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Alpha Phenyl-N-Tert-Butyl Nitrone (PBN) Protects Rat Cortical Cultures from Aluminium Cytotoxicity

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Dissociated zero day old rat cortical cells were grown up to six days in vitro. On the sixth day, they were exposed to either aluminium chloride (600µM) or co-treated with aluminium chloride and spin trap agent, phenyl butyl nitrone - PBN (3mM) for forty-eight hours. Aluminium cytotoxicity was assessed by microscopic examination of cell morphology and by measuring lactate dehydrogenase (LDH) activity in the culture medium. The normal morphology of smooth, phase bright soma with profused net work of neurites was considerably distorted in cultures exposed to aluminium. Instead the cells appeared swollen with beaded and fragmented neurites. In addition, the LDH activity was significantly high in aluminium exposed cultures indicating cell lysis. The PBN co-treatment has significantly attenuated both the morphological alterations and LDH efflux induced by aluminium. The study has demonstrated the neuroprotective role of spin trap; PBN in aluminium induced cytotoxicity.

Key words - *Aluminium, Cytotoxicity, Cortical cultures, PBN, Neuroprotection.*

Aluminium is an established neurotoxin. In recent years, the neurobehavioural toxicity of aluminium has been described in great detail [1,2,3]. Clinically, aluminium neurotoxicity has been studied extensively in dialysis encephalopathy[4]. Brain aluminium accumulation has been claimed to lead to Alzheimer's disease[5]. Experimentally, various aspects of aluminium neurotoxicity have been demonstrated using different animals[6,7]. Although many adverse effects of aluminium on the brain have been described, the mechanism of aluminium neurotoxicity has not been understood clearly. Recent studies[8,9,10] suggest that aluminium potentiates the free radical mediated oxidative stress and these studies may have implications for the mechanisms of aluminium toxicity. Free radical species such as super oxide ($O_2^{-\bullet}$) and hydroxyl ($OH^{\bullet}$) radicals being highly toxic and reactive, can induce oxidative damages to critical biological molecules[11] (proteins, aminoacids, nucleic acid bases) and initiates chain reactions such as lipid peroxidation[12]. Many laboratory and clinical studies suggest that oxygen radical formation and resultant cell damage contribute to neuro-degeneration. Accordingly, antioxidants represent a viable therapeutic approach for management of CNS oxidative damages. Recently, a spin trapping compound phe-

nyl butyl nitrone (PBN) appears promising for treatment for conditions of free radical mediated oxidative stress. Initially, PBN was used to characterize the in vitro and in vivo production of free radicals[13]. PBN being highly lipophilic, diffuses easily into the cell and quenches a variety of oxygen and carbon based free radicals by forming more stable nitroxide adducts which can be detected by electron spin resonance [14,15]. Several investigators have reported that spin trap PBN reduces mortality of rats exposed to trauma[16] or endotoxin[17] or protects against oxidative damages[18,19,20]. However the neuroprotective role of PBN has not been reported in aluminium toxicity. The primary goal of this study was to evaluate the protective effects of PBN on aluminium induced cytotoxicity in vitro.

Material and Methods

Culturing of cortical neurons were done as per the method described by Munirathinam et al [21]. In brief, the cerebral cortex was removed from zero day old Wistar rat pups, freed from meninges and cut into pieces and placed in a sterile petridish containing DMEM-F10 (GIBCO, USA) medium (Dulbecco's modified Eagles medium with 10% fetal calf serum). The tissue was dissociated into cell aggregates by gentle trituration. 200µl of dissociated cell suspension was plated at a cell density of 100,000 to 150,000 on the cover slip (13mm diameter) placed in individual wells containing 400µl of preincubated medium in a 24 well multicavity tray (Laxbro, India). Cultures were maintained at 37°C in a humidified incubator (Heraues Germany) at 95% air and 5% CO_2.

Exposure to aluminium and PBN

After 3 days in vitro, culture medium was replaced with fresh serum free medium. On the sixth day, the cultures were exposed to either 600µ M aluminium chloride (NICE, India) and 3mM concentration of PBN (Sigma, USA) (prepared in serum free medium) for forty-eight hours. The effect of varying concentrations of (100µM to 1000µM) of aluminium on primary cortical cultures had been reported from the same laboratory[21]. Complete cytotoxicity was observed only when the cultures were exposed to 600µM aluminium chloride for forty-eight hours. In the present study also same experimental conditions were adopted. The dose of PBN used in the present study replicated those used in previous studies[22]. Control cultures were incubated with only serum free medium. After forty-eight hours, cell morphology was examined under the inverted phase contrast microscope (Olympus). Culture medium was removed for LDH assay and protein estimation. For each experiment, quadruplicate wells were used and experiments were repeated six times.

Lactate dehydrogenase (LDH)

For quantitative assessment of cell injury, LDH assay was performed on the culture supernatant and its activity was expressed as n moles of NADH oxidized/

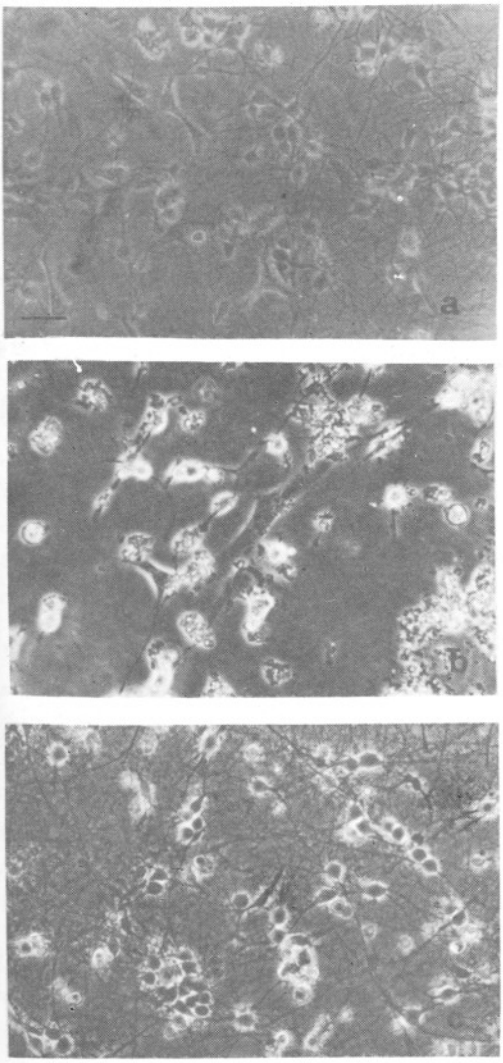

Figure 1. Photomicrographs showing primary cortical cultures 8 days in vitro (8DIV). (a) Control cultures: neurons with phase bright cell bodies and long neurites can be seen. (b) Aluminium (600μM) treated cultures: note the lack of phase bright neurons with disrupted neurites. (c) After co-exposure with PBN (3mM) and aluminium (600 μM): cells appear similar to controls with extended neurites and phase bright cell bodies. Scale Bar = 50 μM

min/mg protein. The LDH assay was carried out by the spectrophotometric method of Yoshida and Freeze[23] and Protein content was estimated using the dye (Coomassie blue) binding method of Bradford[24].

The data obtained were statistically analyzed by one way analysis of variance (ANOVA) followed by Newman Kuel's test[25].

Results

Morphological Observation

Eight days old control cultures showed phase bright neuronal soma with long smooth neurites profused branched to form an extensive network as shown in fig(1a). This normal morphology of culture was totally disrupted in aluminium exposed cultures. The neuronal soma was swollen, filled with vacuoles and the neural network was disrupted considerably due to the fragmentation of neurites as shown in fig(1b). This aluminium induced morphological alterations were significantly attenuated in cultures co-treated with aluminium and PBN and the cells retained their normal morphology (fig 1c).

LDH Assay

Table I explains the LDH activity measured from culture medium exposed to different agents. The LDH activity was significantly increased in aluminium ex-

posed cultures indicating the disruption of membrane integrity following aluminium exposure. The increase in LDH activity was nearly two folds from that of control cultures. The LDH activity from cultures cotreated with aluminium and PBN were comparable to that of control cultures.

Table I

Lactate Dehydrogenase (LDH) efflux assayed as per Yoshida and Freeze[23]. Values represent mean±SD of six independent experiments.

	Control cultures	Cultures exposed to aluminium	Cultures exposed to aluminium and PBN
LDH activity (n moles of NADH oxidised/ min/mg protein).	263.54±52.03	401.03±84.40*	262.17±58.65

*P<0.0005 as compared to control and aluminium and PBN co-exposed cultures by one-way ANOVA followed by Newman Kuel's test

Discussion

The study demonstrated the cytotoxic effects of aluminium in primary cortical cultures. Aluminium induced cell lysis was accompanied by distorted morphology and increased LDH activity. The results further suggest that spin trap PBN are effective in ameliorating the aluminium-induced cytotoxicity.

Aluminium cytotoxicity has been demonstrated in-vitro using different neuronal cultures[8,21,26,27]. The present study has demonstrated the cytotoxicity of aluminium (600μM) when the cultures were exposed for forty-eight hours. However other studies have demonstrated aluminium toxicity with different concentrations of aluminium. Brenner and Yoon[26] have observed the lysis of rat primary hippocampal cultures within one hour of exposure to 100μM concentrations of aluminium chloride. Similarly Murphy et al[27] have observed cytotoxicity of mice spinal cord cultures following one hour of exposure to aluminium silicates. Aluminium neurotoxicity appears to be a phenomenon, which occurs in several different cell types with varying degree of effectiveness. When compared to hippocampal and spinal cord cultures, cortical cultures may be comparatively more resistant and required higher concentration of aluminium to induce cytotoxicity.

The PBN co-treatment has brought significant attenuation of aluminium toxicity. PBN being a free radical trapping compound has received attention as a neuroprotective agent in conditions oxidative injuries. Though the neuroprotective role of PBN has been demonstrated in situations of free radical mediated oxidative damages[18,19]; the present study for the first time has demonstrated the neuroprotective ability of PBN in aluminium toxicity. Cao and Phillis[20] have observed significant protective effects of PBN against focal ischemia in rats. They have reported that PBN could ameliorate the cerebral infarct volume and brain edema associated with ischemic injuries. In addition, Phillis and Clough-Helfman[28] and Yue et al [29] have recently reported that PBN treatment has shown to prevent the increased locomotor activity associated with global brain

ischemia in gerbils and reduce hippocampal CA1 pyramidal cell death. PBN also mitigates glutamate induced excitotoxicity in cultured rat cerebellar neurons[29]. Free radical mediated oxidative damages have been reported as an important cause of cell death in ischemic injuries[30,31] and the cerebroprotection brought by PBN has been attributed to its ability to reverse or chronically ameliorate the oxidative stress by scavenging free radicals. In addition, the free radical scavenging ability of PBN has been shown to reverse the age-related dysfunctions associated with oxidative stress and altered membrane integrity[18,32,33]. In the present study, PBN cotreatment has ameliorated the morphological alterations and the LDH efflux induced by aluminium and thus protected the cultures from aluminium cytotoxicity. This result suggests that the neuroprotection may be due to a free radical scavenging mechanism.

Aluminium is a metal with no redox capacity. However, evidences from recent studies suggest that aluminium accelerate the generation of free radicals and subsequent oxidative stress by various means. The pro-oxidant effects of aluminium are likely to be exerted by enhancing the superoxide radical oxidation of NADH[34]. Aluminium forms complexes with superoxide and forms a stronger oxidant than the superoxide itself. In addition, aluminium may facilitate the production of reactive oxygen species and potentially contribute to neurotoxicity induced by other agents such as iron[35] or glutamate[8]. It also promotes the amyloid beta aggregation, which in turn mediates neuronal oxidative stress[36]. Moreover the enhanced oxidative stress has been shown to be induced exclusively by the intraneuronal aluminium[35]. As mentioned, chronic enhancement of oxidative defense either by treating with antioxidants or by free radical trapping may be effective to negate the deleterious effects of aluminium cytotoxicity. Munirathinam et al [21] have reported the neuroprotective effects of (-) deprenyl in aluminium cyto-toxicity using rat primary cortical cultures. The neuroprotective ability of (-) deprenyl, a MAO-B antagonist has been attributed to its potent antioxidant properties[37]. The present study confirms the role of free radical scavenger PBN against aluminium toxicity. The observations substantially support the concept of free radical generation and subsequent oxidative injury following aluminium toxicity.

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