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Silymarin improves the behavioural, biochemical and histoarchitecture alterations in focal ischemic rats: A comparative evaluation with piracetam and protocatachuic acid

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ABSTRACT

Comparative neuroprotective potential of silymarin, piracetam and protocatechuic acid ethyl ester (PCA) was evaluated in focal ischemic rats. Various pharmacological, biochemical (lipid peroxidation, reduced glutathione, catalase, nitrite content, brain water content) and behavioural (memory impairment, motor control, neurological score) including infarct size and histopathological alterations were evaluated. Silymarin (200 mg/kg) and PCA treatment significantly improved behavioural, biochemical and histopathological changes, and reduced water content and infarct size. However, piracetam only improved behavioural and histopathological changes, reduced water content and infarct size. The findings indicate that silymarin exhibits neuroprotective activity better than PCA and piracetam in focal ischemia/reperfusion reflected by its better restoration of behavioural and antioxidant profile.

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1. Introduction

Cerebral ischemia leads to a decreased blood flow resulting in oxidative stress; due to the imbalance between the generations of the reactive oxygen species and body's endogenous defence system (Ozkul et al., 2007; Chong et al., 2005). The order of events occurring after ischemia are: anaerobic glycolysis, excitotoxicity, free radical formation, release of inflammatory mediators, increased nitrite content and calcium, enzyme alterations etc. (Alexandrova and Bochev, 2005; Bemeur et al., 2007; Rodrigo et al., 2005).

Piracetam is a cyclic derivative of gama-amino-butyric acid (GABA), used clinically as a nootropic agent, known to improve higher cerebral integrative functions like dementia, learning and memory (Giurgea, 1976; Salimov et al., 1995; Waegemans et al., 2002). In addition, it also modulates effectively neuroplasticity, neuroprotection and brain metabolism (Winnicka et al., 2005). Furthermore, the protective effect of piracetam on brain function against hypoxic insults both in experimental animals and humans including amelioration of amnesic effect in rats has been documented (Sara and Lefevre, 1972). Moreover, piracetam was able to restore impaired dopamine release due to hypoxia (Wastmann et al., 1982), improved cortical neuronal responsiveness to cutaneous stimulation after focal brain injury (Coq and Xerry, 1999) and reduced infarct brain volume in permanent middle cerebral artery occlusion in rats (Tortiglione et al., 2002). Protocatechuic acid ethyl ester (PCA) showed neuroprotective activity by promoting endogenous enzyme antioxidants, prevented free radical formation, and hydrogen peroxide (H2O2) induced oxidative damage on cultured PC12 cells (Shi et al., 2006). Silymarin is a polyphenolic flavanoid isolated from fruits and seeds of the 'Milk thistle' (*Silybum marianum*) exhibit strong anti-oxidant activity and are used clinically for the treatment of hepatic disorders (El-Kamary et al., 2009; Koksal et al., 2009; Sangeetha et al., 2010). Anti-oxidant activity of silymarin is attributed to scavenging of free radicals, activation of anti-oxidative defensive mechanism by elevating cellular glutathione (GSH) content, and improving superoxide dismutase activity (Rauen and de Groot, 1998; Valenzuela et al., 1989; Müzes et al., 1991). Furthermore, silymarin reported to ameliorate oxidative stress in rat brain (Nencini et al., 2007; Galhardi et al., 2009). The recent documented report enumerates protective effect silymarin on neurons in focal cerebral ischemia through the inhibition of neurological deficits as well as oxidative damage (Raza et al., 2011).

With such background information about antioxidant activity profile of silymarin, piracetam and PCA, the present work was undertaken to demonstrate neuroprotective potential of silymarin in focal ischemia/reperfusion using behavioural, biochemical and histological evaluation, and its comparison with piracetam and PCA.

2. Material and methods

2.1. Animals

Male Wistar rats (150–180 g) were obtained from National Toxicological Centre (NTC), Pune. The animals were kept under standard

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animal house conditions: 12 h light/dark cycle, temperature (24 ± 2 °C) and relative humidity (60–70%), and had free access to food and water ad libitum. Rats were acclimatized for 2 weeks in the laboratory conditions before the experiment. Experimental protocols were reviewed and approved by the Institutional Animal Ethics Committee (DYPIPSR/IAEC/09-10/p-07), and conform to the Indian National Science Academy Guidelines for the Use and Care of Experimental Animals in Research [which is similar to the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication No. 85–23, revised 1985)]. Institutional animal house is registered (198/99/CPCSEA) with the Govt. of India.

2.2. Drugs and chemicals

The drugs, silymarin (Serum Institute of India Ltd., Pune), piracetam (UCB India Pvt. Ltd.) and PCA ethyl ester (Sigma Aldrich, St. Louis, Mo, USA) were procured. The chemicals like 5, 5-dithiobis-2-nitrobenzoic acid (DTNB), eserine and triphenyl tetrazolium chloride (TTC) were obtained from Sigma Aldrich, St. Louis, Mo, USA.

2.3. Treatment schedule

Twenty rats were randomly divided into 7 groups and treated in the following way:

Group I—served as Sham operated group
Group II—served as vehicle treated ischemic control, received carboxy methyl cellulose (CMC) 1% w/v [1 ml/100 g, body weight (b.w.), orally (p.o.)] for 7 days.
Groups III, IV and V—received silymarin 50/100/200 mg/kg, b.w., p.o. daily for 7 days, respectively.
Groups VI and VII—received PCA [200 mg/kg, b.w., intraperitoneally (i.p.)] daily for 4 days and piracetam (500 mg/kg, b.w., i.p.) after 22 h of focal ischemia/reperfusion respectively.

Brain samples were collected from all the groups, 24 h after the last dose treatment for biochemical and histological studies.

Silymarin was prepared in 1% (w/v) CMC, PCA in polyethylene glycol 400 (PEG 400) and piracetam was dissolved in saline solution. All rats underwent surgery for middle cerebral artery occlusion (MCAO) followed by reperfusion, in sham operated rats, all surgical procedures were performed except occlusion.

2.4. Induction of focal cerebral ischemia and reperfusion

Transient middle cerebral artery (MCAO) occlusion was induced in rats by the method of Nagasawa and Kogure (1989). Briefly, Rats were given atropine sulphate (0.5 mg/kg i.p.) and then anaesthetized with pentobarbital injection (3 mg/kg, i.p.), thereafter, the left common carotid artery was exposed after a midline cervical incision. The external carotid artery and the extra-cranial branch of the internal carotid artery were ligated. The origin of MCAO was then occluded by introducing 4–0 monofilament nylon suture with a tip rounded by heating through the stump of the external carotid artery. The nylon filament was secured in place with a ligature, and the wound was closed. After 1 h of occlusion, the nylon filament was removed to restore blood flow.

2.5. Behavioural assessment

2.5.1. Elevated plus maze

The maze had 2 open arms (dimensions: 50 × 10 cm) and 2 closed arms (50 × 10 × 40 cm) facing each other and was elevated at a height 50 cm. Each rat was placed at the end of the open arm facing the central platform. The time taken by the rat to enter from open arm with all the 4 legs into the enclosed arm was taken as the transfer latency time (TLT). If the animal does not enter the closed arm within 90 s. then TLT of that animal was assigned as 90 s. After entering in the closed arm, the animal was allowed to explore for 30 s. Each animal received a single trial per day for 3 days. The rats then underwent for focal cerebral ischemia/reperfusion and TLT was measured again on day 4 (Bora and Sharma, 2010).

2.5.2. Rota-rod test

In this test, rats were placed on the rotating rod (speed: 8 rpm) until they learnt to maintain themselves on the rotating rod for a period of 2 min. After the training, rats received a single trial per day for 3 days during which the animals were placed on the rotating rod with varying speed from 4 to 40 rpm during a 2 min period. The fall off latency time (FTL) i.e. the time for which they remained on the rotating rod was measured. The animals then underwent focal cerebral ischemia/reperfusion and the FLT was measured again on day 4 (Dohare et al., 2008).

2.5.3. Neurological scoring system

After 24 h of reperfusion, following MCAO the rats were neurologically evaluated based on the scoring system previously described by the Elango et al. (2009), viz 0 — for no observable neurological deficit (normal); 1 — for failure to extend left forepaw when whole body was lifted by the tail (mild); 2 — for circling to the contra-lateral side at rest (moderate); 3 — for leaning to the contra-lateral side at rest (severe); and 4 — for no spontaneous motor activity (very severe) (Elango et al., 2009).

2.5.4. Lateral push test

Lateral push test was selected to assess grip strength of animals. The animals were placed on a rough surface and were evaluated for the resistance to lateral push from either side of the shoulder. Animals with increased or decreased resistance to lateral push after focal cerebral ischemia/reperfusion were assigned '+' or '−' score respectively (Bederson et al., 1986).

2.6. Biochemical studies

2.6.1. Preparation of sample solution (for MDA, GSH, SOD, catalase and nitrite content)

At the end of the drug treatment, 6 rats from each group were sacrificed with urethane. Brains were removed, washed with ice cold saline to remove as much blood as possible and weighed. Brain homogenates (10%, w/v) were prepared in cold 0.1 M Phosphate buffer (pH 7.4) using Remi homogenizer. The unbroken cells and cell debris were removed by centrifugation at 10,000 × g for 20 min at 4 °C using a Remi C-24 refrigerated centrifuge. The clear supernatant was used for various assays.

2.6.2. Lipid peroxidation

The Extent of malondialdehyde (MDA) formation was measured in tissue homogenates; equal volumes (2 ml) of the tissue homogenate and trichloroacetic acid (10% w/v) were mixed. The mixture was then cooled for 15 min and centrifuged. To the supernatant (0.5 ml), 3 ml of (0.67%) thiobarbituric acid was added, the reaction mixture was then kept in boiling water for 10 min., cooled and thereafter absorbance was measured against blank at 535 nm on Shimadzu 1700 UV spectrophotometer. The amount of MDA formed was expressed as nM of MDA/g of wet tissue (Slater and Sawyer, 1971).

2.6.3. Estimation of reduced glutathione (GSH)

Equal volumes of tissue supernatant and 20% trichloroacetic acid (TCA) were mixed. Aliquot 0.25 ml of supernatant was taken in test tube and, 2 ml of DTNB [5, 5′-dithiobis (2-nitro benzoic acid), 0.6 mM] reagent was added. The final volume was made up to 3 ml with phosphate buffer (0.2 M, pH 8.0). The colour developed was read at 412 nm against a reagent blank on Shimadzu 1700 UV

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