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Original Article

Dendritic processes as targets for arsenic induced neurotoxicity: Protective role of curcumin

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ABSTRACT

Introduction: Microtubule associated protein2 (MAP2) plays a vital role in morphological stabilization and plasticity of the neuronal dendritic processes. Any alteration in the expression of this protein following exposure to environmental contaminants such as arsenic (*iAs*) could induce functional deficits in neurons. In India, over 1.5 million people are exposed to *iAs* with over 200,000 reported cases of arsenicosis. Oxidative stress has been identified as one of the key factors underlying *iAs* induced toxicity. Hence, the need of the hour is to identify cost effective and safe therapeutic approaches for combating *iAs* induced adverse effects. The present study aimed at determining the ameliorative potential of Curcumin (Cur) supplementation on dendritic profile of cerebellar Purkinje cells in rats subjected to *iAs* exposure during postnatal period.

Methods: Mother reared rats were divided into control and experimental groups (receiving $NaAsO_2$ alone or along with Cur by intraperitoneal route from postnatal day (PND) 1–21. Cerebellar tissue obtained from perfusion fixed animals was processed for Cresyl Violet staining and immunohistochemical localization of MAP2.

Results: An overall decrease in molecular layer thickness (MLT) of cerebellar cortex along with disrupted morphology of Purkinje dendritic processes was evident in *iAs* alone treated animals as compared to controls and Cur co-treated animals. Also, decrease in MAP2 immunostained area (%) was noted in the ML of *iAs* alone treated animals.

Discussion: Preliminary observations suggest modulating effect of Cur on MAP2 expression and dendritic morphology of cerebellar Purkinje cells in rats following $NaAsO_2$ exposure.

1. Introduction

Exposure to various environmental contaminants is emerging as a matter of great concern all over the globe. Millions of people across various continents get exposed to *iAs* by consuming ground water contaminated with high levels of *iAs*.[1] There have been reports of adverse effects on various organ systems in humans and animal models following exposure to *iAs*.[2] Besides contaminated water, consumption of grains (especially rice) grown in areas where *iAs* contaminated water is used for irrigation, constitutes additional medium of exposure to *iAs*.[3,4] In India, over 1.5 million people are exposed to high levels of *iAs* with over 200,000 reported cases of arsenicosis. It is worthwhile to mention here that pregnant women comprise a significant segment of this population, especially in the endemic areas.[1]

Developmental exposure to environmental contaminants during critical periods of Central Nervous System (CNS) development is seen as an open challenge. A number of cellular processes ranging from neurite formation (neuritogenesis) to neuro-morphogenesis[5] are highly susceptible to various exogenous as well as endogenous insults. Amongst various cell organelles, stability of cellular skeleton is considered critical for healthy maintenance of cellular architecture, intracellular movements, cell division etc.[6,7] Being extensively localized in the dendrites of the nerve cells, Microtubule Associated Protein 2 (MAP2), plays a key role in neural transmission by facilitating the assembly of microtubules.[8] MAP2 contributes actively in morphological stabilization and plasticity of the dendritic processes[9] hence, considered as one of the important markers for structural integrity. The observations of our earlier study suggested decreased cognitive and exploratory ability in rat pups exposed to sodium arsenite ($NaAsO_2$) during postnatal

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period.[10] For understanding the structural basis underlying *iAs* induced functional deficits, we studied the effect of postnatal *iAs* exposure on expression pattern of MAP2 in cerebellar cortex of rat pups. Since, oxidative stress has been reported as one of the factors underlying *iAs* induced toxicity, the aim of the present study was to determine the effects of curcumin (Cur) (bioactive component of turmeric) supplementation on *iAs* induced effects on MAP2 expression (marker of neuronal structural integrity) in cerebellar cortex of these animals.

2. Material and methods

Ethical clearance from the Institute Animal Ethical Committee (IAEC 594/11) was obtained for procuring pregnant Wistar rats (gestation day 18–19). The animals were housed in temperature (20 °C-25 °C) and humidity (50-60%) controlled rooms within Central Animal Facility with 12 *h* light/dark cycle and fed on standard rodent diet with *ad libitum* access to drinking water. The guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals) were strictly followed for the care of the animals. The delivery status of the animals was checked daily at 10 A M and 4 PM and the day of delivery of pups was designated as postnatal day (PND) 0. The mother reared pups were grouped as normal controls (Ia- with no treatment) and sham controls [Ib- sterile water; Ic- dimethyl sulfoxide (DMSO Sigma Aldrich D5879)]. Animals belonging to the experimental groups received $NaAsO_2$ alone [IIa- 1.5; IIb- 2.5 mg/kg body weight (bw)] (Sigma Aldrich 71,287); whereas animals in groups IIIa and IIIb received Cur (150 mg/kg bw) (Sigma Aldrich C1386) along with 1.5 and 2.5 mg/kg bw $NaAsO_2$ (Fig. 1). The test substances ($NaAsO_2$ and Cur) were administered once daily by intraperitoneal (i.p.) route from PND 1–21 with half an hour interval in between.[11] i.p. route was considered for ensuring the requisite dose delivery of the test substance and to avoid variation in exposure doses. To the best of our knowledge, there are no reports suggestive of Cur induced toxicity at the dosage used in the study. During the entire experimental period, the animals were observed for signs of normal developmental features. The animals were sacrificed on PND 22 by perfusion fixation (0.9% saline followed by 4% paraformaldehyde in 0.1 M phosphate buffer) and the cerebellar tissue obtained was processed for paraffin and cryo sectioning (n = 12/group).

2.1. Morphology and morphometry

The fixed cerebellum was dehydrated in ascending grades of ethanol [70% (overnight), 80% [30 min], 90% (30 min), 96% (15 min) and 96% (15 min)]. Tissue was cleared in xylene (30 min) and embedded in paraffin.7 μm thick serial sections were cut (Rotary microtome- Shandon AS325) and processed for CV staining. Briefly, the sections were dewaxed in xylene (5 min) and hydrated in decreasing concentrations of ethanol (96%, 90%, 80% and 70%) (5 min each). The slides were immersed in CV stain (Sigma Chemicals) (5 min) and then dehydrated by passing through increasing grades of ethanol 30%, 50%, 70%, 90%, 96% (3 min each). The slides were differentiated in acid alcohol, cleared in xylene and mounted in DPX. The stained sections (vermal and paravermal regions) were observed under bright field Nikon E-600 microscope fitted with Nikon Digital Camera System (DS-Fil-U2) for morphological features. Morphometric parameters were determined by the attached image analysis system (Nikon Imaging Software, NIS-Elements-AR 3.10). In the cerebellar cortex, Molecular Layer thickness (MLT) was measured (μm) as a perpendicular line drawn from the outer edge of ML to outer edge of Purkinje cell layer (PCL). A total of 10 reference areas/ section were randomly selected and 5 sections per animal were analysed (n = 6/ group). The first section was randomly chosen and the subsequent sections were every 20th from that.[12]

2.2. Immunohistochemistry and semi quantitative analysis

Fixed cerebellar tissue was subjected to cryoprotection in graded sucrose solution (15% and 30%), at 4 °C. Cryocut (HS 525, Microm GmbH, Germany) sagittal sections (30 μm) were collected in 0.1 M PB and processed by free floating immunohistochemical technique following the standardized protocol.[10] Antigen retrieval was done using 0.5% Sodium Dodecyl Sulphate (SDS) and quenching of endogenous peroxidase was carried out in 0.3% hydrogen peroxide. After washing (0.1 M PBS), blocking was done in normal goat serum (Jackson Laboratories, USA) (1 *h*, at RT) followed by overnight incubation in primary antibody (mouse monoclonal anti-microtubule associated protein 2 MAP2, Santa Cruz, USA 56561, 1:200). Ultravision Plus Detection system kit (Thermo Scientific TP-060-HLX) and DAB kit (Bio SB. BSB 0017) were used for visualization of immune complexes. The sections were dehydrated and mounted on gelatin coated slides.

The mid-sagittal sections (vermal and para-vermal regions) presenting well defined lobules were considered for semi-quantitive analysis of IHC expression. The mean cumulative grey values were calculated on a grey scale of 0–255 where (0) represented black and 255 represented white.[13,14] A rectangular grid (200 μm x 50 μm) was superimposed on randomly selected reference areas in ML and the area surrounding the grid was cropped off. The percentage of MAP2 immuno-stained area (reflecting mainly the dendritic arborization of the PCs in the ML) was determined in the region of interest (ROI) and expressed

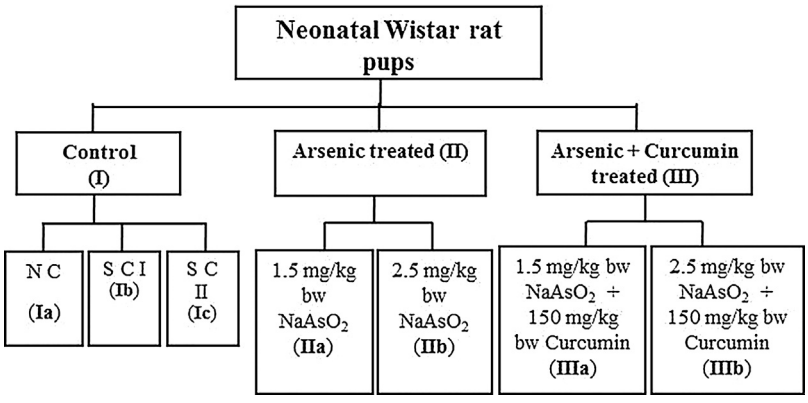

Fig. 1. Showing the grouping of mother reared rat pups.

with respect to the control.[15,16] Ten reference areas/ section and a total of 5 sections/ animal (n = 6 animals/ group) were analysed.[12] The values were pooled together for obtaining the mean values.

2.3. Statistical analysis

Graphpad prism was used for statistical analysis. One Way ANOVA followed by Newman Keuls posthoc test were applied and p value <0.05 was considered significant. As the parameters among the normal controls and the vehicle treated animals did not show significant difference, the values of sham control (sterile water) were considered for comparison.

3. Results

All along the experimental period (PND 1– 21), the animals belonging to various groups exhibited normal developmental milestones.

3.1. Morphology and morphometry

A well-defined layering pattern of the cerebellar cortex with distinct demarcation of outer ML, intermediate Purkinje Cell Layer (PCL) and inner Granule Cell Layer (GCL) was observed in CV stained sections of the controls as well as the experimental animals (Fig. 2A–E). ML, the most superficial layer of the cortex, comprised of small widespread interneurons, rich dendritic arborization of PCs and a number of fibre bundles. The PCL was demarcated as a monolayer of large and densely stained PC bodies between the ML and GCL, with their apices pointing towards the ML. The GCL was identified as a densely packed zone of overlapping GCs with darkly stained Nissl substance. The GCs were uniformly dispersed in the GCL of the control and Cur co-treated groups, whereas, in GCL of *iAs* alone treated groups disrupted dispersal of GCs was observed. Also, density of PCs in the PCL of *iAs* alone treated groups was decreased as compared to the controls and the animal groups receiving Cur with *iAs* (Fig. 2B,C). The thickness of ML in the cerebellar sections of *iAs* alone treated animals was significantly ($p < 0.05$) decreased in comparison to MLT of control and Cur co-treated animals (Fig. 2F).

3.2. Immunohistochemical observations

MAP2 expression was localized in all the three cerebellar cortical layers of the control and the experimental animals (Fig. 3A–E). The overall expression was well defined in the PCL with more intensity in the dendritic processes (lying in the ML) as compared to that in the cell body of PCs. MAP2 immunoreactivity, evident in the GCL, could possibly correspond to the expression in the dendrites of Golgi and Granule Cells (Fig. 3A–E). Qualitative observations revealed down-regulated expression in *iAs* alone treated group (Fig. 3B,C) as compared on to control (Fig. 3A) and Cur co-treated groups (Fig. 3D, E).

Semi quantitative analysis showed a significant decrease ($p < 0.05$) in the % of MAP2 immunostained area in the ML of *iAs* alone treated animals (IIa, IIb), as compared to the controls. Animals receiving Cur with *iAs* (IIIa, IIIb) exhibited significant ($p < 0.05$) increase (23.41% and 22.89% respectively) in the % of MAP2 immunostained area (Fig. 3F).

4. Discussion

Neurotoxic effects induced by exposure to *iAs* have largely been reported in adults,[16,17,18] however, the meagre data pertaining to various effects induced by exposure to *iAs* during the vulnerable periods of development raises special concern. As impaired postnatal growth and development are sensitive measures of central neurotoxicity, the primary focus of the present work was to evaluate the role of Cur administration during early postnatal period in amelioration of *iAs* induced adverse effects on cerebellar developmental.

During cerebellar development in rats, the complete disappearance of the external granular layer (EGL) by PND 21, makes the ML the most superficial layer. The thickness of ML is determined by the residing cells and afferent fibre bundles.[19] Parallel fibres (PF), axonal processes of the GCs, form synapses with PC dendritic spines. The climbing fibres reach the PC dendrites from the inferior olivary complex.[20] This rich synaptic zone (ML), plays an important role in determining the status of overall cerebellar functioning by providing for fine tuning between the inputs to PCs and output of

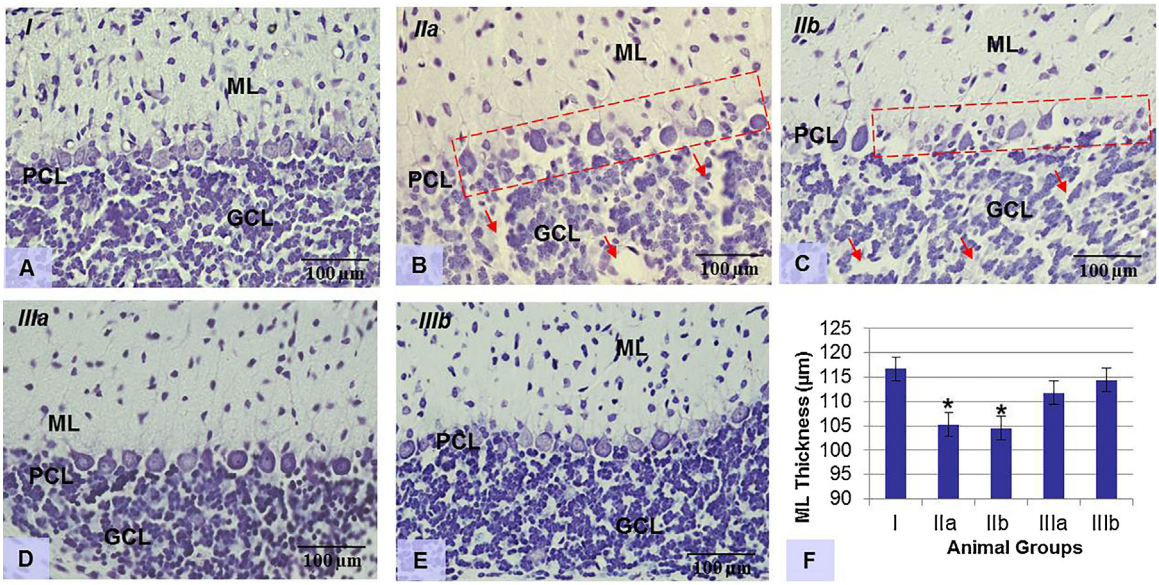

Fig. 2. Photomicrographs (40X) of CV stained sections of rat cerebellar cortex from control (A) & experimental (B,C,D,E) groups showing three layered pattern i.e. molecular layer (ML), Purkinje cell layer (PCL) & granule cell layer (GCL). Note: Disrupted dispersal of GCs in GCL (→) along with an apparent decrease in PCLD (box) in *iAs* alone treated groups (B & C) as compared to A (controls) and Cur co-treated groups (D & E). F: The bar diagram shows significant decrease in the MLT in the *iAs* alone treated groups as compared to the controls. Note substantial recovery in the MLT of the Cur co-treated groups.

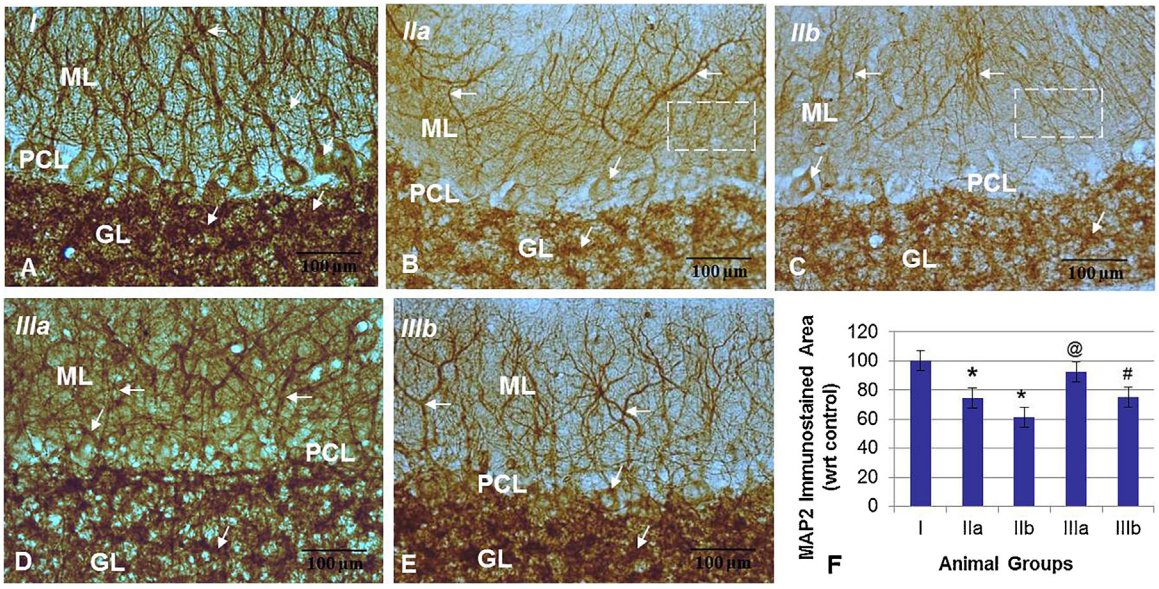

Fig. 3. Photomicrographs (40X) showing IHC localization of MAP2 (→) in cerebellar cortical layers (ML, PCL & GCL) of control (A) & experimental (B,C,D,E) groups. (Box) areas of weak immuno-expression in ML of the *iAs* alone treated groups. F: The bar diagram is showing the MAP2 immunostained area (%). Note significantly more MAP2 immunostained area in the ML of animals co-treated with Cur and *iAs* as against the *iAs* alone treated animals. Significant at $p < 0.05$ levels: * compared to I; @ compared to IIa; # compared to IIb respectively.

PCs to deep nuclei. The significant decrease in the thickness of ML in *iAs* alone treated animals could be associated with poor development of the PC dendritic arborization along with decreased number of incoming PF in these animals. The alterations in cyto-architecture of the *iAs* exposed groups observed in the present study indicate structural modifications and alterations in neuronal maturation. Since these changes are dependent to a large extent on the intricate inter-neuronal chemical communication and various neurotrophic factors, any interference (*iAs* induced) in these signalling pathways could have induced structural alterations leading to functional deficits in the exposed animals. Maintenance of cerebellar morphology following co-treatment with Cur evidences its protective role.

The MAP2 expression pattern in the cerebellum of the control and the experimental animals (present study) is in coherence with the MAP2 expression pattern in the cerebellum reported by earlier investigators.[9,21,22] The reduction in the MAP2 immunostained area (%) in the ML of the *iAs* alone treated animals (present study) could be associated with decrease in dendritic arborization of PCs following exposure to *iAs*. *iAs* induced interference with cytoskeletal protein synthesis has been reported earlier.[23] Li and Chou observed altered morphology of microtubules and microfilaments in Swiss 3 T3 mouse cells cultured in modified Eagles medium and exposed to 2.5 and 20 micro molar $As3^{+}$ for 16 h. These investigators suggested affinity of *iAs* for sulphydryl groups of proteins as the underlying factor for the same.[23] This thiol attacking nature of *iAs* seems to be an important factor for its multipronged action. Since, maintenance in reduced form of certain cystine residues in dimeric tubulin molecule is crucial for microtubule stability, increased affinity of *iAs* for thiol groups leads to decreased availability of tubulin thiol groups, thereby impairing the assembly of microtubules.[24] Impaired assembly was also observed in microtubules isolated from aged rat brain and the same was correlated with decrease in MAP2 content.[25] Harada and co-workers while working with MAP2 deficient mice noted decrease in the length of the dendrites as well as decrease in microtubule density within these processes.[26] Besides, MAP2 is one of the major Protein kinase A (PKA) anchoring proteins in dendrites,[27] thereby assuming a central role in PKA signal transduction involved in the regulation of synaptic strength and long-term potentiation.[28] On the contrary, recent study by Aung and co-workers did not reveal any significant decrease in the mRNA levels of MAP2 in SCAT3 cells exposed to 5 and 10 u M $NaAsO_2$.[29] The variation in the levels/ expression of MAP2 reported in the earlier studies and the present study could be due to the differences in the study model used (in vivo and in vitro), dose of *iAs* administered, duration of exposure as well as the techniques used for evaluation of effects.

Observations of the present study indicate that exposure of rat pups to *iAs* during the critical window period of cerebellar development induces dose dependent adverse effects as evidenced by impaired cytoarchitectural features and down-regulation of one of the key proteins (MAP2) associated with dendritic arborization and maturation. Administration of Cur along with *iAs* was helpful in restoration of these changes to a substantial extent. The multipronged approach of Cur in providing neuroprotective efficacy by its chelating action, modulation of redox potential and up-regulation of crucial markers with critical role in neuronal maturation such as MAP2 could be suggested, however, it is difficult to decipher the precise mechanism underlying the ameliorating role of Cur against *iAs* induced developmental neurotoxicity. Hence, it could be proposed that, administration of Cur as dietary adjuvants in the endemic areas might hold a definitive promise and can be augmented as a safe and cost effective antidote amongst populations inhabiting areas with high *iAs* contamination.

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