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Original Research Article (Experimental)

Ameliorative role of antioxidant supplementation on sodium-arsenite induced adverse effects on the developing rat cerebellum

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ABSTRACT

Background: Arsenic is an environmental contaminant of global concern. Consumption of ground water contaminated with inorganic arsenic (iAs) continues to be the major source of its exposure. The developing nervous system is especially vulnerable to environmental insults due to its higher rate of oxygen consumption and provision of weaker antioxidant (AOX) machinery.
Objective: Since oxidative stress has been reported as one of the major factors underlying iAs induced toxicity, the aim of the present study is to study the effect of two AOXs i.e., Alpha Lipoic Acid (ALA) and Curcumin (Cur) in developing cerebellum of rats exposed to arsenic during postnatal period.
Materials and Methods: The study was carried out on mother reared neonatal rat pups grouped as normal (Ia) and sham (vehicle) controls (Ib,c,d), while the experimental groups IIa/ IIb received sodium arsenite (NaAsO2) [(1.5/2.5 mg/kg body weight (bw)] alone or along with ALA (70 mg/kg bw)- IIIa/ IIIb or along with Cur (150 mg/kg bw)- IVa/ IVb. Behavioural, biochemical and immunohistochemical procedures were carried out to understand the underlying mechanisms.
Results: The observations indicated deficits in locomotor function, accumulation of iAs, increased levels of oxidative stress markers along with downregulation of the expression of proteins closely associated with synaptic functioning (Synaptophysin and Postsynaptic density protein95) in the cerebellum of iAs treated animals. Substantial recovery in all these parameters was observed in AOX co-treated groups.
Conclusion: Our results support the potential of ALA and Cur in amelioration of iAs induced developmental neurotoxicity. ALA and Cur can be proposed as dietary adjuvants amongst populations inhabiting areas with high iAs contamination as a safe and cost effective antidotes.

1. Introduction

The developmental processes in the nervous system are highly susceptible to disruption by various environmental contaminants even at doses that may not be toxic to mature systems [1]. Accordingly, the pre-natal and early postnatal periods have been identified as the critical periods in nervous system development. In humans, extensive development of brain occurs during the period of brain growth spurt (third trimester of pregnancy to early infancy) and the developing nervous system is highly vulnerable to environmental influences during this period. In laboratory animals, such as the rat, the brain growth spurt occurs primarily during the first 2–3 weeks after birth, with a peak around postnatal day (PND) 6–8 [2].

Cerebellum, an integral component of hindbrain, is involved in the control of posture, balance and fine coordination of motor movements. It is one of the initial regions of the brain to undergo differentiation and one of the last to attain maturity. This protracted developmental period makes it highly vulnerable to exogenous and endogenous insults, thereby targeting rapidly ongoing complex processes such as neuronal proliferation, their migration, myelination and synaptogenesis [3]. Thus, exposure to toxic substances during this vulnerable period could have an adverse effect on cytogenetics, morphogenesis and synaptic connectivity [4]. Also, cerebellum is an easy target for harmful environmental agents owing to its propensity towards oxidative stress, based on provision of relatively poor antioxidant (AOX) machinery and redox–active transition metal ions [5].

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Arsenic *(As)* is a metalloid and ranks first in the USEPA (United States Environmental Protection Agency) list of prioritized pollutants [6]. Inorganic arsenic (*iAs*) induced toxicity has been described on a vast scale, with approximately 140 million people suffering from it in 70 countries across the globe [7]. Consumption of *iAs* contaminated groundwater continues to be the chief source of *iAs* exposure. Exposure to *iAs* via consumption of poultry, sea-food, rice and rice products (the later being major dietary supplements amongst infants) has been described [8]. Also, the pregnant women are often exposed to high levels of *As* due to its omnipresence in the environment. Thus, early postnatal exposure to *iAs* could influence various neurodevelopmental processes and subsequently result in functional deficits in later life [9]. The paucity of data pertaining to various effects induced by *iAs* exposure during the vulnerable periods of development raises special concern, though *iAs* induced neurotoxic effects have largely been reported in adult models [10–12].

Oxidative stress has been identified as one of the major mechanisms underlying *iAs* induced toxicity [13] and few studies have suggested the role of various AOXs in combating *iAs* induced adverse effects [11,12]. Among the phytochemicals, the availability of exogenously administered alpha lipoic acid (ALA) and Curcumin (Cur) is ensured by their ability to cross blood brain barrier (BBB) [14,15]. In the present study, the role of ALA and Cur was evaluated on neurobehavioral, biochemical and synaptic alterations in cerebellum of rats exposed to sodium arsenite ($NaAsO_2$) from PND 1–21 (critical window period of cerebellar development).

2. Material and methods

2.1. Ethical approval

The ethical clearance for the study was obtained from the Institute Ethical Animal Committee (IEAC 594/11). Pregnant Wistar rats (gestation day 18–19), obtained from the Experimental Animal Facility (EAF) were housed in temperature (20°C–24 °C) and humidity (50–60%) controlled rooms with 12 h light/dark cycle and fed on standard rodent diet (Ashirwad Industries, India) with *ad libitum* access to drinking water. The study was carried out in accordance with the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).

2.2. Experimental design and treatments

The animals were checked for delivery status daily at 10 AM and 4 PM and the day of delivery of pups was considered as PND 0. Animals from each litter were assigned to different groups so that no two animals from the same litter belonged to the same group and average litter size was 6 animals/lactating mother. The protocol for evaluation of ameliorative role of test substance in toxicity model was adopted from previously reported literature [16,17]. The mother reared pups were divided as normal controls receiving no treatment (Ia) and the sham controls (Ib, Ic, Id) receiving sterile water, ethanol (Merck 1009830511) and Di methylsulfoxide (DMSO) (Sigma Aldrich D5879) (vehicles for $NaAsO_2$, ALA and Cur) respectively. The pups belonging to experimental groups IIa/IIb received only $NaAsO_2$ (Sigma Aldrich 71287) (1.5/2.5 mg/kg body weight (bw) whereas pups in groups IIIa/IIIb and IVa/IVb received 1.5/2.5 mg/kg bw $NaAsO_2$ along with ALA (Sigma Aldrich 62320) (70 mg/kg bw) and Cur (Sigma Aldrich C1386) (150 mg/kg bw) respectively (Fig. 1). As the study design involved intervention from PND 1, the animals were randomly assigned to different groups irrespective of sex with each group having mixed population (sex determination based on anogenital distance though reported, continues to be illusive at the time of birth) [18].

LD50 of $NaAsO_2$ in adult Wistar rats when administered intraperitoneally (i.p.) has been reported as 15.86 mg/kg bw [19]. In the present study, the dose of $NaAsO_2$ (1.5 and 2.5 mg/kg bw) approximately equivalent to 9.5 and 16% of its LD50 was used. Similar doses for evaluation of *iAs* induced toxicity have been used in previous studies [19–24]. The doses of ALA (70 mg/kg bw) and Cur (150 mg/kg bw) were adopted from reported literature [22,25]. To the best of our knowledge, there are no reported toxic effects for these AOXs at these dose levels which make them suitable candidates for trial use in toxicological studies [11,12]. The AOXs (ALA and cur) were administered half an hour later to $NaAsO_2$ [26]. The i.p. route was chosen to ensure rapidity in uptake and uniformity in exposure to the test substance(s) that were administered on daily basis (once a day from PND 1–21).

2.3. Rota-rod test

To investigate the status of motor coordination, postural balance and cerebellar dependent learning in control and experimental animals, automated rota-rod test (TSE Systems, Germany) was carried out (n = 6/group). To familiarize the animals to the procedure, four practice sessions, with the rod moving at a fixed speed of 5 rotations per minute (rpm) for 5 min each were carried out on PND 19. For test trials, rats were placed on the rod and the initial rpm adjusted at 5 was steadily increased to 25 rpm over a period of 300 s. The animals were tested for 3 trials per day for three consecutive days (PND 20, 21, 22) representing day 1, 2 and 3 respectively, with 1 h inter-trial interval to avoid fatigue. The time spent by the animal in balancing itself on the rotating rod before falling off from the rod (latency to fall) was recorded as detected by the photo-beam sensor [27].

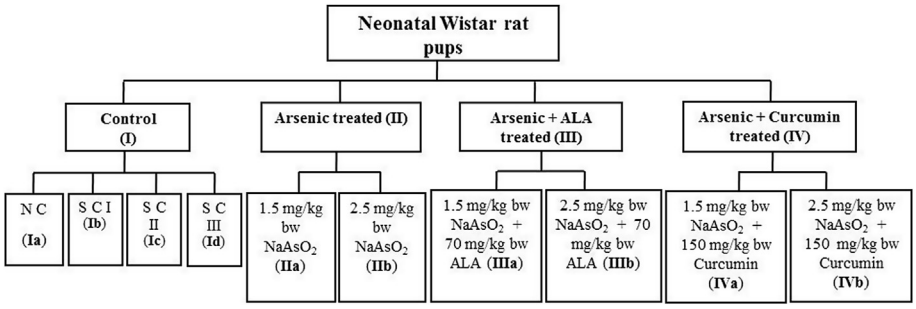

Fig. 1. Grouping of animals based on the exposure pattern.

2.4. Tissue collection

The animals were either perfusion fixed (4% paraformaldehyde) or sacrificed by cervical dislocation on PND 22. The cerebella obtained from perfusion fixed animals (n = 6/group) were processed for immunohistochemical localization of synaptic proteins. The freshly (unfixed) obtained cerebella (n = 6/group/technique) were either snap frozen (−80 °C) and processed for estimation of oxidative stress marker levels, synaptic protein levels (Western Blotting) or digested for analysis of tissue *iAs* levels (Atomic absorption spectrophotometer- AAS).

2.5. Tissue arsenic level

The tissue samples were weighed and subjected to acid (15 mL of 50% HNO_3) digestion (Aurora Biomed Microwave Digester). The final volume of the digested sample was made upto 50 mL (Mili Q) and analyzed by Atomic Absorption Spectrophotometer (AA 7000, Lab India) with working range between 0.1 and 0.8 absorbance for estimation of *iAs* levels. The values were expressed in μg/gm of wet tissue weight [28].

2.6. Reduced glutathione (GSH) and malondialdehyde (MDA) levels

GSH level was estimated by the method of Ellman [29]. In brief, 150 μL of the tissue homogenate (10% w/v in 0.3 M Na_2HPO_4) and 150 μL of 5% TCA (trichloro-acetic acid) were centrifuged (5000 rpm for 10 min at 4 °C) and 100 μL of the supernatant was added to the cuvette with 4 mL of 0.3 M Na_2HPO_4; 400 μL of 5% TCA and 500 μL of Ellman's reagent (DTNB -5, 5′-dithiobis 2-nitrobenzoic acid). Immediately after addition of DTNB, the absorbance was read at 412 nm (Biomate 3S Spectrophotometer, Thermo Scientific) against a reagent blank. A standard curve was plotted using known concentrations of GSH and the tissue GSH level was expressed as μg/gm of tissue [30].

MDA, a by-product of LPO and Thiobarbituric acid reactive substance (TBARS) was estimated by the method of Ohkawa et al. [31]. Briefly, a mixture of 500 μL of tissue homogenate; 50 μL of 8.1% SDS (sodium dodycyl sulfate); 1500 μL of 20% acetic acid in aqueous solution (v/v) pH 3.5; 1500 μL of 0.8% thiobarbituric acid; and 700 μL of distilled water was vortexed, the reaction being carried out in a hot water bath (80–90 °C) for 1 h. After cooling and further centrifugation at 4000 rpm for 10 min, the pink staining in the supernatant was read at 532 nm (Biomate 3S Spectrophotometer, Thermo Scientific). A calibration curve was generated using 1,1,3,3-tetramethoxypropane as a standard and the calculated MDA level was expressed as nMol/gm of tissue.

2.7. Immunohistochemistry

Cryocut (HS 525, Microm GmbH, Germany) sagittal sections (30 μm) of **the** cerebellum were processed for standardized free floating imunohistochemical technique [24]. Specific monoclonal antibodies: Synaptophysin (Syp: SantaCruz Biotechnology. sc9116) and Post Synaptic Density marker (PSD 95: Pierce Biotechnology, Inc. MA1045) in dilutions of 1:200 were used for primary incubation whereas Ultravision Plus Detection system kit (Thermo Scientific TP-060-HLX) was used as secondary antibody. 3′,3' diaminobenzidine (DAB) was used as chromogen for visualizing immunoreactivity. Negative and positive controls were processed simultaneously by carrying out incubation with specific normal serum instead of primary antibody for the former and staining of hippocampus for the later.

2.8. Western Blotting

The standardized protocol [24] was adopted for Western Blotting. Specific primary antibodies- Syp (Santa Cruz Biotechnology. sc9116); PSD95 (Pierce Biotechnology, Inc. MA1045) and loading control (GAPDH & β actin; Bioss, USA) in dilutions of 1:1000 were used for primary incubation followed by overnight incubation with secondary antibody (Goat anti mouse sc 2005 or Goat anti rabbit sc 2004, SantaCruz Biotechnology; dilution- 1:2000). Bands were visualized using DAB. The blots were scanned for densitometric analysis using Quantity 1 software of Gel Documentation System (Bio-Rad, USA). The result was expressed as change in terms of control as follows: $\frac{OD_{PE}/OD_{LE}}{OD_{PC}/OD_{LC}}$ where, $OD_{PE, LE, PC, LC}$ referred to OD of experimental group, loading control in experimental group; control group; loading control in control group respectively.

2.9. Statistical analysis

One Way ANOVA followed by Newman Keuls posthoc test was applied for statistical analysis using Graphpad prism 6. p value < 0.05 was considered significant. As the data among the normal controls and the sham controls did not show significant difference, the values of corresponding sham controls were considered for comparison.

3. Results

3.1. Rota-rod testing

A significant ($p < 0.05$) impairment in the motor coordination, evident by quicker fall of the animals from the rotating rod, was observed in *iAs* alone treated groups, the performance being significantly impaired on day one and two of the trial in group IIb as compared to the controls. An overall improvement in the performance was noted across the AOX (ALA, Cur) supplemented groups on day two and three of the trial. The motor co-ordination of the AOX (ALA, Cur) co-treated groups increased significantly ($p < 0.05$) from day 1 to day 3 as compared to the *iAs* alone treated groups, the increase being 71.44% and 99.18% in the IIIa and IIIb and 67.87% and 94.31% in IVa and IVb as compared to IIa and IIb respectively (Fig. 2).

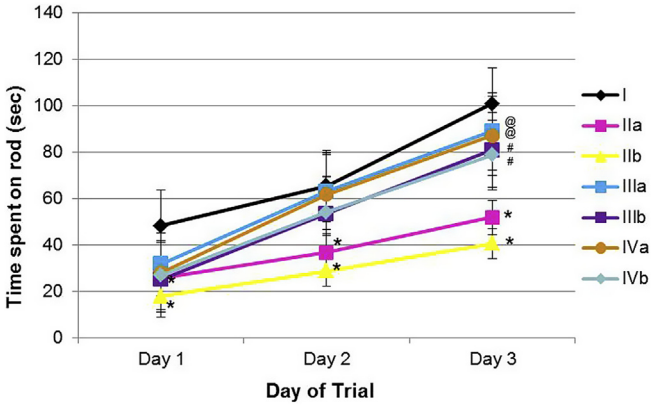

Fig. 2. Time spent on the rotating rod (rota-rod apparatus) by the control (I) and the experimental (IIa, b; IIIa, b; IVa, b) animals on day 1 (PND 20), 2 (PND 21) & 3 (PND 22) of trial. Significant at $p < 0.05$ values × compared to I, @ compared to IIa and # compared to IIb respectively. Note: Improved performance was evidenced by increased time spent by the animals receiving antioxidants (ALA or Cur) with $NaAsO_2$ on the rotating rod.

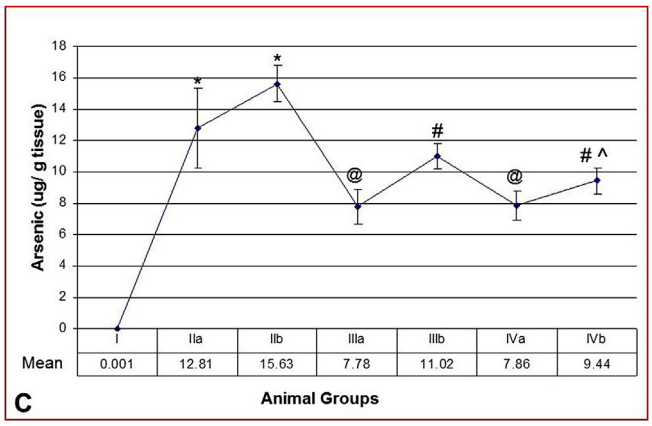

Fig. 3. Cerebellar *iAs* levels (μg/g tissue) in the control (I) and the experimental (IIa, b; IIIa, b; IVa, b) animals. Values are Mean ± SD. Significant at $p < 0.05$ levels × compared to I, @ compared to IIa, # compared to IIb & ^ compared to IIIb respectively. Note: Significant decrease in *iAs* levels of antioxidant co-treated groups (IIIa, b; IVa, b) as compared to *iAs* alone (IIa, b) treated animals.

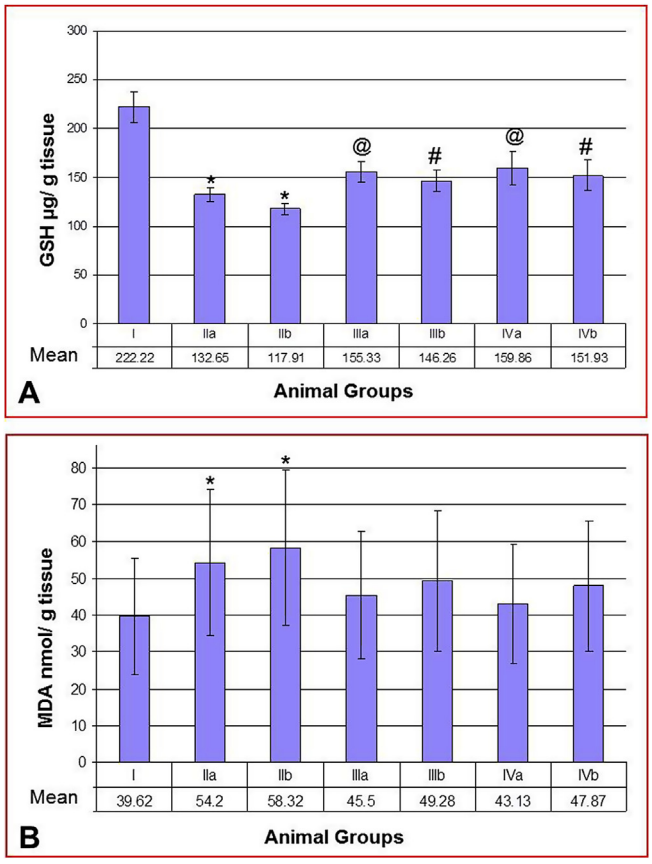

Fig. 4. Levels of GSH (A) and MDA (B) in cerebellum of control (I) and experimental (IIa, b; IIIa, b; IVa, b) groups. Values are Mean ± SD. Significant at $p < 0.05$ levels × compared to I, @ & # compared to IIa & IIb respectively. Note: Significant recovery in GSH & MDA levels in the antioxidant co-treated groups.

3.2. Cerebellar arsenic levels

A significant ($p < 0.05$) dose dependent accumulation of *iAs* (μg/g cerebellar tissue) was observed in animals belonging to group IIa and IIb. The cerebellar *iAs* levels in the animals receiving ALA with low (IIIa) and high (IIIb) dose of *iAs* decreased significantly by 39.26% and 29.49% respectively. The animals co-treated with Cur and *iAs* (IVa, IVb) presented a significant ($p < 0.05$) decrease by 38.64% and 39.60% in cerebellar *iAs* levels as compared to *iAs* alone treated groups (IIa, IIb). The cerebellar *iAs* levels were comparable among the animal groups receiving either of the two AOXs with lower dose of *iAs* (IIIa, IVa), whereas, in animal groups receiving either of the AOXs with higher dose of *iAs* (IIIb, IVb), the tissue *iAs* levels were comparatively ($p < 0.05$) lower in group co-treated with Cur as against group receiving ALA (Fig. 3**).**

3.3. Biochemical parameters (GSH and MDA)

The cerebellar GSH levels revealed a significant ($p < 0.05$) dose dependent decrease (40.30%, 46.93%) in the animals exposed to *iAs* alone (IIa, IIb) as compared to the controls (Fig. 4A). Co-administration of ALA/Cur with *iAs* led to significant increase ($p < 0.05$) in cerebellar GSH by 17.09% and 20.51% in ALA co-treated groups (IIIa, IIIb) and 24.4% and 28.8% in Cur co-treated groups (IVa, IVb) as compared to *iAs* alone treated groups (IIa, IIb). A significant ($p < 0.05$) and dose dependent increase (36.79%, 47.19%) in cerebellar MDA levels was observed in *iAs* alone treated groups as compared to the controls (Fig. 4B). Simultaneous administration of ALA with *iAs* decreased MDA levels by 16.05% and 20.42% (IIIa, IIIb) whereas Cur co-treatment with *iAs* led to 15.55% and 17.91% decrease in MDA level as compared to *iAs* alone treated animals (IIa, IIb).

3.4. Syp and PSD95 expression

The pattern of Syp immuno expression was somewhat uniform across all the groups with intense immuno-reactivity in the molecular layer (ML) [possibly depicting synaptic zones between fibres (parallel and climbing) with Purkinje cell dendrites and interneurons and substantial immunoreactivity around the Purkinje cells (PCL) and the glomeruli in the Granule cell layer (GL). The Syp immunoexpression was less intense in *iAs* alone treated animals as compared to the controls as well as AOX co-treated animals (Fig. 5). These observations were substantiated by Western blot data analysis, which showed a significant ($p < 0.05$) dose dependent decrease (28% and 37%) in the Syp level of *iAs* alone treated groups (IIa, IIb). Cotreatrment of *iAs* and AOXs (ALA/Cur) increased the Syp levels by 23.40% and 30.76% (IIIa, IIIb) and 23.07% and 32.40% (IVa, IVb) respectively (Fig. 6).

On the whole, PSD95 immunoreactivity presented a punctate appearance in the ML (possibly denoting the synapses of Purkinje cell dendrites with parallel and climbing fibres) and GCL (possibly denoting glomeruli) whereas substantial immunoreactivity was noted around the Purkinje cells. PSD95 immunoexpression was less intense in the cerebella of *iAs* alone treated animals as compared to the controls and animals co-treated with *iAs* and AOX (ALA/Cur) (Fig. 7). These results were validated by Western Blot analysis which revealed significant ($p < 0.05$) down-regulation (24% and 22%) in PSD95 level in *iAs* alone treated animals (IIa, IIb) and significant ($p < 0.05$) up-regulation in ALA (20% and 10.34%) and Cur (16.4% and 14.28%) co-treated groups (Fig. 8).

4. Discussion

The preliminary observations of the present study have demonstrated *iAs* induced adverse effects on behavioral, biochemical and immunohistochemical parameters associated with developing rat cerebellum. Also, evaluation of the role of exogenously administered AOXs (ALA, Cur) revealed their ameliorating effect on *iAs* induced neurotoxicity.

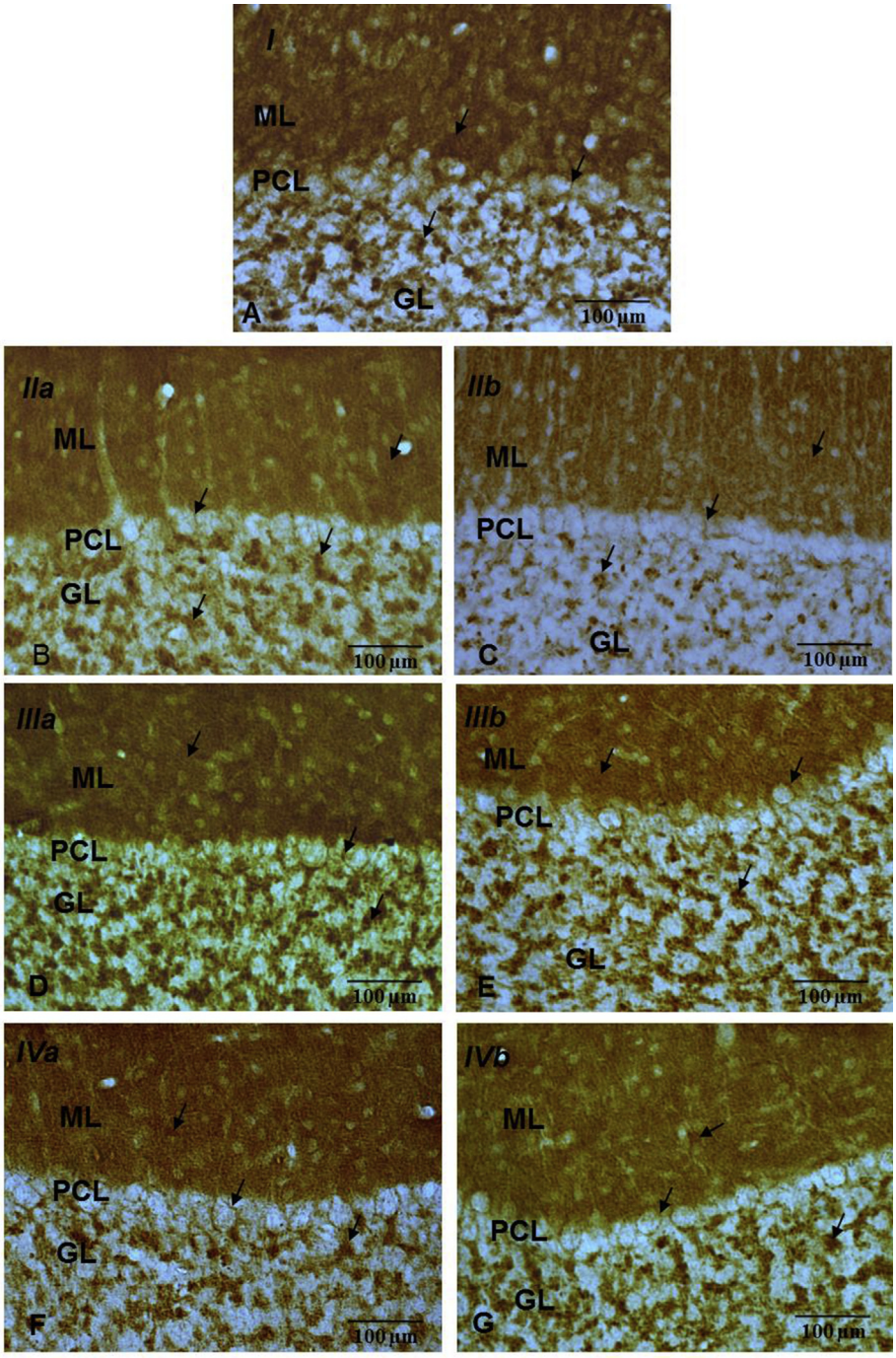

Fig. 5. Immuno-histochemical localization of Syp (→) in cerebellar cortical layers (ML, PCL & GL) of control (A) & experimental (B, C, D, E, F, G) groups (40X). Note: Decreased Syp immunoreactivity in B (IIa) & C (IIb) as compared to group A (I), D (IIIa), E (IIIb), F (IVa) & G (IVb).

Our observations of dose dependent decrease in rota-rod performance of *iAs* alone exposed animals (IIa, IIb) could be suggestive of deranged motor coordination and the same is in agreement with previous reports [12,32–34]. Although there are conflicting reports in literature of increased as well as decreased motor activity following *iAs* exposure in rats and mice [35], yet, the role of various factors such as dose of *iAs* as well as duration of exposure have been suggested as important determinants of altered response in locomotor activity. Association has been drawn between various plausible mechanisms being involved in *iAs* induced effects on locomotor activity. Optimal Calbindin (a vital calcium binding protein) level with in the Purkinje cells is considered an essential determinant of normal motor coordination and sensory integration [36]. *iAs* induced decreased expression of Calbindin could be associated with deficits in the precision of motor coordination. A number of investigators have also linked behavioral alterations with *iAs* induced disturbances in various neurotransmitter systems such as inhibition of cholinesterase [37] and glutamate decarboxylase [38]. Besides, *iAs* induced alterations in the AOX status and dopaminergic elements of the nervous system have been suggested as underlying factors in behavioral alterations [33]. In the present study, the

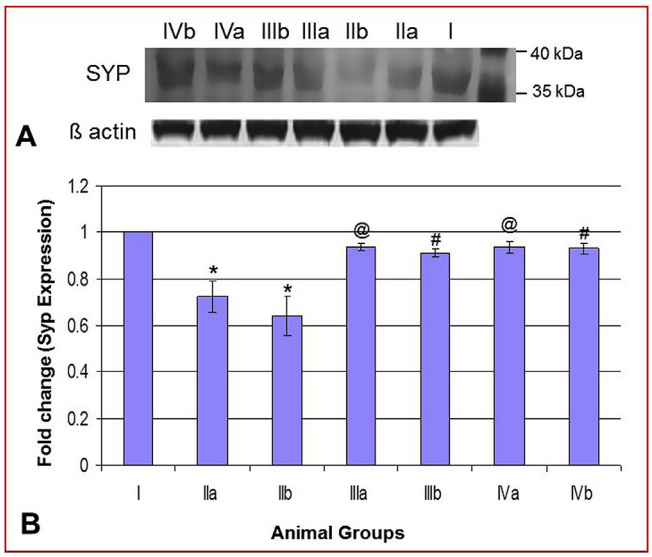

Fig. 6. (A) Immunoblots of Syp (cerebellum) of control (I) & the experimental (IIa, b; IIIa, b; IVa, b) animals along with loading control (β actin). (B) Bar diagram showing fold change in Syp expression in the experimental (IIa, b; IIIa, b; IVa, b) vs control group. Values are Mean ± SD. Significant at $p < 0.05$ levels × compared to I, @ & # compared to IIa & IIb respectively.

animals co-treated with ALA/Cur along with *iAs* spent significantly more time on the rotating rod. Although, improvement in the motor function has been previously reported [12] following co-exposure to *iAs* (20, 100 mg/kg bw $NaAsO_2$) and Cur (orally for 28 days) in adult rats, yet the present study demonstrated enhanced motor coordination of juvenile rats following exposure to *iAs* and Cur postnatally. The observations of the earlier reports as well as of the present study suggest the scope for recovery in *iAs* induced altered motor activity, provided AOXs are supplemented during the period of exposure.

Our observations of dose dependent accumulation of *iAs* at tissue level are in coherence with the earlier studies whereby increased tissue levels of *iAs* have been reported in various animal models following exposure to *iAs* [11,12,32–35]. However, the variation in the tissue *iAs* levels, as observed in the present study and the previously reported studies, could be due to adoption of varied routes for *iAs* administration (i.p. v/s oral) and different durations of exposure (acute vs chronic), the difference in the age groups of the animal models (pups v/s adults) as well as due to differential affinity for *iAs* at tissue level.

Accumulation of *iAs* at tissue level (cerebellum) could be traced to *iAs* induced structural and functional alterations of the defensive barriers (blood brain and blood Cerebrospinal fluid barrier) of the central nervous system. Furthermore, accumulation of *iAs* at tissue level could be reinforced by its binding to molecules containing thiol groups such as GSH, Cysteine etc., though variation in thiol content of the tissues could be the determining factor for its differential accumulation [39]. Our data of significant dose dependent decrease in cerebellar *iAs* levels of animals co-treated with AOXs is in coherence with earlier reports [11,12,40]. AOX induced decrease in cerebellar *iAs* levels could be correlated with the metal chelating properties of these two AOXs. The chelating ability of ALA could be linked to its dithiol groups and the resultant decrease in the metallic load on the biological system [41]. On the other hand, the role of Cur in chelation is linked to its unique conjugated structure of two methoxylated phenols and an enol form of beta diketone whereby it is able to trap as well as donate H atoms from methylene and phenolic groups [42]. From the observations of the present study, it could be proposed that these AOXs possess the potential of decreasing the tissue *iAs* levels, thereby protecting the tissues from its toxic effects. However, there is the need to explore whether it is the chelating activity of AOXs alone or any other action in combination with chelation that enables these to lower tissue *iAs* levels.

Oxidative stress resulting from deranged balance of pro-oxidant/AOX system has been reported as the major mechanism underlying *iAs* induced neurotoxicity [13]. In the present study, we focused on the levels of GSH as this AOX is closely associated with the metabolism of *iAs*, while MDA levels were estimated to evaluate *iAs* induced damage to the cell membranes. We noted decrease in the levels of GSH, while MDA levels were found to be elevated in the cerebellar tissue of *iAs* alone treated animals, thereby indicating *iAs* induced depletion of AOX machinery as well as induction of lipid peroxidation (LPO) respectively. Neuronal tissue is highly vulnerable to oxidative stress with direct impact on synaptic plasticity, dendritic morphology and neurogenesis, thereby highlighting the importance of optimal levels of AOXs in the nervous system. Accumulation of *iAs* and its metabolites directly or indirectly promote ROS induction which in turn induces COX-2 expression through activation of the mitogen-activated protein kinase (MAPK) pathway [43]. Furthermore, compromised levels of GSH could adversely affect processes like cellular detoxification capacity, methylation of *iAs* and stress protein gene expression, besides rendering the cells more sensitive to cell death by Fas ligand and 12-lipooxygenase mediated apoptotic pathways [44,45]. Also, *iAs* induced LPO results in loss of transmembrane potential, alteration in membrane fluidity and permeability, increase in intracellular calcium levels and inhibition of various cell membrane proteins (Na+/K-ATPases and glutamate transporters) [46]. *iAs* induced activation of the Nuclear factor (erythroid-derived 2)-like 2 (Nrf2) has been implicated in regulation of expression of several AOX enzymes such as NADPH, quinine oxido-reductase-1, Heme oxygenase-1 and glutathione S-transferase (GST) [47].

The significant recovery in oxidative stress of animals co-treated with ALA or Cur is in coherence with the reports put forth by earlier investigators [11,12,48]. The decrease in oxidative stress could either be due to direct ROS scavenging property of these AOXs or by chelating action. The neuroprotective role of ALA and Cur could be attributed to their potential of increasing the de-novo synthesis of GSH [49,50]. The up-regulatory effects of Cur on Nrf2 and phase II detoxifying enzymes, also plays a major role in decreasing oxidative stress [51]. Furthermore, it was interesting to note substantial restoration of redox status in the groups receiving AOXs (ALA and Cur) along with higher dose of *iAs* as evidenced by 20.51% and 28.8% increase in GSH and 47.19% and 20.42% decrease in the MDA levels. In the groups receiving low dose of *iAs* along with ALA and Cur, the alteration in redox status was less substantial such as 17.09% and 24.4% increase in GSH and 36.79% and 16.05% decrease in MDA levels. This response could be linked to rigorous activation of cellular defense machinery following exposure to higher dose of *iAs*. It could thus be hypothesized that *iAs* induced decrease in GSH beyond a threshold level might initiate the feed-back activation for its further formation. Various investigators have reported *iAs* induced increased uptake of cystine by the cells as a secondary stress response to compensate the loss of GSH [52]. Thus, the cumulative effect of the cell's intrinsic protective mechanisms and the additive effect of exogenously administered AOX could have boosted the cellular ability to combat *iAs* induced oxidative stress.

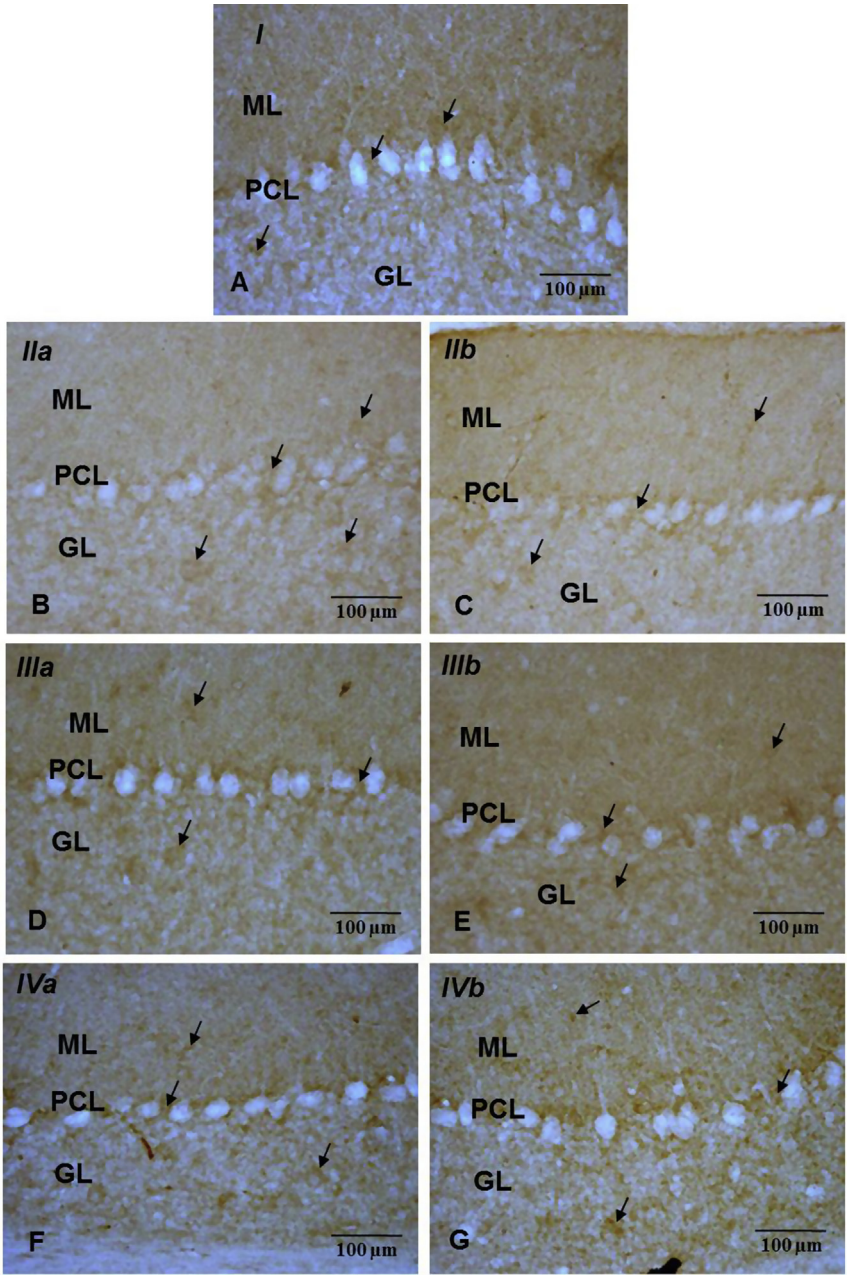

Fig. 7. Immuno-histochemical localization of PSD95 (→) in ML, GCL & areas intervening between PCs of cerebellar cortex from control (A) & experimental (B, C, D, E, F, G) groups (40X). Note: Decrease in the cerebellar PSD95 immunoreactivity in B (IIa) & C (IIb) as compared to group A (I), D (IIIa), E (IIIb), F (IVa) & G (IVb).

Synapses, the highly specialized sites for quick signaling amongst neurons, are rapidly formed during the postnatal period. Since, Syp and PSD95 are amongst the important proteins associated with structural and functional aspects of pre and post synaptic membranes, we studied their expression in the developing rat cerebellum following exposure to *iAs* alone or in combination with AOXs (ALA, Cur) during the peak period of synaptogenesis.

The pattern of Syp and PSD95 immuno-expression observed in our study is similar to that reported by earlier investigators [53]. The decreased expression of Syp in *iAs* alone treated animals is indicative of *iAs* induced derangement in the neurotransmission and neuronal plasticity along with poor nerve terminal differentiation and synaptogenesis [54]. Fujimura and coworkers [55] noted decreased levels of Syp in the cerebellum of rats exposed perinatally to methyl mercury (5 ppm, orally) and associated it with motor dysfunction (Rota-rod testing), thereby reiterating the vital role of optimal Syp expression in maintenance of neurotransmission at chemical synapses. Syp is crucial in synaptic vesicle (SV) exocytosis [54] and modulation of synaptic plasticity [56]. Besides, it plays an important role in release of neurotransmitters by binding to calcium [57]. Changes in the expression of Syp have also been associated with external stimuli such as constraint stress and internal factors like deficits of neurotrophic factors [58].

Our observation of *iAs* induced decrease in expression of PSD95 is in congruence with the recent study of Luo and coworkers [59], who, reported decreased expression of PSD95 in hippocampus of adult rats exposed to $NaAsO_2$ (2.72, 13.6,68 mg/l orally) for 3 months. These investigators hypothesized, *iAs* induced alterations in the expression of various proteins such as PSD95, NR2A, CaMKII, synaptic Ras GTPase-activating protein (SynGAP) and nuclear activated extracellular signal regulated

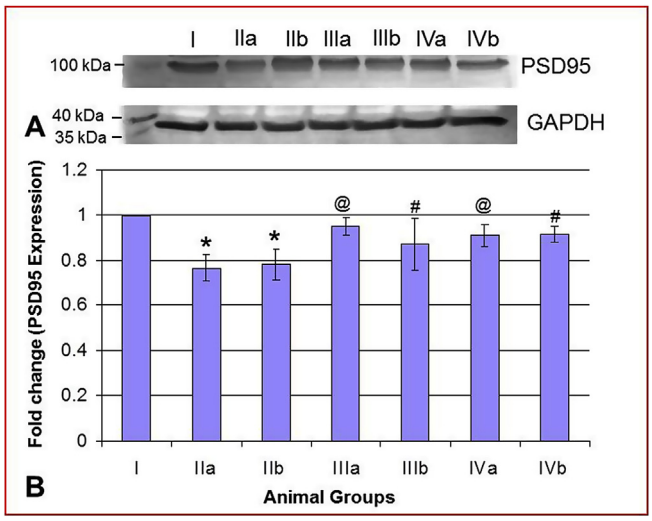

Fig. 8. (A) Immunoblots of PSD95 (cerebellum) of control (I) & the experimental (IIa, b; IIIa, b; IVa, b) animals along with loading control (GAPDH). (B) Bar diagram showing fold change in PSD95 expression in the experimental (IIa, b; IIIa, b; IVa, b) vs control group. Values are Mean ± SD. Significant at $p < 0.05$ levels × compared to I, @ & # compared to IIa & IIb respectively.

kinase (ERK1/2) as the molecular basis of *iAs* induced neurotoxicity. Decreased level of PSD95 in *iAs* alone treated animals is of immense relevance in the present study as this protein is implicated to play a crucial role in the formation and maturation of excitatory synapses during synaptogenesis, the period corresponding to the experimental period of the present study [60]. Furthermore, PSD95 (a PDZ domain scaffold protein) being a prominent component of post synaptic structure is involved in synaptic development, neurotransmission, signal transduction and synaptic plasticity [61]. By virtue of its location and interaction with a number of other proteins including NMDA and AMPA receptors, it plays a central role in post synaptic transmission [60]. Thus, the neurochemical basis of *iAs* induced neurotoxicity could be linked to down-regulated expression of two vital synaptic proteins, Syp and PSD95. However, further studies focusing on the up- and down-stream molecules required for the expression of these vital proteins of synaptogenesis would be helpful to decipher various mechanisms underlying *iAs* induced neurotoxicity.

5. Conclusion

The major observations of our study suggest that exposure of animals to *iAs* during the critical window period of cerebellar development results in neurobehavioral alterations which could be associated with *iAs* induced decrease in levels of Synaptic proteins (Syp and PSD95) and increase in oxidative stress. The neuroprotective activity of the supplemented AOXs (ALA/Cur) could be linked to their role in up-regulation of synaptic proteins, augmentation of AOX defense system and chelation of *iAs* at tissue level. Hence, these AOXs can be proposed as dietary adjuvants among the populations inhabiting areas with high *iAs* contamination as safe and cost effective antidotes.

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Conflict of interest

None

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