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Prophylactic neuroprotective property of *Centella asiatica* against 3-nitropropionic acid induced oxidative stress and mitochondrial dysfunctions in brain regions of prepubertal mice

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ABSTRACT

Despite the increasing popularity of *Centella asiatica* (a well known plant in ayurvedic medicine) globally, evidence demonstrating its protective efficacy against neurotoxicants in animal models is limited. 3-Nitropropionic acid (3-NPA), a fungal toxin is a well known neurotoxicant which induces selective striatal pathology similar to that seen in Huntington's disease. The present study aimed to understand the neuroprotective efficacy of a standardized aqueous extract of *C. asiatica* (CA) against 3-NPA-induced early oxidative stress and mitochondrial dysfunctions in striatum and other brain regions. We determined the extent of oxidative stress in cytosol and mitochondria of brain regions of male mice (4 wk old) given CA prophylaxis (5 mg/kg bw) for 10 days followed by 3-NPA administration (i.p., 75 mg/kg bw/d) on the last 2 days. The neurotoxicant elicited marked oxidative stress in the untreated mice as evidenced by elevated levels of malondialdehyde, ROS levels and hydroperoxides in the striatum (cytosol and mitochondria), while CA prophylaxis completely attenuated the 3-NPA-induced oxidative stress. 3-NPA also caused significant oxidative stress and protein oxidation in cytosol/mitochondria of other brain regions as well which were predominantly abolished by CA prophylaxis. Significant depletion of GSH levels, total thiols and perturbations in antioxidant enzymic defences in striatum and other brain regions discernible among 3-NPA administered mice were also protected with CA prophylaxis. Interestingly, CA prophylaxis offered varying degree of protection against 3-NPA-induced mitochondrial dysfunctions viz., reduction in the activity of succinic dehydrogenase, ETC enzymes and decreased mitochondrial viability. Collectively these findings clearly suggest that short-term oral intake of a standardized aqueous extract of CA confers marked resistance against the 3-NPA-induced oxidative stress and mitochondrial dysfunctions in brain. Although the precise mechanism/s underlying the prophylactic efficacy of CA merit further investigation, based on these findings, it is hypothesized that it may be wholly or in part related to the enhancement of GSH, thiols and antioxidant machinery in the brain regions of prepubertal mice.

1. Introduction

Centella asiatica (L) Urban (Umbelliferae) a plant native to countries like India, Srilanka, Madagascar, South Africa and Malasia (Kartnig, 1988) is used in the ayurvedic system of medicine to treat various ailments (Shukla et al., 1999). Recent studies have shown various neuropharmacological effects with *C. asiatica* (CA) and the major effects described in experimental animals comprise of memory enhancement and cognitive function (Veerendra kumar and Gupta, 2002; Wijeweera et al., 2006), increased neurite elongation *in vitro* and acceleration of nerve regeneration *in vivo* (Soumyanath et al., 2005). Notable bioactive compounds of CA are the triterpene saponins, madecassocide and asiaticoside with their respective ursane type sapogenins viz., madecassic and asiatic acid (Mangas et al., 2006). CA contains numerous caffeic acid derivatives and flavonols (Zainol et al., 2003) some of which have been shown to be potent antioxidants (Hussin et al., 2007). Despite its wide usage, precise the mechanism/s by which CA exerts its neuropharmacological effects are not well understood.

Oxidative stress is a major mechanism for cellular damage associated with a wide variety of neurotoxicants (Gitto et al., 2002; Halliwell, 2006). It is suggested that the developing brain may be relatively more sensitive to oxidative damage not only because of

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Abbreviations: CA, *Centella asiatica*; Ct, cortex; Cb, cerebellum; Hc, hippocampus; LPO, lipid peroxidation; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances; MDA, malondialdehyde; DCF, 2′7′-dichloro-fluorescein; DCF-DA, 2′,7′-dichloro-fluorescein diacetate.

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the increased metabolic demand associated with growth, but also because of lower reserves of protective enzymes/antioxidants (such as glutathione) and is deficient in glia (relative to the adult brain), which ordinarily protects neurons from oxidative molecules (Tanaka et al., 1999; James et al., 2005). More importantly, prepubertal period and adolescence is a time when substantial culling of synaptic connections occurs with close to 50% of the synaptic connections are lost in some cortical regions (Spear, 2007).

3-Nitropropionic acid (3-NPA), a complex II inhibitor of the electron transport chain, is found to effectively induce specific behavioral changes and selective striatal lesions in rats and non-human primates mimicking those in Huntington's disease (Mandavilli et al., 2005; Lee and Chang, 2004). Although various parts of the brain are affected, 3-NPA induces specific striatal damage, a characteristic exploited to produce an experimental model of Huntington's disease (Perez De la Cruz et al., 2006). However, the primary mechanism of action of 3-NPA *in vivo* is not clearly understood, but various mechanisms including oxidative injury, transcriptional deregulation, glutamate receptor excitotoxicity (Li et al., 2003; Jarabek et al., 2004), apoptotic signals, mitochondrial dysfunction and energy depletion have been proposed (Beal and Ferrante, 2004). Since the toxin produces depleted levels of reduced glutathione (GSH), alters the antioxidant enzyme profiles and enhances ROS levels in the lesioned striatum (Binienda et al., 1998; Nam et al., 2005), oxidative stress is hypothesized to play a vital role in 3-NPA-induced neurotoxicity (Fu et al., 1995; Fontaine et al., 2000). Recently, in experimental models, the therapeutic efficacy of few exogenous antioxidants (e.g., L-carnitine, *S*-allyl cysteine and ginseng saponins) have been assessed against 3-NPA-induced oxidative stress in brain (Binienda et al., 2004; Kim et al., 2005; Herrera-Mundo et al., 2006).

The relevance of understanding the neurotoxic implications during adolescence in experimental models animals has been recently emphasized (Spear, 2007). To the best of our knowledge, studies on the 3-NPA-induced oxidative stress and mitochondrial dysfunctions in brain regions of prepubertal rodents are scarce. More importantly, no attempts have been made to explore the possible amelioration of 3-NPA-induced oxidative stress in brain employing natural bioactive compounds. Earlier we have demonstrated the ability of CA (whole leaf powder) supplementation to markedly diminish the endogenous oxidative markers and enhancement of antioxidant defenses in both cytosol and mitochondria of striatum and other brain regions of prepubertal mice (Shinomol and Muralidhara, 2008). Further, we also showed that the aqueous extract of CA possess good antioxidant activity in chemical test systems and markedly mitigates 3-NPA-induced oxidative stress in mitochondria *in vitro*. Based on our earlier findings, we hypothesize that CA prophylaxis among prepubertal mice is likely to confer significant protection against neurotoxicant induced brain oxidative stress. The hypothesis was examined by investigating the degree of protection conferred by short-term CA prophylaxis against 3-NPA-induced oxidative stress and mitochondrial dysfunctions in striatum and other brain regions such as cortex, cerebellum and hippocampus in prepubertal male mice.

2. Experimental procedures

2.1. Chemicals

Thiobarbituric acid (TBA), 1,1,3,3-tetramethoxypropane, 2′,7′-dichloro-fluorescein (DCF), 2′,7′-dichloro-fluorescein diacetate (DCFH-DA), 3-nitropropionic acid and other fine chemicals were procured from M/s Sigma Chemical Co. St. Louis, USA. All other chemicals used were of analytical-grade.

2.2. Animals and care

Prepubertal male mice (CFT-Swiss, 4 wk old) drawn from the stock colony of our animal house facility were housed in rectangular polypropylene cages (three per cage) kept on racks built of slotted angles, in a controlled atmosphere with a 12 h light/dark cycle. They were acclimatized for 3 days prior to the start of the experiment. The animals were maintained on a commercial powdered diet and tap water *ad libitum*. The experiments were conducted strictly in accordance with approved guidelines by the "Institute Animal Ethical Committee" regulated by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Social justice and Empowerment, Government of India, India.

2.3. Preparation of aqueous extract of CA leaves

C. asiatica plant material was collected during early summer from the state of Kerala, India. The plant was authenticated by Dr. C.M Joy, Department of Botany, Sacred Heart College, Thevara, Mahatma Gandhi University, Kerala, India. The CA leaves along with the petiole were shade dried and powdered and was extracted with eight parts of water under boiling for 5 h and filtered to collect the extract. The filtrate was flash evaporated and finally dried to powder by lyophilization. The triterpene content of the standardized extract analyzed by HPLC following the conditions described earlier (Inamdar et al., 1996) was found to be in the range of 8.0–8.6% on dry basis, while the asiaticoside content was in the range of 0.8%.

2.4. Experimental design

Dosages of 3-NPA and CA aqueous extract were selected on the basis of a previous study conducted in our laboratory. Prepubertal male mice (4 wk old) were orally administered (5 mg/kg bw) with an aqueous extract of *C. asiatica* for a period of 10 days (prophylaxis group). Both normal and mice given CA prophylaxis were injected 3-NPA (i.p., 75 mg/kg bw/d) on days 9 and 10 and sacrificed on day 11. Mice given physiological saline served as the normal controls. During the experimental period, food intake and individual body weights were monitored daily. The induction of oxidative stress and mitochondrial dysfunctions were determined in striatum and other brain regions.

2.5. Preparation of mitochondria

Mitochondria were prepared by differential centrifugation as described by Moreadith and Fiskum (1984) with minor modifications. Briefly, a 10% homogenate of the brain regions was prepared in ice-cold Tris–Sucrose buffer (0.25 M, pH 7.4) using a glass–teflon grinder at 4 °C. The homogenate was centrifuged at 1000 × *g* for 10 min at 4 °C to obtain the nuclear pellet. Mitochondria were obtained by centrifuging the post-nuclear supernatant at 10,000 × *g* for 20 min at 4 °C. The pellet was washed three times in Mannitol–Sucrose–HEPES buffer (pH 7.4), resuspended in the same buffer and stored at 4 °C until further use.

2.6. MTT assay

MTT reduction by mitochondria was determined as per the method of Cohen et al. (1997). Briefly, an aliquot of mitochondrial protein (~10 μg) was added to a reaction mixture of Mannitol–Sucrose–HEPES, pH 7.4 containing 20 mM sodium succinate, 1 mM NADH followed by the addition of 15 μL of MTT (5 mg/mL). The tubes were then incubated at 37 °C for 2 h and the formazan