ACTA NEUROBIOLOGIAE EXPERIMENTALIS

Research paper Acta Neurobiol Exp 2015, 75: 399–411

Metabolic enhancer piracetam attenuates rotenone induced oxidative stress: a study in different rat brain regions

Dinesh Kumar Verma[#1], Neeraj Joshi[#2], Kunumuri Sivarama Raju[3], Muhammad Wahajuddin[3], Rama Kant Singh[1], and Sarika Singh[1]*

[1] Toxicology Division, CSIR-Central Drug Research Institute, Lucknow, India; [2] Department of Biochemistry and Biophysics, University of California, San Francisco, San Francisco, California, USA; [3] Pharmacokinetics & Metabolism Division, CSIR-Central Drug Research Institute, Lucknow, India; *Email: sarika_singh@cdri.res.in
[#] Authors contributed equally to this work

Piracetam is clinically being used nootropic drug but the details of its neuroprotective mechanism are not well studied. The present study was conducted to assess the effects of piracetam on rotenone induced oxidative stress by using both *ex vivo* and *in vivo* test systems. Rats were treated with piracetam (600 mg/kg b.w. oral) for seven constitutive days prior to rotenone administration (intracerebroventricular, 12µg) in rat brain. Rotenone induced oxidative stress was assessed after 1 h and 24 h of rotenone administration. *Ex vivo* estimations were performed by using two experimental designs. In one experimental design the rat brain homogenate was treated with rotenone (1 mM, 2 mM and 4 mM) and rotenone+piracetam (10 mM) for 1 h. While in second experimental design the rats were pretreated with piracetam for seven consecutive days. On eighth day the rats were sacrificed, brain homogenate was prepared and treated with rotenone (1 mM, 2 mM and 4 mM) for 1h. After treatment the glutathione (GSH) and malondialdehyde (MDA) levels were estimated in brain homogenate. *In vivo* study showed that pretreatment of piracetam offered significant protection against rotenone induced decreased GSH and increased MDA level though the protection was region specific. But the co-treatment of piracetam with rotenone did not offer significant protection against rotenone induced oxidative stress in *ex vivo* study. Whereas *ex vivo* experiments in rat brain homogenate of piracetam pretreated rats, showed the significant protection against rotenone induced oxidative stress. Findings indicated that pretreatment of piracetam significantly attenuated the rotenone induced oxidative stress though the protection was region specific. Piracetam treatment to rats led to its absorption and accumulation in different brain regions as assessed by liquid chromatography mass spectrometry/mass spectrometry. In conclusion, study indicates the piracetam is able to enhance the antioxidant capacity in brain cells in region specific manner. The study is also revealing the rationale for its clinical use in cognitive impairment and other neurological diseases.

Key words: rotenone, piracetam, oxidative stress, mid brain, hippocampus, frontal cortex

INTRODUCTION

Piracetam, a low molecular weight derivative of γ-aminobutyric acid, is widely used in treatment of cognitive disorders, ischemia, epilepsy, hypoxia and palatal myoclonus (Leuner et al. 2010). Protective effects of piracetam on brain function against hypoxic insults have also been reported in experimental animals and in cultured cells (He et al. 2008). It increases the oxygen consumption thus mitochondrial activity in rat brains (Keil et al. 2006) and its efficacy is usually associated with conditions of disturbed brain functions as observed in aging (Muller et al. 1997). Cognitive impairment is age related disease in which piracetam offered clinically proved protection (Leuner et al. 2010). In aged individuals the decreased level of anti-oxidants thus increased oxidative stress has been reported (Rybka et al. 2011) which might be the target for piracetam but such reports are scarce.

Piracetam offered anti-oxidative effects in blood cells and peritoneal macrophage at high concentration (Horvath et al. 2002, Singh et al. 2011) but information regarding the effect of piracetam in brain cells is limited. The continuous clinical use of piracetam with

Correspondence should be addressed to S. Singh
Email: sarika_singh@cdri.res.in

Received 26 February 2015, accepted 14 October 2015

fewer side effects led us to explore its neuroprotective mechanisms. The adverse effects of piracetam like anxiety, insomnia, drowsiness and agitation are of short duration and reversible (Malykh and Sadaie 2010). Fedi and others (2001) reported the long term use of piracetam and suggested that it may be safe for up to 18 months in humans at doses of 3.2 gm daily.

In one of our previous study we have showed that piracetam provide significant protection against 6-hydroxydopamine induced DNA damage in human neuroblastoma SHSY5Y cells (Goswami et al. 2011). He and others (2014) have also showed that piracetam ameliorate the oxygen and glucose deprivation induced oxidative injury in cortical neurons. Recently we have reported that piracetam induced protective pathway involve the translocation of endonuclease G in non-neuronal cells (Gupta et al. 2014). The present study was conducted to evaluate the anti-oxidative effect of piracetam in different brain regions related to Alzheimer's (frontal cortex and hippocampus) and Parkinson's (mid brain) diseases. Recently Sendrowski and others (2015) have also reported that piracetam could offer protection against amyloid beta induced neuronal damage.

Rotenone is an environmental pesticide that induces oxidative stress, disturbs mitochondrial function and causes morphological alterations in neurons (Testa et al. 2005). In previous study we have showed the rotenone induced impaired mitochondrial function and oxidative stress in time (1 and 7 day) and concentration (3 µg, 6 µg and 12 µg of rotenone) dependent manner (Swarnkar et al. 2010, 2011). Based on the previous reports, in the present study the rotenone was selected to induce the oxidative stress in brain cells. For *in vivo* study the 12 µg rotenone was administered in intracerebroventricular (icv) region of rat brain which is sufficient to cause impaired mitochondrial function and oxidative stress (Swarnkar et al. 2010).

MATERIALS AND METHODS

Chemicals – Rotenone, piracetam, DMSO (dimethyl sulphoxide) 5, 5-dithiobis 2-nitrobenzoic acid (DTNB), thiobarbituric (TBA) from Sigma Chemicals Co. (St. Louis, MA, USA). Anesthetic ether and folin's reagent was purchased from Sisco Research Laboratories Limited, Mumbai, India. Trichloroacetic acid (TCA) was purchased from Qualigens India and hydrochloric acid (HCl) from Thermo Fisher Scientific India Pvt. Ltd. Mumbai, India.

Animals

The experiments were carried out with adult male Sprague–Dawley rats (170–220 g). The animals were kept in polyacrylic cages and maintained under standard housing conditions (room temperature 22±1°C and humidity 60–65%) with 12 h light and dark cycle (lights on at 6:00 AM). The food in form of dry chow pellets and water were available ad libitum. The animals were procured from the National Laboratory Animal Center of Central Drug Research Institute and experiments were performed according to internationally followed ethical standards and approved by the animal research ethics committee of CSIR – Central Drug Research Institute (IAEC/2011/16/01). The number of rats in each group was 5–8.

Ex vivo experiments in brain homogenate

Ex vivo experiments were performed in brain homogenate of different brain regions by using two experimental designs.

Experimental design 1: The animals were sacrificed and brain homogenate was prepared. Brain homogenate was treated with rotenone (1 mM, 2 mM, 4 mM; Swarnkar et al. 2009) and rotenone+piracetam (10 mM; Singh et al. 2011) for 1 h. After treatment the homogenate was processed for estimation of enzymatic activity. The experimental sets were control, vehicle, rotenone and rotenone+piracetam.

Experimental design 2: The animals were pretreated with piracetam (600 mg/kg, oral) for seven consecutive days (He et al. 2008). On eighth day rats were sacrificed and brain homogenate was prepared and treated with rotenone (1 mM, 2 mM, 4 mM) for 1 h and processed for estimation of GSH and MDA level.

Preparation of brain homogenate and treatment for *ex vivo* study

Rats were anesthetized with diethyl ether as described by Candelario-Jalil and others (2000), intra cardiac perfusion with saline was done and rats were sacrificed by decapitation and whole brain was removed. The brain regions mid brain, hippocampus and frontal cortex were isolated and processed for different estimations. Homogenates of different brain regions, 10% (w/v) were prepared in sodium phosphate buffer (30 mmol/l, pH 7.0) using Ultra-Turrax T25

(USA) homogenizer. Rotenone was dissolved in DMSO, PEG and brain homogenate was treated with different concentration of rotenone (1 mM, 2 mM and 4 mM). Then reaction mixture of brain homogenate was incubated in a water bath at 37˚C for 1 h. Piracetam (10 mM) dissolved in saline and concurrently incubated with rotenone in separate set. Rest of the volume was made up to 1ml by DMSO, PEG. Vehicle and doses of rotenone and piracetam were selected on the basis of earlier reports (Facino et al. 1995, Singh et al. 2011). After incubation the homogenate was used for GSH and MDA assays. Each experiment was done separately in duplicate and number of animals in each group was 5–8. The DMSO concentration was adjusted in such a manner that the final concentration was not above 0.1% for the experiment.

In vivo experiments

Groups and treatment – Pre-treatment of piracetam (600 mg/kg, oral) was given to rats for seven consecutive days. On eighth day the rotenone (12 µg, 6+6 bilateral intracerebroventricular) was administered in the rat brain and rats were sacrificed after 1 h and 24 h of rotenone administration. The groups were control, vehicle (DMSO treated), per se piracetam, rotenone 1 h, rotenone 24 h, rotenone 1 h + piracetam (pretreated), rotenone 24 h + piracetam (pretreated).

Rotenone injection in rat brain

Rat was anesthetized with chloral hydrate (300 mg/kg, i.p.) and placed on a stereotaxic frame (Stoelting, USA) for surgery. Rotenone was dissolved in DMSO and injected in intracerebroventricular region of rat brain bilaterally (total 12 µg, 6 µg each side) for its better diffusion in different brain regions. The stereotaxic coordinates for intracerebroventricular regions were AP – 0.8 mm, L – 1.6 mm and DV – 3.5 mm, from the bregma point (Paxinos and Watson 1982). Proper postoperative care was done till the animals recovered completely.

Preparation of brain homogenate for biochemical estimations

Rats were anesthetized with mild ether anaesthesia and perfused with pre chilled saline by intra-cardiac injection. After perfusion the brain was dissected to isolate the mid brain, frontal cortex and hippocampus (Glowinski et al. 1966). Rat brain homogenate (10%, w/v) of different regions was prepared in sodium phosphate buffer (30 mM/l, pH 7.0) by using Ultra-Turrax T25 (USA) at a speed of 9500xrpm. The homogenate was collected and immediately processed for biochemical estimations.

Glutathione (GSH) estimation

Reduced glutathione (GSH) as a marker of oxidative stress was estimated (Sharma and Gupta 2003). Homogenates were deproteinized using 1% Trichloroacetic acid (TCA) and centrifuged for 5 min at 5000xrpm at 4˚C. GSH was estimated in supernatant which yielded yellow color with DTNB and potassium phosphate buffer (0.1 M, pH 8.4). Absorbance was read immediately at 412 nm using ELISA plate reader (BIO-TEK Instruments). The assay for each sample was run in duplicate. The GSH concentration in the samples was extrapolated from the standard curve obtained by plotting the OD of the standard GSH concentrations. Results are expressed as GSH µg/mg protein.

Malondialdehyde (MDA) estimation

Estimation of MDA was done as a marker of lipid peroxidation (Colado at al. 1997). Homogenate was deproteinized with 30% TCA and 5N HCl, followed by 2% TBA in 0.5 N sodium hydroxide. The reaction mixture was incubated on a water bath at 90˚C for 15 min and after cooling at room temperature it was centrifuged in Biofuge Fresco (Heraeus, Germany) at 5000xrpm for 10 min. The supernatant was collected and the absorbance was measured at 532 nm, using ELISA plate reader (BIO-TEK Instruments). The assay for each sample was run in triplicate. The MDA concentration in the samples was extrapolated from the standard curve obtained by plotting the O.D. of the standard concentrations of tetraethoxypropane (TEP). Results are expressed as MDA nmol/mg protein.

Estimation of protein content

Protein concentration in the homogenates of different brain regions was estimated at 660nm wavelength (Lowry et al. 1951). Bovine serum albumin (BSA) 0.01–0.1 mg/ml was used as standard.

Estimation of piracetam level in brain regions

Rats were orally administered with piracetam (600 mg/kg) for seven consecutive days. On eighth day the rats were sacrificed after one hour of piracetam-dosing, to isolate the brain for estimation of piracetam in different regions. The piracetam levels in different brain regions were estimated by liquid chromatography – mass spectrometry/mass spectrometry (LC-MS/MS). To 100 µl of the tissue homogenate, 200 µl of acetonitrile was added and vortexed for 10 min followed by centrifugation for 10 min at 13000×g at room temperature. The supernatant (200 µl) was separated and was injected onto analytical Water X bridge C18 column (4.6×50, 5 µm).

Analysis was carried out using a HPLC system consists of Shimazdu LC-20AD pumps and SIL-HTc auto sampler with temperature controller on a XBridge RP18 column (4.6×50 mm, 5.0 µM). The system was run in isocratic mode with mobile phase consisting of acetonitrile and 10 mM ammonium acetate in the ratio of 10:90 (v/v) at a flow rate of 0.80 ml/min. Mass spectrometric detection was performed on an API 4000 Qtrap mass spectrometer (Applied Biosystems, MDS Sciex Toronto, Canada) equipped with an API electrospray ionization (ESI) source. The mass spectrometer was operated at ESI positive ion mode and detection of the ions was performed in the multiple reaction monitoring (MRM) mode, monitoring transition of m/z 143.0 precursor ion $(M+H)^+$ to the m/z 98.0 product ion. Data acquisition and quantitation were performed using analyst software version 1.4.1 (Applied Biosystems, MDS Sciex Toronto, Canada). The lower limit of quantification of the method was 100 ng/ml and linearity in the calibration curve standards were demonstrated up to an upper limit of 20 µg/ml.

Statistics analysis

Results are expressed as the mean ±S.E.M. The data was analyzed by one way analysis of variance (ANOVA) followed by post-hoc Dunnett's test or Newman-Keuls test to determine the significance of differences between the groups, $P<0.05$ was considered as level of significance.

RESULTS

***Ex vivo* study**

Experimental design 1. Effect of piracetam on rotenone induced altered GSH and MDA level in homogenate of different rat brain regions after rotenone and rotenone+piracetam treatment:

1) Mid brain. The GSH level in rat brain homogenate of control rat (untreated) was 14.7±0.9 µg/mg protein. After treatment with different concentration of rotenone (1 mM, 2 mM and 4 mM) for 1 h, the GSH and MDA levels were significantly altered (Fig. 1). Rotenone (4 mM) treatment to brain homogenate caused significant ($p<0.01$) decrease in GSH level (3.01±0.7 µg/mg protein) which is significantly decreased in comparison to control. Co treatment of piracetam offered significant ($p<0.05$) protection against rotenone induced decreased GSH level and level reverted to 5.0±0.3 µg/mg protein (Fig. 1A). The MDA level in brain homogenate of control set was 0.14±0.01 nmol/mg of protein while in rotenone treated (4 mM) brain homogenate the MDA level was 1.04±0.002 nmol/mg of protein, which was significantly ($p<0.01$) increased in comparison to control values (Fig. 1B). The MDA level in rotenone+piracetam treated set was 0.7±0.01 nmol/mg of protein which was significantly ($p<0.01$) less in comparison to rotenone treated set.

2) Frontal cortex. The GSH level in brain homogenate of control rat was 13.2±1.03 µg/mg protein. Rotenone treatment caused significantly ($p<0.01$) decreased GSH level with increased concentration of rotenone. The GSH level in highest concentration (4 mM) of rotenone was 3.3±0.5 µg/mg protein. Co treatment of piracetam did not offer significant protection (Fig. 1A). Rotenone treatment to brain homogenate caused significant ($p<0.01$) increase of MDA level in comparison to brain homogenate of control rats. The MDA level in rat brain homogenate of control rat was 0.3±0.02 nmol/mg of protein while in rotenone (4 mM) treated brain homogenate the level was 0.94±0.003 nmol/mg of protein which was significantly ($p<0.01$) higher in comparison to control rats (Fig. 1B). Co treatment of piracetam did not offer significant protection in MDA level (0.8±0.01 nmol/mg of protein).

3) Hippocampus. The GSH level in brain homogenate of control rats was 14.6±1.03 µg/mg protein which was significantly ($p<0.01$) decreased with rotenone treatment in concentration dependent manner. The GSH level in rotenone (4 mM) treated brain homogenate was 2.5±0.03 µg/mg protein. Co treatment of piracetam did not offer significant protection at highest concentration of rotenone (4 mM). However, at lower concentration of rotenone (1 mM) significant

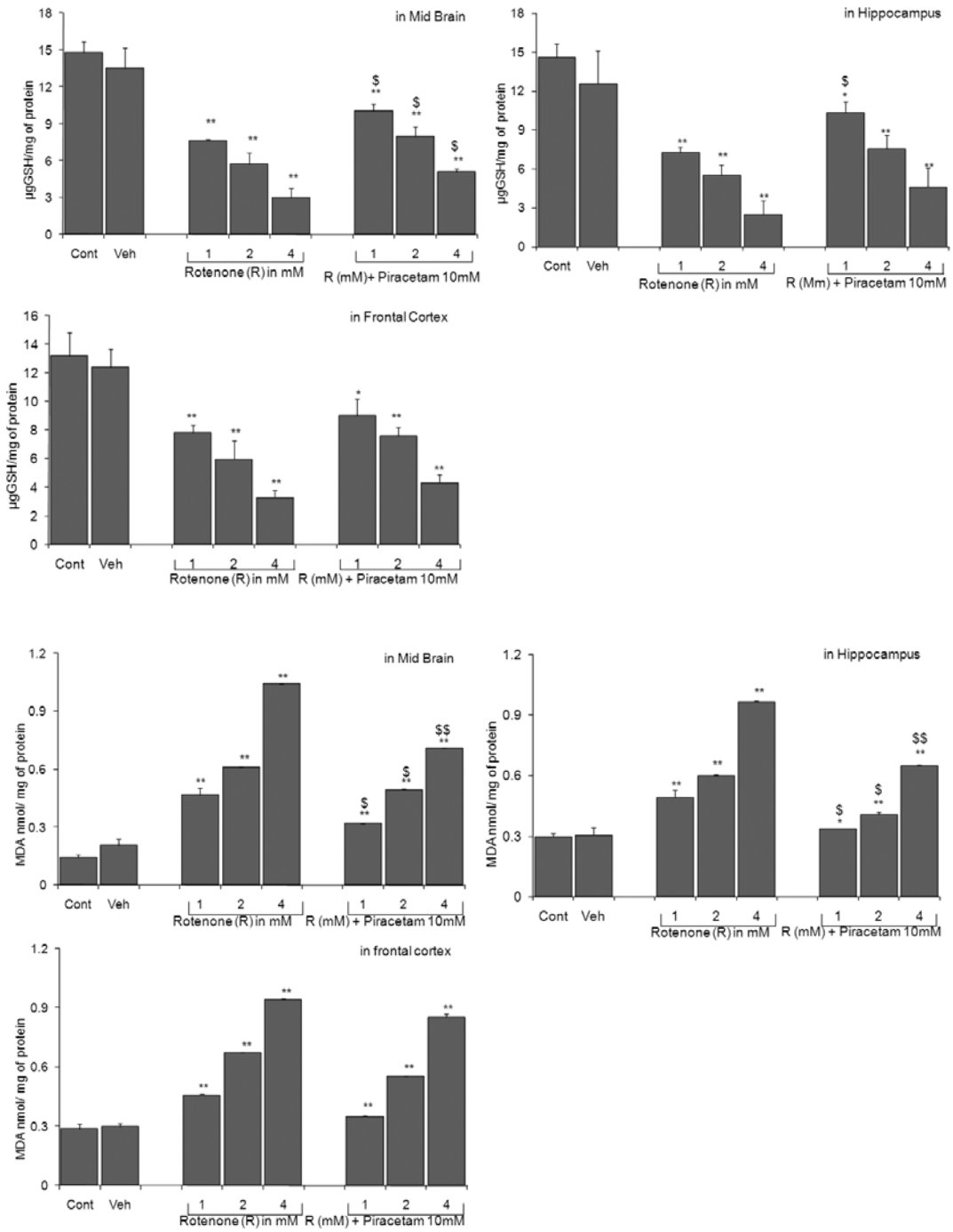

Fig. 1. (A) Graphs illustrating the levels of GSH in brain homogenate. The brain homogenate of mid brain, frontal cortex and hippocampus was prepared as described in method section. The brain homogenate was treated with rotenone (1 mM, 2 mM and 4 mM) and rotenone+piracetam (P=10 mM) for 1 h. After treatment the homogenate was processed for GSH estimation. *=p<0.05 control *vs.* rotenone treatment. $=p<0.05 rotenone *vs.* rotenone+piracetam treatment. Data is presented as mean ±SEM. (B) Graphs illustrating the levels of MDA in brain homogenate. The brain homogenate of mid brain, frontal cortex and hippocampus was prepared as described in method section. The brain homogenate was treated with rotenone (1 mM, 2 mM and 4 mM) and rotenone+piracetam (P=10 mM) for 1 h. After treatment the homogenate was processed for MDA estimation. **=p<0.01 control *vs.* rotenone treatment. $=p<0.05, $$=p<0.01 rotenone *vs.* rotenone+piracetam. Data is presented as mean ±SEM.

($p<0.05$) protection was observed (Fig. 1A) and the GSH level was 9.06±1.1 μg/mg protein. MDA level was significantly increased after rotenone treatment in concentration dependent manner. The MDA level in brain homogenate of control rats was 0.30±0.01 nmol/mg of protein while in rotenone (4 mM) treated brain homogenate the level was 1.0±0.02 nmol/mg of protein which was significantly ($p<0.01$) higher in comparison to control rats. Co treatment of piracetam offered significant protection against rotenone (4 mM) induced increased level of MDA and level was 0.64±0.004 nmol/mg of protein (Fig. 1B).

Experimental design 2. Effect of piracetam on rotenone induced altered GSH and MDA level in control and piracetam pretreated rats in different brain regions:

1) Mid brain. The GSH level in mid brain of control rats was 9.25±0.3 μg/mg protein. Pretreatment of piracetam to rats for seven days offered significantly increased levels of GSH (12.2±1.9 μg/mg protein) which is in concordance to previous reports. However, piracetam pretreatment to rats did not offer significant protection against rotenone induced oxidative stress in brain homogenate. The GSH level in control and piracetam pretreated rat brain homogenate, after rotenone (4 mM) treatment was 3.04±0.4 and 3.99±0.5 μg/mg protein respectively (Fig. 2A). The MDA level in brain homogenate of control rats was 0.48±0.00 nmol/mg protein while the level in brain homogenate of piracetam treated rats was 0.42±0.00 nmol/mg protein. Rotenone (4 mM) treatment to rat brain homogenate of control and piracetam treated rats caused significant increase in MDA level and the level was 1.08±0.001 and 1.0±0.001 nmol/mg protein respectively (Fig. 2B). Piracetam treatment did not offer significant protection against rotenone induced increased MDA levels.

2) Frontal cortex. The GSH level in frontal cortex of control rats was 14.1±2.9 μg/mg protein while in piracetam pretreated rats the level was 12.8±3.7 μg/mg protein which was not significantly different in comparison to level of control rats. Rotenone (4 mM) treatment to brain homogenate of control (piracetam untreated) and piracetam pretreated rats, caused significant ($p<0.01$) decrease in GSH level and the level was 2.8±0.5 and 3.54±0.4 μg/mg protein respectively (Fig. 2A). The MDA level in brain homogenate of control rats was 0.42±0.00 nmol/mg protein while in brain homogenate of piracetam treated rats the level was 0.34±0.001 nmol/mg protein. Rotenone (4 mM) treatment to rat brain homogenate of control (piracetam untreated) and piracetam treated rats caused significant increase in MDA level and level was 1.0±0.002 and 1.0±0.001 nmol/mg protein respectively. Piracetam pretreatment did not offer significant protection against rotenone induced increased MDA level (Fig. 2B).

3) Hippocampus. The GSH level in hippocampus of control rats was 7.85±0.5 μg/mg protein while in piracetam pretreated rats the level was 9.6±0.3 μg/mg protein which was higher in comparison to control sets. Rotenone (4 mM) treatment to brain homogenate of control (piracetam untreated) and piracetam pretreated caused decreased GSH level. The GSH level in control (piracetam untreated) and piracetam pretreated rats was 3.2±0.2 and 3.2±0.1 μg/mg protein respectively. Piracetam pretreatment did not offer significant protection at higher concentration of rotenone (4 mM). However, at lower concentration of rotenone, piracetam offered significant ($p<0.05$) protection against rotenone induced augmented GSH level. Piracetam-pretreatment offered significant protection ($\$=p<0.05$) against rotenone induced oxidative stress (Fig. 2A). The MDA level in brain homogenate of control rats was 0.38±0.00 nmol/mg protein while in brain homogenate of piracetam pretreated rats the level was 0.37±0.004 nmol/mg protein. The MDA level in rotenone (4 mM) treated brain homogenate of control and piracetam pretreated rats was 1.11±0.001 and 0.93±0.00 nmol/mg protein respectively (Fig. 2) which was not significantly different (Fig. 2B). However, pretreatment of piracetam offered significant ($p<0.01$) protection against rotenone (1 mM and 2 mM) induced augmented MDA level (Fig. 2B).

In vivo study

Effects of piracetam on rotenone induced altered GSH and MDA level in rat brain regions after 1 h and 24 h of rotenone administration:

1) Mid brain. In control rats the GSH level was 9.98±1.6 μg/mg protein. In piracetam pretreated rats the significant increase in GSH level was observed and level was 19.8±2.6 μg/mg protein ($p<0.01$). After 1 h of rotenone administration no significant alteration in GSH level was observed and the level was 10.7±1.2 μg/mg protein. However, after 24 h of rotenone administration the level was decreased significantly and level was 3.7±0.9 μg/mg protein ($p<0.05$). In piracetam pretreated rats the level of GSH in rotenone administered

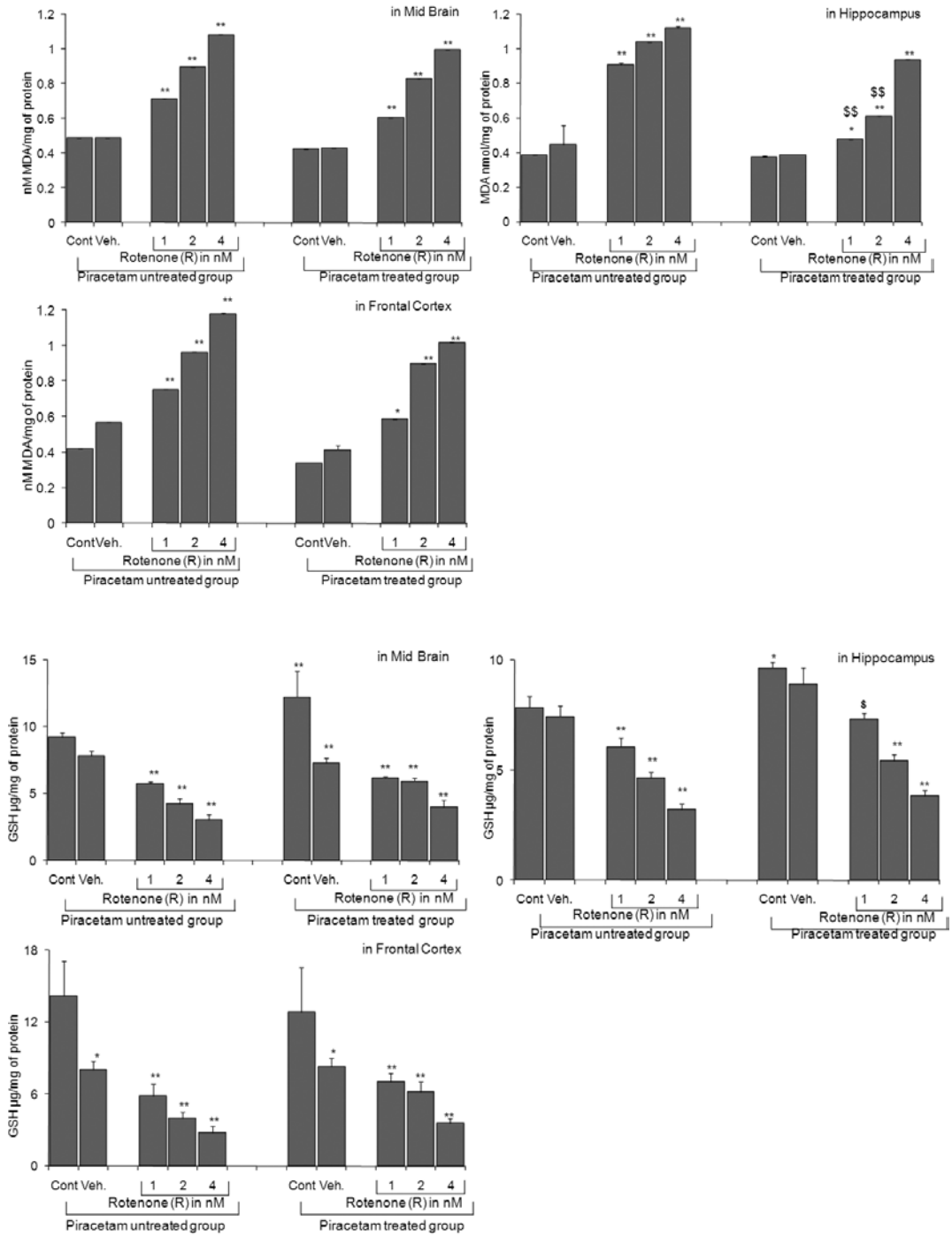

Fig. 2. (A) Graphs illustrating the GSH level in brain homogenate in piracetam pretreated and piracetam untreated rats. After constitutive 7 days of piracetam treatment rats were sacrificed. The brain homogenate of mid brain, frontal cortex and hippocampus was prepared as described in method section. The brain homogenate was treated with rotenone (1 mM, 2 mM and 4 mM) for 1 h. After treatment the homogenate was processed for GSH estimation. **=p<0.01 control *vs.* rotenone treatment. Data is presented as mean ±SEM. (B) Graphs illustrating the MDA level in brain homogenate of piracetam pretreated and piracetam untreated rats. After constitutive 7 days of piracetam treatment rats were sacrificed. The brain homogenate of mid brain, frontal cortex and hippocampus was prepared as described in method section. The brain homogenate was treated with rotenone (1 mM, 2 mM and 4 mM) for 1 h. After treatment the homogenate was processed for MDA estimation. **=p<0.01 control *vs.* rotenone treatment. Data is presented as mean ±SEM.

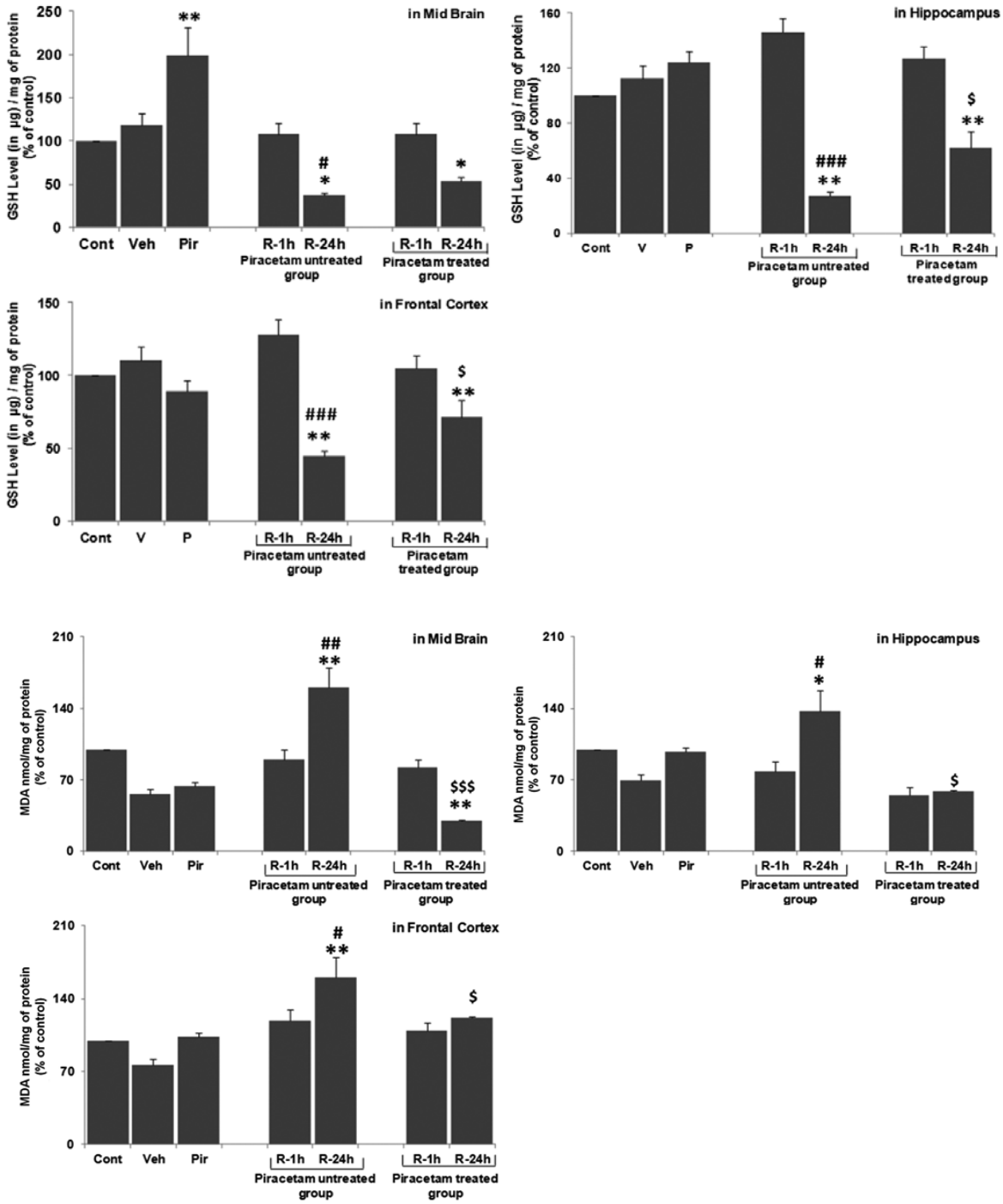

Fig. 3. (A) Graphs illustrating the levels of GSH in different regions of rat brain after rotenone (icv) and rotenone (icv)+piracetam (oral) administration. Pretreatment of piracetam offered significant protection against rotenone induced oxidative stress. R-rotenone *=p<0.05 control *vs.* rotenone treated, $=p<0.05 rotenone *vs.* rotenone+piracetam, #=p<0.05, ###=p<0.001 rot – 1 h *vs.* rot – 24 h. Data is represented as mean ±SEM. (B) Graphs illustrating the levels of GSH in different regions of rat brain after rotenone (icv) and rotenone (icv)+piracetam (oral) administration. Pretreatment of piracetam offered significant protection against rotenone induced oxidative stress. R-rotenone *=p<0.05, p<0.01 control *vs.* rotenone treated, $=p<0.05, $$$=p<0.001 rotenone *vs.* rotenone+piracetam, #=p<0.05, ##=p<0.01 rot – 1 h *vs.* rot – 24 h. Data is represented as mean ±SEM.

Table I

Level of piracetam in different brain regions of control and piracetam treated rats. Concentration expressed as mean ±SEM

Brain parts	Control (μg/g)	Treated (μg/g)
MB	0	33.50±0.44
STR	0	31.89±0.35
HP	0	42.40±0.56
FC	0	45.40±0.53
CC	0	49.40±0.52

rats after 1 h and 24 h was 10.8±1.3 μg and 5.3±1.9 μg/mg protein showed the significant attenuation against rotenone induced decreased GSH level (Fig. 3A). In control rats the MDA level was 0.17±0.08 nmol/mg of protein. The MDA level was not significantly altered in piracetam pretreated rat brain regions. In rotenone 24 h group the significant increase in MDA level was observed and level was 1.60±0.2 nmol/mg of protein ($p<0.01$). While in rotenone 1 h group no significant alteration was observed in comparison to control rats. Piracetam pretreatment offered significant ($p<0.001$) protection against rotenone induced increased MDA level in rotenone 24 h group and level was 0.10±0.12 nmol/mg of protein (% of control) (Fig. 3B).

2) Frontal cortex. In control rats the GSH level was 10.9±1.6 μg/mg protein. No significant alteration in GSH level was observed in piracetam pretreated rats. After 24 h of rotenone administration significant ($p<0.01$) decrease in GSH level and level was 4.47±0.4 μg/mg protein ($p<0.01$). However, no significant alteration was observed in rats of rotenone 1 h group. In piracetam pretreated rat brain significant ($p<0.05$) attenuation against rotenone induced decreased GSH level was observed after 24 h of rotenone administration and the level was 7.1±1.5 μg/mg protein. In rotenone 1 h group with and without piracetam treatment no significant alteration in GSH level was observed in comparison to control rats (Fig. 3A). In control rats the MDA level was 0.15±0.04 nmol/mg of protein. MDA level in piracetam treated rats was 0.18±0.08 nmol/mg of protein. After 24 h of rotenone administration the significant ($p<0.01$) increase in MDA level was observed and the level was 1.6±0.2 nmol/mg of protein. However in rotenone 1 h group, no significant increase was observed. Piracetam pretreated rats showed significant ($p<0.05$) protection against rotenone induced increased MDA level in rotenone 24 h group and level was 0.19±.03 nmol/mg of protein (Fig. 3B).

3) Hippocampus. In control rats the GSH level was 11.9±1.5 μg/mg protein. Piracetam pretreated rats did not show significant alteration in GSH level and level was 12.4±1.2 μg/mg protein. After 24 h of rotenone administration significant decrease in GSH level was observed and level was 3.6±0.21 μg/mg protein ($p<0.01$). However, no significant alteration was observed in rats of rotenone 1 h group in comparison to control rats. In piracetam pretreated rat brain significant ($p<0.05$) attenuation against rotenone induced decreased GSH level was observed and level was 3.2±0.4 μg/mg protein. In rotenone 1 h group with and without piracetam treatment no significant alteration in GSH level was observed (Fig. 3A). In control rats the MDA level was 0.18±0.03 nmol/mg of protein. MDA level in piracetam treated rats was 0.17±0.04 nmol/mg of protein. After 24 h of rotenone administration the significant ($p<0.01$) increase in MDA level was observed and the level was 1.1±0.05 nmol/mg of protein. However in rotenone 1 h group no significant increase was observed. Piracetam pretreated rats showed significant ($p<0.05$) protection against rotenone induced increased MDA level in rotenone 24 h group and level was 0.16±0.02 nmol/mg of protein (Fig. 3B).

Level of piracetam in different brain regions

The representative chromatogram for the LC-MS/MS analysis of the piracetam in the brain tissue is given in Fig. 4. The mean concentration of piracetam in each brain regions (μg/gm of tissue) is shown in the table I. In frontal cortex the level of piracetam was 45.40±0.53 μg/gm of tissue which was higher in comparison to mid brain and hippocampus region though it was not significantly different.

DISCUSSION

In the present study piracetam pretreatment exhibited the significant protection against rotenone induced oxidative stress in different brain regions though the

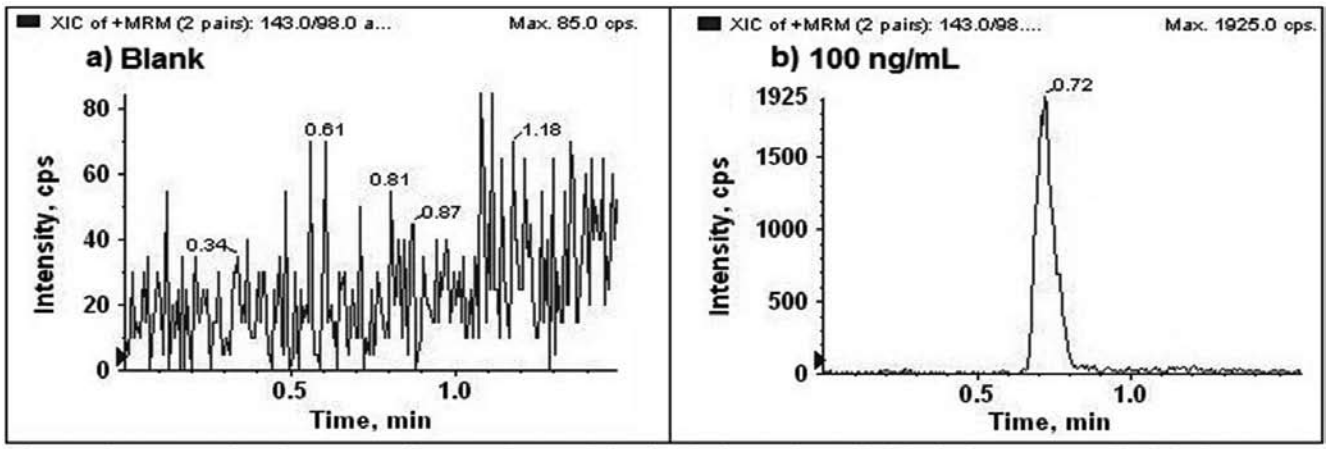

Fig. 4. The representative chromatogram for LC-MS/MS analysis of the brain samples: (A) blank brain homogenate and (B) brain homogenate spiked with 100 ng/mL of piracetam.

pattern varied with respect to different brain regions. Previously we have showed that rotenone (12 µg) is able to cause the mitochondrial impairment and oxidative stress in time dependent manner (Swarnkar et al. 2010). Therefore in the present study 12 µg of rotenone concentration was selected to evaluate the efficient antioxidant like property of piracetam. The depleted tyrosine hydroxylase expression was also reported after 12 µg administration of rotenone in rat brain (Swarnkar et al. 2013). To assess the absorption of piracetam in rat brain after its oral administration, the piracetam level were estimated in studied rat brain regions by LC-MS/MS. Data showed that oral administration of piracetam led to approximate hundred percent absorption of piracetam.

Rotenone is a naturally occurring toxin extracted from various parts of leguminous plants and commonly used as pesticide. It induces the generation of reactive oxygen species (ROS) directly and via the activation of neuronal supportive glial cells which in turn contribute in neuronal death (Swarnkar et al. 2010). It crosses the blood brain barrier thus all the brain regions are equally susceptible to rotenone induced neurotoxicity. Rotenone exposure also causes interefernce in mitochondrial trafficking which lead to initiation of neurodegenerative mechanisms (Chaves et al. 2013). In previous study the rotenone was administered intranigrally (Swarnkar et al. 2010) however in the present study it was injected in intracerebroventricularly for its better diffusion to all regions of rat brain. The study was conducted in mid brain, frontal cortex and hippocampus regions of rat brain which are involved in the pathology of Alzheimer's and Parkinson's disease.

To assess the oxidative stress and lipid peroxidation, the glutathione (GSH) and malonaldehyde (MDA) levels were estimated. In physiological conditions, tissues have a functional antioxidant molecule GSH that is depleted due to oxidative stress. It prevents the damage to important cellular components caused by reactive oxygen species such as free radicals and peroxides (Pompella et al. 2003). Excessive generation of free radicals causes decreased GSH level thus contributes in oxidative stress and consequent brain damage (Jain et al. 1991). MDA level were estimated as an indicator of membrane lipid peroxidation and its elevated level is considered as marker of oxidative stress, which causes cellular damage by peroxidation of membrane phospholipids (Jose et al. 2001, Facino et al. 1995, Radi et al. 1991) suggested that the increase in lipid peroxidation may be due to an insufficiency of the protective antioxidant systems (mainly GSH). Mitochondrial GSH is the main line of defense for the maintenance of mitochondrial redox environment due to physiologic reactions of electron transport chain. Though under physiological conditions the free radicals could be neutralized with antioxidant system of body, thus prevent the free radicals induced oxidative modifications of proteins, mitochondrial dysfunction and oxidative stress mediated cell death. Rotenone causes the inhibition of mitochondrial activity and resulted in increased ROS generation and decreased level of GSH which make cell more vulnerable to oxidative stress (Deneke et al. 1989).

In the present study we have observed the decreased GSH and increased MDA level in all three brain

regions though the protection offered with pre-treatment of piracetam was varied. Piracetam pre-treatment offered significant protection against rotenone induced decreased GSH and increased MDA level in frontal cortex and hippocampus region. However, in mid brain the decreased GSH level was not significantly attenuated with piracetam pre-treatment (in brain homogenate) though in rat brain regions significant attenuation was observed with piracetam pre-treatment. The augmented MDA level was also significantly attenuated with piracetam pre-treatment in different brain regions. The varied response of piracetam in different brain regions suggested the diverse susceptibility of neuronal population against rotenone induced toxic effects. In few regions piracetam offered protection against rotenone induced decreased GSH levels while it did not offer protection against rotenone induced augmented MDA level. Observation showed that piracetam is not solely acts through enhancing GSH level and lipid peroxidation rather it includes other protective signalling mechanisms. In concordance to this last year we have reported that piracetam induced cell protection mechanisms involve the endonuclease G mediated caspase independent pathway (Gupta et al. 2014). In addition the findings by others also indicate the potential use of piracetam in region and disease specific manner (Leuner et al. 2010, He et al. 2008). The GSH level in mid brain region of piracetam per se treated group was significantly higher in comparison to control while in frontal cortex and hippocampus no significant alteration was observed confirming the findings of the previous reports (Keil et al. 2006) and suggesting the effect of piracetam on mitochondrial activity. In the *ex vivo* brain homogenate experiments also significantly increased GSH level was observed in piracetam per se treated sets indicating the region specific response of piracetam. Observations from *in vivo* experiments showed that piracetam pre-treatment offered significant protection against rotenone induced (when rotenone administered in rats by icv route) oxidative stress in different brain regions. Piracetam increases the cerebral blood flow and consequently increases glucose and oxygen availability (Jordaan et al. 1996) which is required for neuronal survival and this might be the reason for region specific effect of piracetam. Most of the neurodegenerative diseases are related with progressive decrease of oxygen and glucose consumption with a reduction of cerebral blood flow (CBF), which could be responsible for age-related neurodegenerative disorders and this might be the rationale and significant efficacy of piracetam in neurological diseases. In agreement to our study, it has been reported that piracetam induces augmented GSH level and inhibition of cytokine production in mice brain and prostate cancer cells (Navarro et al. 2013, Costa et al. 2013).

Previously we have reported that piracetam offered protection against oxidative DNA damage in peripheral blood leukocytes (Singh et al. 2011). It showed the membrane fluidity effect of piracetam at the sub-cellular level for membranes of brain mitochondria (Giurgea et al. 1982). It also showed the protective effect towards mitochondrial function (Keil et al. 2006), which gets altered in most of the neurological disorders. Piracetam has protective action for the mitochondrial functions and mitochondrial GSH act as key survival antioxidant (Marí et al. 2009). Thus piracetam might act through enhancing the level of mitochondrial GSH and provide protection towards oxidative stress mediated cell death.

From *in vivo* observations it appears that mid brain and hippocampus are more susceptible to rotenone induced oxidative stress in comparison to frontal cortex. Piracetam offered significant protection only in hippocampus and frontal cortex while in mid brain no significant protection was observed. However *in vitro* experiments showed the more protection in brain homogenate of mid brain in comparison to homogenate of other regions indicating the test system dependent responses of piracetam reflecting the test system dependent findings. Though the *in vivo* findings are more relevant as reflecting the complex signaling mechanism of human body and the study is for the ultimate use of piracetam for the welfare of mankind. However, both test system showed the significant protection in case of regions affected in cognitive patients and Alzheimer's revealing its potential therapeutic effect in memory related disorders. Findings also suggested that use of piracetam in preventive mode might provide better efficacy and protective effects in comparison to curative mode.

In conclusion results indicated that piracetam pretreatment offered significant protection against rotenone induced oxidative stress in mid brain, hippocampus and frontal cortex regions of rat brain. Findings reflect the antioxidant like property of piracetam in region specific manner which might be one of the neuroprotective mechanisms of piracetam in cognitive patients and its rationale for its clinical use.

REFERENCES

Candelario-Jalil E, Ajamieh HH, Sam S, Martinez G, Fernandez OSL (2000) Nimesulide limits kainate-induced oxidative damage in the rat hippocampus. Eur J Pharmacol 390: 295–298.

Chaves RS, Melo TQ, D'Unhao AM, Farizatto KL, and Ferrari MF (2013) Dynein c1h1, dynactin and syntaphilin expression in brain areas related to neurodegenerative diseases following exposure to rotenone. Acta Neurobiol Exp (Wars) 73: 541–556.

Colado MI, O'Shea E, Granados R, Misra A, Murray TK, Green AR (1997) A study of the neurotoxic effect of MDMA ("ectasy") on 5-HT neurons in the brains of mothers and neonates following administration of the drug during pregnancy. Br J Pharmacol 121: 827–833.

Costa RA, Fernandes MP, de Souza-Pinto NC, Vercesi AE (2013) Protective effects of L-carnitine and piracetam against mitochondrial permeability transition and PC3 cell necrosis induced by simvastatin. Eur J Pharmacol 701: 82–86.

Deneke SM, Fanburg BL (1989) Regulation of cellular glutathione. Am J Physiol 1: 163–173.

Facino RM, Carini M, Aldini G, Saibene L, Morelli R (1995) Differential inhibition of superoxide, hydroxyl and peroxyl radicals by nimesulide and its main metabolite 4-hydroxynimesulide. Arzneimittelforschung 45: 1102–1109.

Fedi M, Reutens D, Dubeau F, Andermann E, D'Agostino D, Andermann F (2001). Long-term efficacy and safety of piracetam in the treatment of progressive myoclonus epilepsy. Arch Neurol 58(5): 781–786.

Giurgea CE (1982) The nootropic concept and its prospective implications. Drug Dev Res 2: 441–446.

Glowinski J, Iversen LL (1966) Regional studies of catecholamines in the rat brain. The disposition of [3H] norepinephrine [3H] dopamine and [3H] DOPA in various regions of the brain. J Neurochem 13: 655–670.

Goswami P, Swarnkar S, Harsoliya MS, Singh S, Nath C, Sharma S (2011) Attenuation of DNA damage, A key step in neuroprotective effect of piracetam against 6-hydroxydopamine induced neuronal death in SHSY5Y cells. J Pharm Res 4: 1716–1719.

Gupta S, Verma DK, Biswas J, Rama Raju KS, Joshi N, Wahajuddin, Singh S (2014) The metabolic enhancer piracetam attenuates mitochondrion-specific endonuclease G translocation and oxidative DNA fragmentation. Free Radic Biol Med 73: 278–290.

He Z, Liao Y, Zheng M, Zeng FD, Guo LJ (2008) Piracetam improves cognitive deficits caused by chronic cerebral hypoperfusion in rats. Cell Mol Neurobiol 28: 613–627.

He Z, Hu M, Zha YH, Li ZC, Zhao B, Yu LL, Yu M, Qian Y (2014). Piracetam ameliorated oxygen and glucose deprivation-induced injury in rat cortical neurons via inhibition of oxidative stress, excitatory amino acids release and P53/Bax. Cell Mol Neurobiol 34(4): 539–547.

Horvath B, Marton Z, Halmosi R, Alexy T, Szapary L, Vekasi J, Biro Z, Habon T, Kesmarky G, Toth K (2002) In vitro antioxidant properties of pentoxifylline, piracetam, and vinpocetine. Clin Neuropharmacol 25: 37–42.

Jain A, Martensson J, Stole E, Auld PA, Meister A (1991) Glutathione deficiency leads to mitochondrial damage in brain. Proc Natl Acad Sci U S A 88: 1913–1917.

Jordaan B, Oliver DW, Dormehl IC, Hugo N (1996) Cerebral blood flow effects of piracetam, pentifylline, and nicotinic acid in the baboon model compared with the known effect of acetazolamide. Arzneimittelforschung 46: 844–847.

Jose LM, Olivenza M, Moro MA, Lizasoain I, Lorenzo P, Rodrigo J, Leza JC (2001) Glutathione depletion, lipid peroxidation and mitochondrial dysfunction are induced by chronic stress in rat brain. Neuropsychopharmacology 24: 420–429.

Keil U, Scherping I, Hauptmann S, Schuessel K, Eckert A, Müller WE (2006) Piracetam improves mitochondrial dysfunction following oxidative stress. Br J Pharmacol 147: 199–208.

Leuner K, Kurz C, Guidetti G, Orgogozo JM, Müller WE (2010) Improved mitochondrial function in brain aging and Alzheimer disease – the new mechanism of action of the old metabolic enhancer piracetam. Front Neurosci 7: 44.

Lowry OH, Rosebrough NJ, Farr AL (1951) Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265–275.

Malykh AG, Sadaie MR, (2010). Piracetam and piracetam-like drugs: from basic science to novel clinical applications to CNS disorders. Drugs 70(3): 287–312.

Marí M, Morales A, Colell A, García-Ruiz C, Fernández-Checa JC (2009) Mitochondrial glutathione, a key survival antioxidant. Antioxid Redox Signal 11: 2685–2700.

Muller WE, Koch S, Scheuer K, Rostock A, Bartsch R (1997) Effects of piracetam on membrane fluidity in the aged mouse rat and human brain. Biochem Pharmacol 53: 135–140.

Navarro SA, Serafim KG, Mizokami SS, Hohmann MS, Casagrande R, Verri WA Jr (2013) Analgesic activity of piracetam: effect on cytokine production and oxidative stress. Pharmacol Biochem Behav 105: 183–192.

Paxinos G, Watson C (1982) The Rat Brain in Stereotaxic Coordinates (2nd ed.) Academic Press, New York, NY, USA. p. 83–97.

Pompella A, Visvikis A, Paolicchi A, De Tata V, Casini AF (2003) The changing faces of glutathione, a cellular protagonist. Biochem Pharmacol 66: 1499–1503.

Radi R, Beckman JS, Bush KM, Freeman BA (1991) Peroxynitrite oxidation of sulfhydryls. The cytotoxic potential of superoxide and nitric oxide. J Biol Chem 266: 4244–4250.

Rybka J, Kupczyk D, Kędziora-Kornatowska K, Pawluk H, Czuczejko J, Szewczyk-Golec K, Kozakiewicz M, Antonioli M, Carvalho LA, Kędziora J (2011) Age-related changes in an antioxidant defense system in elderly patients with essential hypertension compared with healthy controls. Redox Rep 16: 71–77.

Sendrowski K, Sobaniec W, Stasiak-Barmuta A, Sobaniec P, Popko J (2015) Study of the protective effects of nootropic agents against neuronal damage induced by amyloid-beta (fragment 25–35) in cultured hippocampal neurons. Pharmacol Rep 67(2): 326–331.

Sharma M, Gupta YK (2003) Effect of alpha lipoic acid on intracerebroventricular streptozotocin model of cognitive impairment in rats. Eur Neuropsychopharmacol 13: 241–247.

Singh S, Goswami P, Swarnkar S, Singh SP, Wahajuddin, Nath C, Sharma S (2011) A study to evaluate the effect of nootropic drug-piracetam on DNA damage in leukocytes and macrophages. Mutat Res 726: 66–74.

Swarnkar S, Goswami P, Kamat PK, Patro IK, Singh S, Nath C (2013) Rotenone-induced neurotoxicity in rat brain areas: a study on neuronal and neuronal supportive cells. Neuroscience 230: 172–183.

Swarnkar S, Singh S, Mathur R, Patro IK, Nath C (2010) A study to correlate rotenone induced biochemical changes and cerebral damage in brain areas with neuromuscular coordination in rats. Toxicology 272: 17–22.

Swarnkar S, Singh S, Sharma S, Mathur R, Patro IK, Nath C (2011) Rotenone induced neurotoxicity in rat brain areas, a histopathological study. Neurosci Lett 501: 123–127.

Swarnkar S, Tyagi E, Agrawal R, Singh MP, Nath C (2009) A comparative study on oxidative stress induced by LPS and rotenone in homogenates of rat brain regions. Environ Toxicol Pharmacol 27: 219–224.

Testa CM, Sherer TB, Greenamyre JT (2005). Rotenone induces oxidative stress and dopaminergic neuron damage in organotypic substantia nigra cultures. Brain Res Mol Brain Res 134: 109–118.