 nm). The assay was performed in a thermostated cuvette at 37 °C. Mitochondrial Ca^{2+} was expressed as percentage of vehicle control.

2.8.3. Mitochondrial membrane potential (MMP)

Rhodamine123 (R-123) was used to determine membrane potential of isolated mitochondrial preparation. R-123 dye displayed a red shift in their excitation and emission fluorescence spectra upon driven mitochondrial uptake (Duchen and Biscoe, 1992). The extent of the wavelength shift was used to determine the distribution of the dye across the mitochondrial membrane and to quantitate MMP using a ratiometric approach. Mitochondrial preparation was mixed with R-123 (0.5 µM) in reaction mixture and incubated at 37 °C in water bath for 30 min. Absorption was read at excitation and emission wavelengths of 490/535 nm respectively. The data are expressed as percentage of vehicle control.

2.9. Brain synaptosomal preparation

Brain synaptosomal preparation was done as described by McGovern et al. (1973). The brain tissue was homogenized in 10 vol 0.32 M sucrose in a Potter-Elvehjem homogenizer, and the homogenate was centrifuged at 4–8 °C for 10 min at 600 *g* in a 3K300 centrifuge. The supernatant was then diluted 1:1 with 1.3 M sucrose, to yield a suspension at a final concentration of 0.8 M sucrose. This suspension was further centrifuged at 20,000 *g* for 30 min at 4 °C. The supernatant was discarded, and the pellet consisting of synaptosomes and free (extrasynaptosomal) mitochondria was resuspended in HEPES buffer (pH 7.4). The resulting synaptosomal preparation was kept in −70 °C till the experiment was performed to estimate ATP, SDH, caspase-3, caspase-9, lipid peroxidation and total nitrite.

2.9.1. Assessment of ATP production

ATP was estimated in synaptosomal preparation by using ATP colorimetric/fluorometric assay kit (Biovision, Catalogue-K354-100) according to manufacturer instructions. Absorbance was measured at 570 nm after 30 min incubation in Elisa plate reader (BIOTEK). The concentration of ATP was calculated using ATP standard curve and expressed in nmol/mg protein.

2.9.2. Assessment of mitochondrial metabolic function

Mitochondrial metabolic function was assessed by the conversion of the dye methylthiazoletetrazolium (MTT) to formazan (Castoldi et al., 2000) This assay is based on the ability of the mitochondrial enzyme succinate dehydrogenase (SDH) to metabolize MTT into formazan, a reaction that takes place only in functionally intact mitochondria. This assay is an index of the bioenergetics behavior of synaptosomes in brain. Synaptosome preparation was treated with solution of 100 µl of MTT (5.0 mg/ml) in HEPES buffer which was added to each tube. The samples were then incubated for 30 min at 37 °C. Formazan crystals were pelletted by centrifugation at 9000 *g* dissolved in DMSO (200 µl) and quantitated spectrophotometrically at 570 nm using a microplate reader (Bio-Tek Instruments, Inc.).The data are expressed as mean of formation of formazan per mg protein basis.

2.9.3. Caspase-3 activity

Caspase-3 activity was measured using the fluorometric assay system, (Promega). Aliquots of the synaptosomal preparation were resuspended in cell lysis buffer [25 mM HEPES (pH 7.5), 5 mM MgCl2, 5 mM EDTA, 2 mM PMSF, 1% cocktail protease inhibitor] and subjected to manual homogenization with Teflon homogenizer. Cell lysates were then incubated with 50 µM inhibitor (Ac-DEVD-CHO for caspase-3) for 30 min at 37 °C prior to addition of 50 µM substrate (Ac-DEVD-AMC for caspase-3). Processed brain synaptosomal sample was mixed with reaction mixture and incubated at 37 °C in water bath for 30 min. The sample was read at excitation and emission wavelengths of 360/460 nm respectively. The caspase-3 activity is expressed in µmol/mg of protein.

2.9.4. Caspase-9 activity

Caspase-9 activity was measured using the ready to use fluorometric LEHD-AFC substrate assay system, (Biovision). Aliquots of the synaptosomal preparation were resuspended in cell lysis buffer [25 mM HEPES (pH 7.5), 5 mM MgCl2, 5 mM EDTA, 2 mM PMSF, 1% cocktail protease inhibitor] and subjected to homogenization with Teflon homogenizers. Processed brain synaptosomal sample was mixed with reaction mixture containing 10 mM DTT and of 50 µM substrate (LEHD-AFC for caspase-9). Finally reaction mixture was incubated at 37 °C in water bath for 1 h. The sample was read at excitation and emission wavelengths of 400/505 nm respectively. The caspase-9 activity is expressed in µmol/mg of protein.

2.9.5. Lipid peroxidation

Lipid peroxidation was measured by the thiobarbituric acid test for malondialdehyde following the method described by (Colado et al., 1997). Brain synaptosomal preparation was deproteinized with 30% trichloro acetic acid (TCA), and 5 N HCl, followed by the addition of 2% (w/v) thiobarbituric acid in 0.5 M NaOH. The reaction mixture was heated in a water bath at 90 °C for 15 min, cooled to room temp and centrifuged at 12,000 *g* for 10 min. The pink chromogen was measured at 532 nm in an Elisa plate reader (Bio-Tek Instruments, Inc). The results are expressed as nM/mg of protein basis.

2.9.6. Total nitrite estimation by nitrate reductase method

Total nitrite was estimated by method of Green et al. (1982). Griess reagent [0.1% N-(1-naphthyl) ethylenediamine dihydrochloride, 1% sulfanilamide, 2.5% phosphoric acid)] i.e. 0.01 g NEDA, 0.1 g sulphanilamide, and 250 µl of phosphoric acid were mixed and volume was made to 10.0 ml. Equal volume of Griess Reagent and brain synaptosomes were treated with regents 1 mM NADH, nitrate reductase 1.0 U/ml and 1 M zinc acetate and incubated at 37 °C for 10 min followed by absorbance reading at 542 nm wavelengths

spectrophotometrically in Elisa plate reader (Bio-Tek Instruments, Inc). The data are expressed as μg/mg protein.

2.10. RT PCR analysis for caspase-3 and caspase-9 mRNA level expression

RNA was isolated from brain using TRIzol reagent (Sigma) as instructions of manufacturer. Concentration of RNA was determined spectrophotometrically using gene quanta and purity of the RNA was determined by A260/A280 ratio. RNA (2 μg) was reverse transcribed using reverse transcriptase (RT) in a 20-μl mixture containing oligo-(dT)-primer, RNase Inhibitor, dNTP mix and 5X reaction buffer (Omniscript RT kit).

The resultant cDNA was amplified separately with specific primer for, caspase-3, caspase-9 and β-actin using Taq PCR core kit (Qiagen USA). Briefly, cDNA was amplified in a 20 μl reaction volume containing 1 U Taq polymerase, 200 μM (each) dNTP mix and 2 μl of 10X 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 different specifications. The primer sets for reverse transcription PCR were: sense primer for caspase-9, 5′-AGCCAGATGCTGTCCCATAC-3′; antisense, 5′-CAGGAGACAAAACCTGGGAA-3′ at annealing temp of 55 °C, sense primer for caspase-3, 5′-CCACTCCCAGTCATTCCTTTAGTG-3′; antisense 5′-ATGGACAACAACGAAACCTCCGTG-3′ at annealing temp of 55 °C, sense primer for β-actin, 5′-GGCTGTGTTGTCCCTGTAT-3′; antisense, 5′-CCGCTCATTGCCGATAGTG-3′ at annealing temp of 55.7 °C. The product length (base pair) for caspase-9, caspase-3 and β-actin were 132 bp, 852 bp and 352 bp respectively. Polymerase chain reaction products were detected by electrophoresis on a 1.5% agarose gel containing ethidium bromide. Band intensities were quantified by computerized densitometry (Alpha Imager Gel Documentation System) and normalized with respect to β-actin.

2.11. Histopathological study by hematoxylin and eosin (HE) staining

A histopathological study in brain tissue was conducted according to Li et al.(1998). Rats were anesthetized under ether anesthesia. The transcardial perfusion with 50 ml of phosphate-buffered saline (0.02 M, pH 7.4), followed by 20 ml of 4% paraformaldehyde in 0.1 M phosphate-buffered saline (pH 7.4) was done for pre-fixation of the brain tissue. Then the brain was dissected out carefully and was kept in 4% paraformaldehyde overnight at room temp for post fixation. After post-fixation the tissue was dehydrated and embedded in paraffin for 4 h in infiltration unit. Block was prepared in block preparation unit (Shandon Histocenter-2) and coronal sections (10 μm) were cut with the help of a microtome (Leica RM 2255, Lab India) and picked up on poly-L-lysine coated slides. Sections from the rostral to the caudal portion of the brain were stained with hematoxylin and eosin (Li et al., 1998). Stained sections were captured (light microscopy) at 100× magnification. Dead cells were identified morphologically by blebbing of plasma membrane, diffused pallor of eosinophilic background, alterations in size and shape of cells, vacuolation, chromatin condensation and condensed nucleus.

2.12. Protein estimation

Protein concentration was measured at 750 nm wavelengths by method of Lowry et al. (1951) in all the brain samples for MDA, and nitrite, caspase-3 activity, metabolic activity and ATP level using Bovine serum albumin (BSA) (1 mg/ml) as standard and measured in the range of 0.01–0.1 mg/ml.

2.13. Statistical analysis

The results are expressed as mean ± S.E.M. Statistical analysis of the Morris water maze was done by two-way ANOVA (repeat measure) and biochemical and molecular data was done by one-way analysis of variance (ANOVA) followed by Tukey test.

3. Result

3.1. Behavioral assessment

3.1.1. Effects of memantine and donepezil on okadaic acid (ICV)-induced memory impairment in the Morris water maze test

The memory function was assessed 13 days after OKA injection in the Morris water maze test. Administration of OKA (ICV) caused a significant impairment in memory as revealed by no significant change in latency time in Morris water maze test. Analysis of latency time of control, aCSF and OKA (ICV) groups by repeat measure two-way ANOVA showed a significant group effect ($F = 10.66$, $P<0.01$), a highly significant session effect ($F = 14.58$, $P<0.001$), no significant interaction between group and session ($F = 0.543$, $P>0.05$) and significant subject matching ($F = 2.78$, $P<0.05$). Further, treatment with donepezil and memantine ameliorated OKA (ICV) induced memory impairment in rats. Comparison of latency time of OKA (ICV), donepezil + OKA and memantine + OKA groups revealed a significant group effect ($F = 5.17$, $P<0.05$), a highly significant session effect ($F = 16.11$, $P<0.001$), no significant interaction between group and session ($F = 1.06$, $P>0.05$) and significant subjects matching ($F = 2.14$, $P<0.05$) (Fig. 1).

3.1.2. Effects on locomotor activity

The spontaneous locomotor activity of rats was assayed on 13th day of OKA (ICV) injection. Locomotor activity of any group did not differ significantly. [Total: $F(4, 25) = 0.6872$, $P>0.05$, Ambulatory: $F(4, 25) = 0.5612$, $P>0.05$ and Vertical: $F(4, 25) = 1.019$, $P>0.05$].

3.2. Estimation of mitochondrial function

3.2.1. Measurement of mitochondrial reactive oxygen species (ROS)

Production of reactive oxygen species (ROS) in brain regions was measured relative to control. There was a significant increase ($P<0.01$) in ROS level in cerebellum, hippocampus, cortex and striatum of OKA 200 ng treated rats as compared to control group. Treatment with memantine significantly ($P<0.05$) reduced the amount of ROS whereas donepezil did not show significant ($P>0.05$) effect in any brain regions (Fig. 2).

3.2.2. Measurement of mitochondrial calcium ion Ca^{2+}

There was a significant ($P<0.01$) increase in Ca^{2+} in hippocampus, cortex, striatum and cerebellum of OKA 200 ng treated rats as compared to control and vehicle treated rats. Treatment with memantine

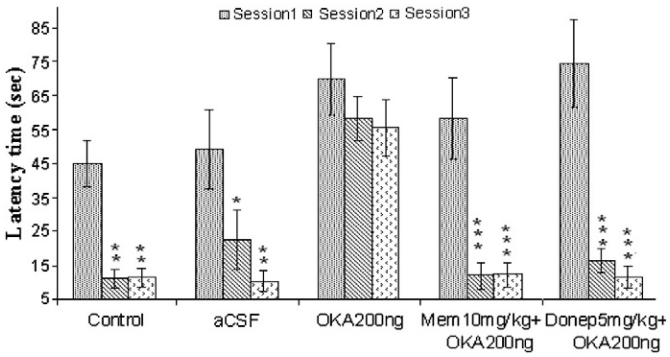

Fig. 1. Water maze graph illustrates that both memantine (Mem) and donepezil (Donep) significantly reduced the latency time on OKA induced memory impairment in rats. OKA 200 ng showing no significant change in latency time from session 1 to session 3. $^{*}P<0.05$, and $^{***}P<0.001$ vs. session 1.

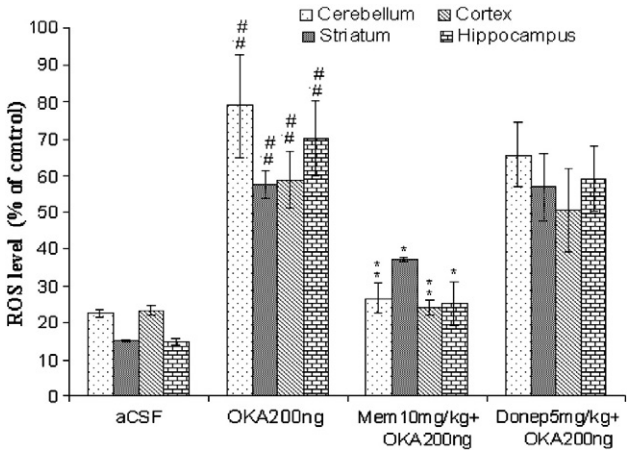

Fig. 2. Figure represents that only memantine (Mem) was able to reduce ROS level in OKA induced ROS generation whereas donepezil (Donep) failed to restore the OKA induced ROS generation in rat. $^{\#\#}P<0.001$ vs. control group and $^{*}P<0.01$, $^{**}P<0.001$ vs. OKA group.

and donepezil significantly ($P<0.01$) reduced amount of Ca^{2+} in OKA treated rat brain regions (Fig. 3).

3.2.3. Measurement of mitochondrial membrane potential (MMP)

There was a significant ($P<0.05$) decrease in MMP in hippocampus and cortex of OKA 200 ng treated rats as compared to control and vehicle treated rats. Treatment with memantine significantly ($P<0.05$) increased MMP in cortex and hippocampus whereas donepezil significantly ($P<0.05$) increased MMP in cortex, hippocampus and striatum as compared to OKA 200 ng treated rat (Fig. 4).

3.2.4. Measurement of ATP level in synaptosomal preparation

Energy metabolism was studied by estimating ATP content in rat brain. OKA administration significantly ($P<0.01$) decreased ATP content in rat brain areas hippocampus, cortex and striatum as compared to control and vehicle groups. Treatment with memantine significantly ($P<0.01$) increased ATP level in hippocampus, striatum, cerebellum and cortex, whereas treatment with donepezil significantly ($P<0.01$) increased ATP level in hippocampus, cortex and striatum as compared to OKA 200 ng treated rat (Fig. 5).

3.2.5. Measurement of mitochondrial SDH activity in synaptosomal preparation

There was significantly ($P<0.05$) less SDH activity in hippocampus and cortex of OKA 200 ng treated rats as compared to control and

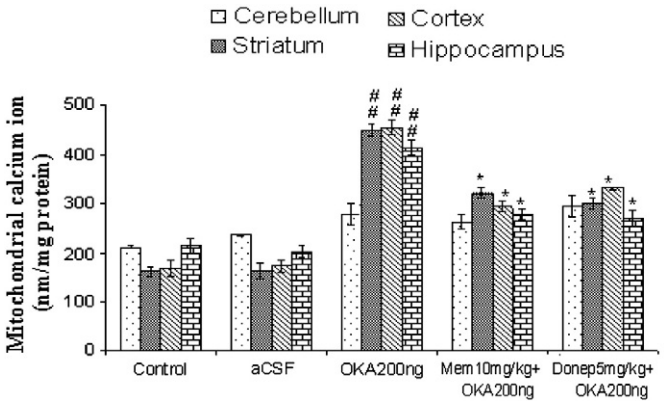

Fig. 3. Okadaic acid significantly increases Ca^{2+} level as compared to control and vehicle groups. Treatment with memantine and donepezil significantly reduced Ca^{2+} level in OKA injected rats. $^{\#\#}P<0.001$ control group and $^{*}P<0.01$, $^{**}P<0.001$ vs. OKA group.

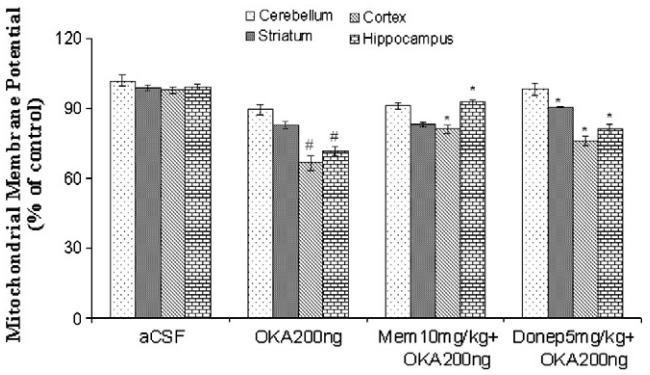

Fig. 4. A significant decrease in MMP level in mitochondrial preparation was observed in cortex and hippocampus of OKA treated group which was reversed by memantine and donepezil. $^{\#}P<0.01$ vs. control group and $^{*}P<0.05$ vs. OKA group.

vehicle treated rats. Preventive treatment with memantine and donepezil restored mitochondrial activity in cortex and hippocampus as shown by significant ($P<0.05$) increase in SDH activity (Fig. 6).

3.2.6. Measurement of malondialdehyde (MDA) level in synaptosomal preparation

OKA administration significantly ($P<0.01$) increased MDA in hippocampus, cerebellum, striatum and cortex as compared to control and vehicle group. Treatment with memantine significantly ($P<0.01$) decreased MDA level in hippocampus, and cerebellum, whereas treatment with donepezil significantly decreased ($P<0.001$) MDA in hippocampus, cerebellum, cortex, and striatum as compared to OKA 200 ng treated rat (Fig. 7).

3.2.7. Measurement of total nitrite in synaptosomal preparation

Nitrite level in the brain regions was measured at the end of the experiment. As shown in Fig. 8, nitrite levels were significantly ($P<0.001$) elevated in cortex and hippocampus of OKA 200 ng treated rat. Both memantine and donepezil significantly prevented ($P<0.001$) this increase in nitrite levels in different brain areas of OKA 200 ng treated rat.

3.2.8. Measurement of caspase-3 activity and mRNA expression

A significant ($P<0.01$) increase in activity and mRNA expression of caspase-3 was observed in hippocampus, striatum and cortex of OKA treated rat brain in comparison to that of control and vehicle groups. Treatment with memantine and donepezil significantly ($P<0.05$)

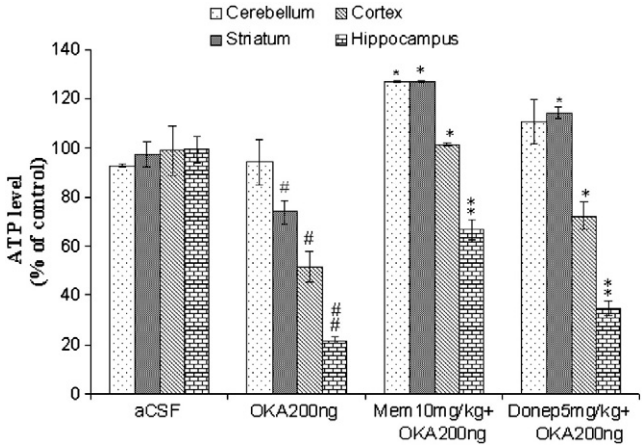

Fig. 5. Figure represents that OKA reduced ATP level in cortex, hippocampus and striatum whereas donepezil and memantine were able to restore the okadaic acid induced ATP depletion in rat. $^{\#}P<0.01$, $^{\#\#}P<0.001$ vs. control group and $^{*}P<0.01$, $^{**}P<0.001$ vs. OKA group.

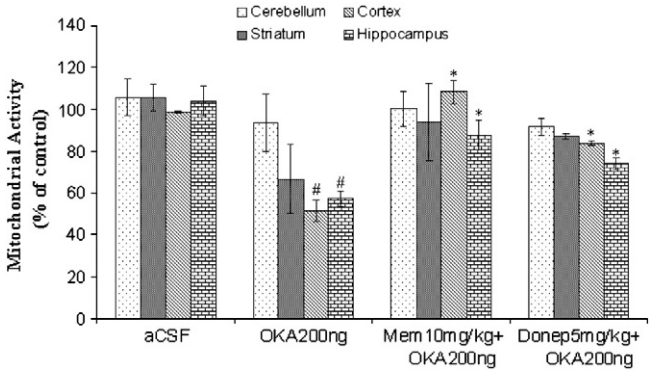

Fig. 6. Data illustrate that OKA reduced mitochondrial activity in cortex and hippocampus which was reversed by donepezil and memantine in rat. $^{\#}P<0.01$ vs. control group and $^{*}P<0.01$ vs. OKA group.

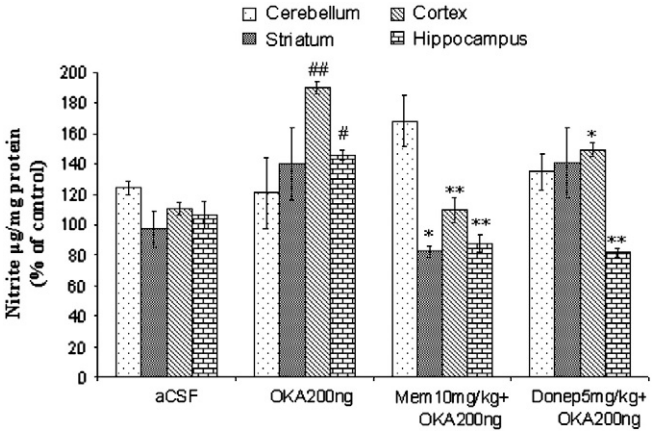

Fig. 8. A significant increase in nitrite level was observed in cortex and hippocampus of OKA treated group which was reversed by memantine and donepezil. $^{\#}P<0.01$ and $^{\#\#}P<0.001$ vs. control group and $^{*}P<0.05$, $^{**}P<0.001$ vs. OKA group.

decreased caspase-3 activity and mRNA level in hippocampus, striatum and cortex of OKA 200 ng treated rat brain (Fig. 9A and B).

3.2.9. Measurement of caspase-9 activity and mRNA expression

A significant ($P<0.01$) increase in activity and mRNA expression of caspase-9 was observed in hippocampus and cortex of OKA treated rats as compared to that of control and vehicle group. Treatment with memantine and donepezil significantly ($P<0.01$) decreased caspase-9 activity and mRNA expression in hippocampus and cortex as compared to OKA 200 ng treated group (Fig. 10A and B).

4. Histopathological changes in hematoxylin and eosin staining

Morphological observations showed that OKA administration caused neurodegeneration in the hippocampal and periventricular cortical regions of the brain as compared to control and vehicle treated rats. Pretreatment with memantine and donepezil prevented neurodegeneration in hippocampal and periventricular cortical regions of OKA treated rat (Fig. 11).

5. Discussion

Recently, we have reported that intracerebroventricular (ICV) administration of okadaic acid (OKA) in rats induces memory impairment that was associated with increased oxidative stress (Kamat et al., 2010). The present study explored the status of mitochondrial function and apoptotic cell death in brain areas related with memory functions in OKA treated rats. Besides memory deficit, OKA caused impairment in mitochondrial function as revealed by alterations in

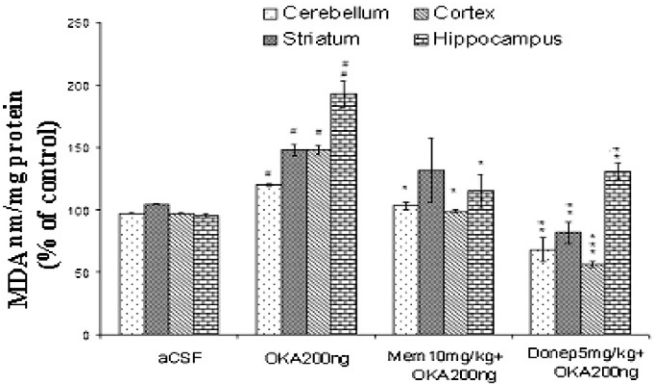

Fig. 7. A significant increase in MDA level was observed in the case of OKA treated rat as compared to control and vehicle treated rats. Both memantine and donepezil significantly reduced MDA level in OKA treated rats. $^{\#}P<0.05$, $^{\#\#}P<0.001$ vs. control group and $^{*}P<0.05$, $^{**}P<0.001$ vs OKA group.

calcium ion, reactive oxygen species (ROS) generation, mitochondrial membrane potential (MMP), SDH activity and ATP level in the brain regions. Further, it was observed that OKA significantly increased activity and mRNA expression of caspase-3 and caspase-9 in rat brain regions indicating involvement of apoptotic pathway in OKA induced neurotoxicity.

Mitochondrial dysfunction is considered to play a cardinal role in the pathogenesis of various neurodegenerative disorders like Alzheimer's disease (AD). Ca^{2+} ion plays a key role as a regulator of numerous cellular functions. It is proposed that 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 nitric oxide radicals and ROS leading to tissue damage (Bonfoco et al., 1995; Halliwell and Gutteridge, 1986). The studies have shown that ROS and Ca^{2+} regulation are tightly bound and disruption in either could affect the other. Elevated ROS level causes an imbalance in Ca^{2+} regulation in mitochondria due to compromised cellular lipid bilayer (Berridge et al., 2000). Therefore, Ca^{2+} and ROS level in mitochondrial are considered as an indicator of mitochondrial function (Mungarro-Menchaca et al., 2002). In the present study OKA caused a significant increase in mitochondrial Ca^{2+} ion and ROS in cortex, hippocampus and striatum of rat brain. Mitochondrial membrane lipids especially the long chain polyunsaturated fatty acid components are highly susceptible to ROS. Lipid peroxidation could cause structural damage to mitochondrial membranes and potentiate their dysfunction. Recently, it has been observed that lipid peroxidation in the brain occurs in early AD (Williams et al., 2006). Therefore, an elevated lipid peroxidation may indicate neuronal degeneration. The rise in level of MDA following OKA administration in synaptosomal preparation of cortex, hippocampus and striatum of rat brain may be linked with neuronal damage. We also observed a significant rise in nitrite levels in brain of OKA treated rat. The concomitant rise in ROS and nitrite may lead to the formation of peroxynitrite, a powerful pro-oxidant (Cosentino et al., 1997), NO and its toxic metabolite, peroxynitrite can inhibit components of the mitochondrial respiratory chain leading to cellular energy deficiency state ultimately activating cell death pathways (Cassina and Radi, 1996).

Further, excessive Ca^{2+} accumulation in mitochondria along with increased free radical formation causes defects in electron transport chain resulting into impaired mitochondrial function due to membrane depolarization and decreased ATP production (Zeevalk et al., 1998; Tota et al., 2011). The enzyme succinate dehydrogenase (SDH) is responsible for reduction of MTT, in the mitochondria of rats (Pollak and Duck-Chong, 1973). In the present study OKA also reduced mitochondrial metabolic function as indicated by less SDH

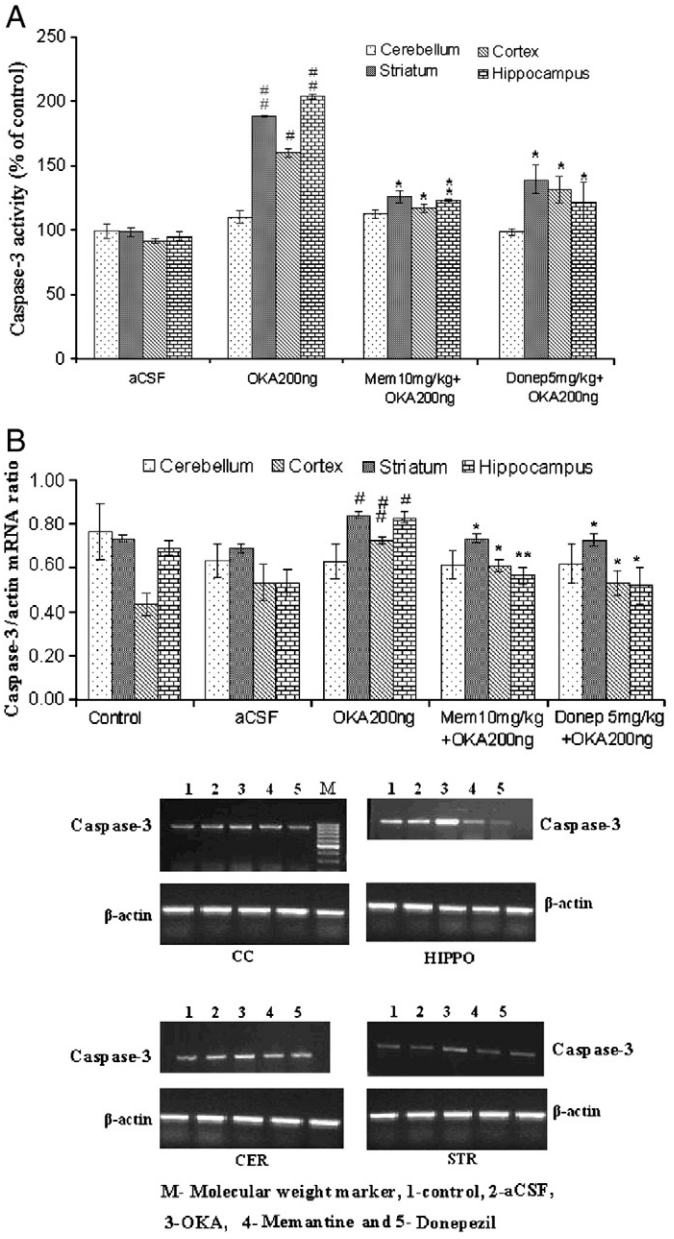

Fig. 9. A. A significant increase in caspase-3 activity was observed in striatum, cortex and hippocampus of OKA treated group which was reversed by memantine and donepezil. $^{\#}P<0.01$ and $^{\#\#}P<0.001$ vs. control group and $^{*}P<0.05$, $^{**}P<0.001$ vs. OKA group. B. Caspase-3 mRNA upregulation was observed in striatum, cortex and hippocampus of okadaic acid treated rat brain which was reversed by memantine and donepezil. $^{\#}P<0.01$ and $^{\#\#}P<0.001$ vs. control group and $^{*}P<0.05$, $^{**}P<0.001$ vs. OKA group.

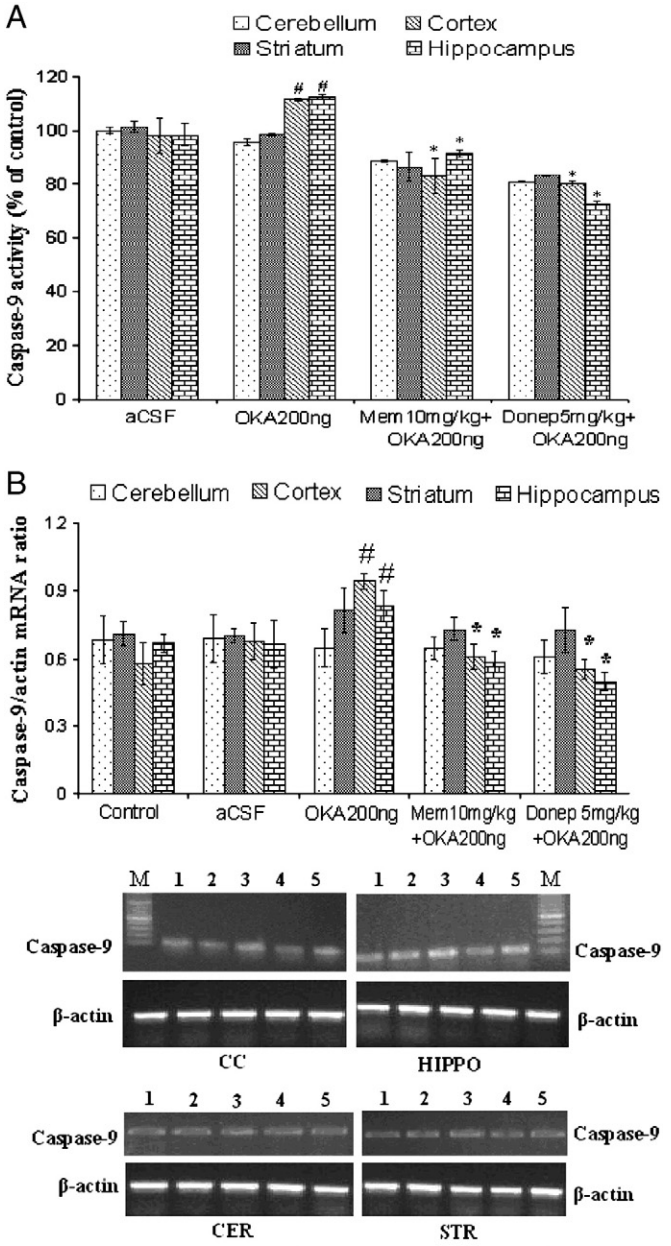

Fig. 10. A. A significant increase in caspase-9 activity was observed in cortex and hippocampus of OKA treated group which was reversed by memantine and donepezil. $^{\#}P<0.01$ vs. control group and $^{*}P<0.05$ vs. OKA group. B. Okadaic acid induces caspase-9 mRNA upregulation in cortex and hippocampus whereas pretreatment with memantine and donepezil reversed the same. $^{\#}P<0.01$ vs. control group and $^{*}P<0.05$ vs. OKA group.

activity in the MTT assay. In this study OKA caused a significantly decreased ATP production, MMP and mitochondrial metabolic activity in cortex, hippocampus and striatum brain regions except cerebellum indicating impairment in energy metabolism and mitochondrial function. This may be due to alteration in mitochondrial Ca^{2+} and free radical formation by OKA.

A decrease in MMP is an early universal event of apoptosis (Wadia et al., 1998). The release of mitochondrial cytochrome c and apoptosis-inducing factor are key events in initiating the cascade of reactions leading to apoptotic cell death (Nicholls and Budd, 2000). It was reported that structural and functional changes in mitochondria play a central role in apoptosis (Desagher and Martinou, 2000).

The extrinsic pathway is activated at the cell surface through death receptor mediated activation of caspase-8 or caspase-10, followed by caspase-3 activation and this pathway may be amplified by caspase-9 activation i.e. intrinsic pathway (Selkoe, 2002). Therefore, caspase-3 and caspase-9 are main initiator caspases which play a pivotal role in the progression of a variety of neurological disorders. Despite the various causes of such disorders, the mechanism of cell death is similar in a broad spectrum of neurological diseases (Yuan and Yankner, 2000). However, the trigger of aberrant caspase activation in majority of these diseases is not well understood. In acute neurological diseases, both necrosis and caspase-mediated apoptotic cell death occur (Emery et al., 1998). By contrast, in chronic neurodegenerative diseases, caspase-mediated apoptotic pathways have the dominant role in mediating cell dysfunction and cell death (Li et al., 2000). In this study, we have investigated the effect of OKA on caspase-3 and caspase-9 level

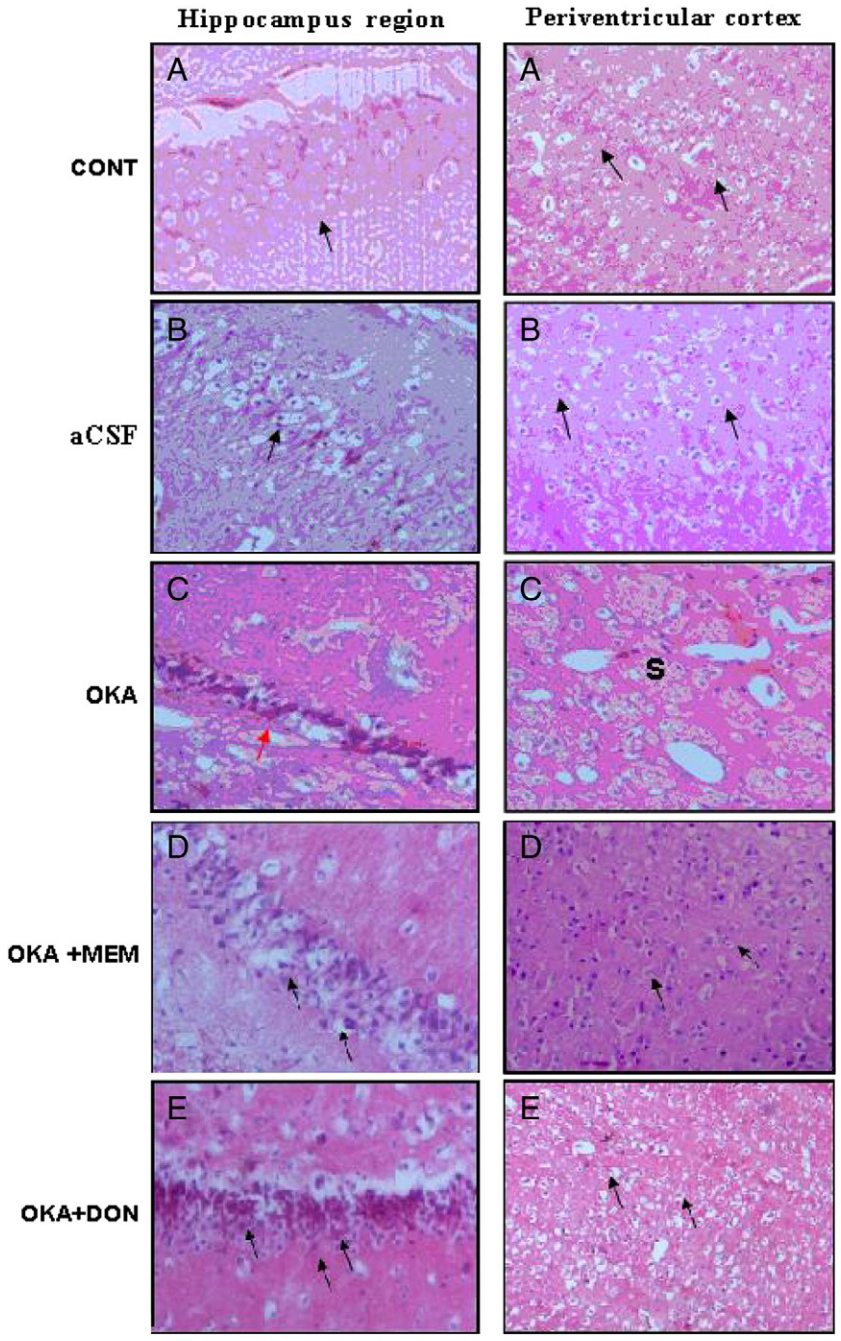

Fig. 11. Micrograph (100×) showing effect of OKA on neurons of the hippocampus: (A) control and (B) aCSF did not reveal difference in cell numbers in the neurons of hippocampus region. (C) Note: shrinkage of hippocampus region in OKA-treated rat brain (red arrow in C) revealed degeneration in the hippocampus of OKA-treated rats. Less degeneration is seen in (D) memantine and (E) donepezil treated rats as compared to OKA treated rats in the hippocampus region. While in periventricular cortex (A) control and (B) aCSF did not reveal difference in cortical cells while (C) OKA showed remarkable degeneration (sponginess of the cell indicated by S) of periventricular cortical neurons. (D) memantine and (E) donepezil treated rats showed less periventricular cortex neuronal degeneration than OKA treated rats as indicated by arrows.

in rat brain. OKA significantly increased activity and mRNA expression of caspase-3 and caspase-9 in rat brain areas indicating involvement of caspase mediated cell death in OKA induced neurodegeneration. Mitochondrial dysfunction induced by OKA could be an important function for initiation of apoptosis. Moreover, histological alteration-vacuoles, sponginess, and cell blebbing, also confirmed neuronal damage and collaborated the expected outcome of mitochondrial dysfunction and caspase apoptotic pathway alteration.

Large number of preclinical studies have suggested facilitatory role of NMDA receptor and acetylcholinesterase enzyme in memory impairment. Earlier we have reported that OKA induced memory impairment and oxidative stress is ameliorated by chronic administration of NMDA receptor antagonist memantine and anticholinesterase drug donepezil (Kamat et al., 2010). Further, this study investigated involvement of NMDA receptor and acetylcholinesterase in OKA induced neuronal dysfunction by using memantine and donepezil as experimental pharmacological tool. Both donepezil and memantine protected against the OKA induced effect on Ca^{2+}, MMP, ATP, and mitochondrial activity and also significantly restored lipid peroxidation and nitrite in synaptosomal preparation. It was also found that pretreatment with donepezil and memantine significantly prevented activities and mRNA expression of caspase-3 and 9 and mitochondrial changes in OKA treated rat.

Further, in histopathological study it was observed that donepezil and memantine reduced the cell loss and neurodegeneration in hippocampus and periventricular cortex regions in OKA treated rats. These drugs were also successful to check memory impairment caused by OKA. Thus, it seems memantine and donepezil diminished neurotoxicity by blocking OKA induced changes in mitochondrial function.

Hippocampus and cortex are the major brain areas implicated in learning and memory function. In the present study, we found that along with memory deficit OKA induces mitochondrial dysfunction, apoptotic cell death and other histological changes predominantly in hippocampus and cerebral cortex in rat brain indicating its selective neurotoxicity. Therefore, the results of the present study also explain the clinical observation that OKA causes memory impairment in human subjects those who used seafood contaminated with dinoflagellate (*Helicondria okadai*) (http://www.aristatek.com/Newsletter/DEC07/DEC07ts.aspx). The currently used antidementic drugs could be effective in OKA containing seafood related cognitive impairment in human.

6. Conclusion

It is evident from this study that mitochondrial dysfunction is associated with neuronal apoptotic cell death in OKA induced memory impairment and clinically used anti-dementic drugs are effective against OKA induced mitochondrial dysfunction and apoptotic cell death.

Acknowledgments

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