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Original Article

Curcumin induced up-regulation of Myelin basic protein (MBP) ameliorates sodium arsenite induced neurotoxicity in developing rat cerebellum

Parul Kaushal [a], *Raj D. Mehra* [b], *Pushpa Dhar* [c,*]

[a] *Research Scholar, Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India*
[b] *Professor, Department of Anatomy, Hamdard Institute of Medical Sciences and Research (HIMSR), Jamia Hamdard, New Delhi, India*
[c] *Professor, Department of Anatomy, All India Institute of Medical Sciences, New Delhi, India*

ARTICLE INFO

Article history:
Received 7 March 2014
Accepted 8 April 2014
Available online 10 May 2014

Keywords:
Arsenic
Postnatal
Antioxidant
Myelin basic protein
Morris Water Maze

ABSTRACT

Introduction: The aim of the present study is to evaluate the modulatory role of Curcumin on cerebellar Myelin basic protein (MBP) expression and associated behavioral parameters by histochemistry and Morris Water Maze (MWM) following exposure of rat pups to sodium arsenite ($NaAsO_2$).

Methods: After obtaining the ethical clearance, pregnant Wistar rats were issued from the Central Animal Facility (CAF) and maintained according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA). The day of birth of pups was designated as postnatal day zero (PND 0). Mother reared pups were divided into: control (normal, sham) and experimental groups receiving $NaAsO_2$ alone or $NaAsO_2$ with Curcumin via intraperitoneal route (i.p.) from PND 1 to 21. Morris Water Maze (MWM) test was carried out on PND 21 to assess the exploratory behavior of the animals. Luxol Fast Blue staining and immuno-localization of MBP was carried out to evaluate the status of myelination in the cerebellar white matter.

Results: Animals subjected to combined exposure to Curcumin and $NaAsO_2$ showed improved exploratory behavior besides maintenance of myelin structure and up-regulation of MBP expression in the cerebellar white matter.

Discussion: The up-regulation of cerebellar MBP expression together with improved exploratory behavior of animals subjected to combined exposure to Curcumin and arsenic (iAs) provides the experimental evidence for mechanistic role of Curcumin in ameliorating $NaAsO_2$ induced neurotoxicity, probably based on the essential properties of Curcumin such as scavenging free radicals and chelation of arsenic.

* *Corresponding author.*
E-mail addresses: dharpushpa@hotmail.com, dharpushpa2013@gmail.com (P. Dhar).
http://dx.doi.org/10.1016/j.jasi.2014.04.001

1. Introduction

Arsenic (*iAs*), a metalloid, is ubiquitously distributed in the air, water and soil.[1] *iAs* induced toxicity is emerging as a major health concern globally, with ground water continuing to be the chief source of exposure for the general population.[2] However, the excessive exposure of children below three years of age to *iAs* via rice has emerged as an equally important issue of concern, as rice products form the main dietary supplements in infants[3] and the accumulation of *iAs* in rice grown in the endemic areas has been reported.[4] Adverse effects following postnatal exposure to *iAs* have been documented in the cerebellum,[5] hippocampus,[6] testes,[7,8] liver[9] etc. Besides, there are reports of deficits in locomotion, neurobehavioral and cognitive functions along with impaired memory and attention following exposure to *iAs* during early childhood.[1,6] Induction of oxidative stress[10] and interference with varied processes such as myelination,[11] cytoarchitecture,[6] neurotransmission[12] etc. have been suggested as the mechanisms underlying *iAs* induced toxicity.

The role of cerebellum in controlling posture, balance, fine coordination of motor movements, adaptation of ocular reflexes and learning some conditioned behaviors is well documented.[13]The development of these modalities along with the underlying structural characterization (neuronal proliferation, migration and synaptogenesis) proceeds at a rapid pace in cerebellum (rats) during the first three weeks of postnatal life, thereby, making it highly vulnerable to various insults.[14] Purkinje cells of the cerebellar cortex are actively involved in integration of the multimodal inputs and play a key role in coordination of a variety of motor and learning tasks. The axons of these cells form the sole source of efferent fibers from the cerebellum, thereby playing an important role in determination of cerebellar circuitry and its functioning.

Various phyto-chemicals are being explored for their potential in ameliorating *iAs* induced toxicity, Curcumin being one of these as it is reported to be several times more potent than vitamin E in scavenging free radicals.[15] Besides, its easy availability and cost effectiveness together with no reported toxic effects, add to its therapeutic potential. In addition, the property of Curcumin to cross the blood brain barrier[16] further enhances its neuroprotective potential. Based on these facts, the present work was designed to determine the role of Curcumin in modulation of Myelin basic protein (MBP) and associated behavioral parameters in the cerebellum of rat pups exposed to sodium arsenite ($NaAsO_2$) during postnatal period (PND 1-21).

2. Methods

Pregnant Wistar rats (gestation day 18–19), procured from the Central Animal Facility (CAF) after obtaining ethical clearance from the Institute Animal Ethical Committee (IAEC 594/11), were housed in temperature (20°C-24 °C) and humidity (50–60%) controlled rooms (CAF) 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. The animals were checked daily at 10 AM and 4 PM for delivery status and the day of delivery of pups was designated as postnatal day (PND) 0. The mother reared pups were divided into normal controls (with no treatment – Ia), and sham controls (receiving either distilled water – Ib or DMSO – Ic). Animals belonging to the experimental groups IIa and IIb received 1.5 and 2.5 mg/kg body weight (bw) $NaAsO_2$ alone whereas animals in groups IIIa and IIIb received Curcumin 150 mg/kg bw along with 1.5 and 2.5 mg/kg bw $NaAsO_2$ (Fig. 1). The test substances were administered once daily by intraperitoneal (i.p.) route from PND 1-21. Animals belonging to group IIIa and IIIb received $NaAsO_2$ and Curcumin injections at half an hour interval in between.[17] The i.p. route was considered for ensuring the requisite delivery of the test substance and to avoid variation in exposure doses.

In Wistar rats, LD50 of $NaAsO_2$ (i.p.) has been reported as 15.86 mg/kg bw.[18] The doses of *iAs* in the present study are approximately equivalent to 9.5 and 16% of its reported LD50. The dose of Curcumin is adopted from the previous studies.[19] To the best of our knowledge, there are no reports suggestive of toxicity induced by the dose of Curcumin used in the study. During the entire experimental period, the animals were observed for signs of normal developmental features.

The exploratory behavior (capacity to solve a spatial problem) was evaluated in cued paradigm of Morris Water Maze (MWM) test on PND 21. The animals were sacrificed on PND 22 under light ether anesthesia 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 ($n = 6$/group). Fresh

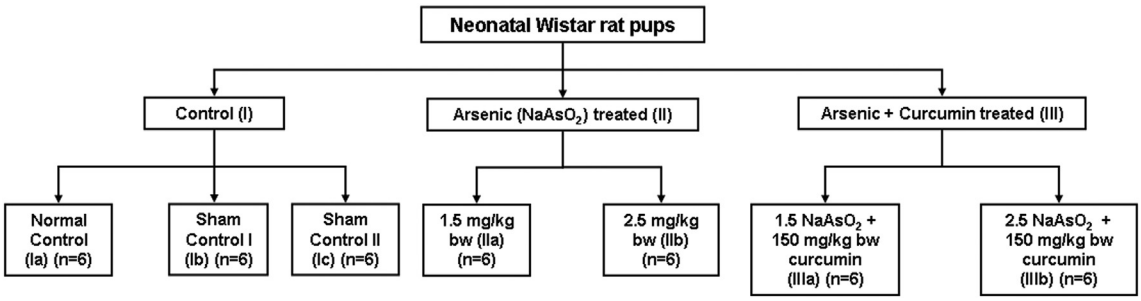

Fig. 1 – Study design showing the various animal groups.

cerebellar tissue from animals sacrificed by cervical dislocation was used for Western blotting.

2.1. *Morris Water Maze (MWM) test*

The protocol for variant of Morris Water Maze testing was adopted from Petrossini et al.[20] Petrossini and co-workers observed exploratory behavior in hemi-cerebellectomized rats after allowing them to observe MWM trials of companion rats and compared their performance with the control rats.[20] In the present study, the visuo-motor and spatial navigational ability of rats was studied by behavioral analysis using MWM. The MWM apparatus consists of a circular pool (1.5 m diameter; 30 cm height) having a circular escape platform made up of plexiglass (10 cm in diameter and 22 cm high). The pool was filled with water up to a level 1 cm above the platform and the temperature of water was maintained at 25–27 °C. The pool was divided into 4 equal quadrants (NE, SE, SW, NW) and the platform was placed at a fixed location in the center of one of the quadrants (SE) (Fig. 2). There was no attempt at enhancing or suppressing the extra maze cues. However, a dominant visual cue was placed on the wall of the pool in the vicinity of the platform. The apparatus was attached to the computer with NI-IMAQ software (Coulbourn instruments, USA) for recording the behavior of the animals. The procedure comprised of two components i.e. observational training and test trial. For observational training, the controls and the experimental animals housed in cages with metallic grid, were allowed to observe the rats (normal rats issued for other ongoing experiments) performing MWM test. Each animal observed 100 trials (32 trials/day on PND 19, 20 and 21) before performing the test. For the test trial (PND21), each animal was gently released from the NE quadrant into the water facing the center of the pool. It was allowed to swim around and find the platform. The paths taken by the animals in the pool to reach the platform were monitored by the mounted video camera. Thigmotaxis i.e. amount of time spent by the animal in repeated circling movements near the periphery of the tank (5 cm from wall of tank) was also recorded to evaluate the problem solving ability. The exploratory behavior of the animal was graded (I, II, III) with reference to use of clue for platform finding, so that grade I pointed to clear usage of the clue, grade II indicated the indifference of the animal to use the clue and grade III indicated no use of the clue at all. Also, the swimming speed of the animals belonging to various groups was recorded.

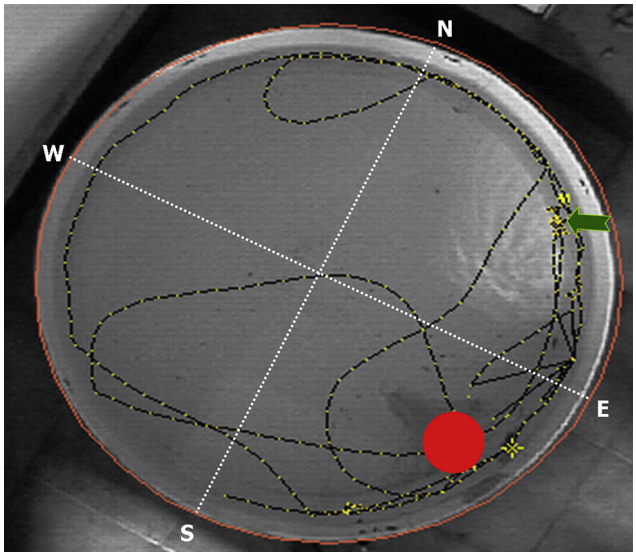

Fig. 2 – Setup for MWM testing. Four quadrants of the pool (SE,NE,NW,SW) (SE – quadrant with platform, NE – quadrant where animal is placed for trial).

2.2. *Luxol Fast Blue (LFB) staining*

Serial sections (7 μm) from paraffin blocks of cerebellar tissue were processed for LFB staining following the standard protocol.[21] The de-waxed sections were immersed in 96% ethanol for 5 min followed by staining in 0.1% LFB solution (overnight at 60 °C) and rinsed in 96% ethanol and distilled water. The differentiation was done in saturated solution of lithium carbonate (0.05%) and continued in 70% ethanol. This was followed by dehydration in increasing concentrations of ethanol (70%, 90%, 96%). Clearing of the sections in xylene (2 changes of 15 min each) was followed by mounting with DPX. The stained sections were observed for morphological features using Nikon E600 microscope fitted with a digital camera (DS-Fi1 Nikon, Melville, NY, USA).

2.3. *Immunohistochemistry*

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) collected in 0.1 M PB were processed by free floating immunohistochemical technique following the standard protocol. Following antigen retrieval by 0.5% Sodium Dodecyl Sulphate (SDS) and washing (0.1 M PBS), 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-Myelin Basic Protein – MBP, sc 71546, 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 semiquantitative estimation of MBP was carried out on mid-sagittal sections (20×) from the vermal and para-vermal regions (presenting well defined lobules) by image analysis using Nikon AR NIS elements 3.1 Software. Immunodensitometric analysis of MBP was observed on a gray scale of 0–255 where (0) represented black and 255 represented white.[22] The first section was randomly chosen and the subsequent sections were every 10th from that. A rectangular grid (200 μm × 40 μm) was superimposed on the lobular fiber tracts and 10 reference areas/section and 5 sections/animal were considered for analysis. The surrounding area was cropped off to confine the intensity of staining in the region of interest (ROI). The mean cumulative gray values ($n = 6$ animals/group) were expressed as mean ± SD.

2.4. Western blotting

Fresh cerebellar tissue, stored at −80 °C, was weighed and homogenized (10% w/v) in Protein lysis buffer (G Biosciences) and Protease inhibitor cocktail (Sigma Chemicals). Homogenate was centrifuged at 1000 rpm (4 °C, 30 min) for protein estimation by Bradford assay[23] in a 96 well plate format using TECAN Infinity F50 plate reader at 595 nm. A standard curve was generated with a range of known concentrations of Bovine Serum Albumin (BSA) for calculation of protein concentration in the supernatant. 20 μl mixture of supernatant and sample loading buffer (G Biosciences) containing 40 μg of protein was denatured with β mercapto-ethanol were loaded in the wells (5% SDS Poly-acrylamide stacking gel) and resolved (12% SDS Poly-acrylamide resolving gel) using electrophoresis apparatus (Mini Protean Tetra Cell, Bio-Rad; USA). The gels were run for $1^1/_2$-2 h at 130 V and pre-stained molecular weight marker (Pierce Biotechnology, Inc.) was run along for identification of bands.

The separated proteins, were transferred on nitrocellulose membranes (Sigma Chemicals, USA) using 25 mM tris glycine buffer pH 8.3, containing methanol and SDS and blocked in 3% BSA (Sigma Chemicals), followed by overnight incubation in respective primary antibody (MBP, Santa Cruz Biotechnology, sc 71546, 1:1000 and β actin Loading control, Bioss, bs 0061R, 1:1000). After washing, the membrane was incubated overnight with secondary antibody (Goat anti mouse sc 2005 or Goat anti rabbit sc 2004, Santa Cruz Biotechnology; dilution – 1:2000). After repeated washings, the bands were visualized by DAB (3,3′diaminobenzidine) (Immunodetector liquid DAB kit, Bio SB, USA). Densitometric analysis of blots was done by Quantity 1 software of gel documentation system (Bio-Rad, USA). All blots were scanned and normalized immediately to avoid the signal loss and to eliminate the differences in the background. The result was expressed as change in terms of control as follows:

$OD_{PE}/OD_{LE}/OD_{PC}/OD_{LC}$; where, OD_{PE} – OD of protein in experimental group; OD_{LE} – OD of loading control in experimental group; OD_{PC} – OD of protein in control group; OD_{LC} – OD of loading control in control group.

2.5. Statistical analysis

GraphPad Prism 6 was used for the data analysis. One Way ANOVA followed by Newman–Keuls posthoc test was used and p value <0.05 was considered significant. As no significant difference was observed among the normal and the sham control animals, the values of corresponding sham controls were considered for comparison.

3. Results

The pattern of general developmental features (ear unfolding, fur development and bilateral eye opening occurring on PND 4, 6 and 14 respectively) was somewhat identical across the control and the experimental groups. However, *iAs* alone treated animals showed a marginal decrease in gaining body weight all along the experimental period, as compared to the controls and the animals receiving Curcumin with *iAs* (Fig. 3).

3.1. Morris Water Maze Test

Analysis of the path traversed in the tank from the starting point up to the platform revealed longer time taken by *iAs* alone treated animals as compared to the controls and the Curcumin co-treated groups. This observation was further reinforced by the variation noted in the swimming speed, wherein *iAs* alone treated groups exhibited marginal decrease in the swimming speed, thereby indicative of impairment in motoric ability and motivation. Further, deficit in exploratory strategy of *iAs* alone treated animals was evident by increased thigmotaxis (increased time spent by the animals in circling movements along peripheral extent of the tank). A downward trend in the exploratory behavior of the *iAs* alone treated animals was evident by the decreased percentage of animals (14.28%) using the clue to reach the platform. On the other hand, the animals receiving Curcumin along with either low or high dose of *iAs* showed improved exploratory behavior being evident by increased percentage of animals (40%) using the clue for finding the platform (Table 1).

3.2. Morphological features

Observations of LFB stained sections revealed well defined pattern of fiber tracts in the cerebellar lobules of all the animal groups. Normal axonal morphology and myelin sheath integrity was evident from uniform staining (LFB) pattern in the cerebellar lobules of control (Fig. 4E) and animal groups co-treated with Curcumin and *iAs* (Fig. 4C,D). In the *iAs* alone treated animals (Fig. 4A,B), diffuse vacuolization and randomly dispersed empty spaces were observed along the fiber tracts, probably indicative of partial loss of myelin sheath.

3.3. Immunohistochemical localization (MBP)

The intensity of MBP immuno-expression, evident in the cerebellar folia along disposition of white matter, primarily depicted the status of myelin sheath of the traversing axons. MBP expression was less intense in the cerebella of *iAs* alone treated animals (Fig. 5A,B) as compared to the controls (Fig. 5E) whereas an intense expression was evident in groups receiving Curcumin along with *iAs* (Fig. 5C,D), the intensity being comparable to that of the controls (Fig. 5E).

3.4. Semiquantitative analysis (MBP)

A significant ($p < 0.05$) dose dependent decrease was observed in the expression of MBP (higher gray values) in the *iAs* alone treated groups as compared to the controls. Animals subjected to a combined exposure of $NaAsO_2$ (1.5 and 2.5 mg/kg bw) and Curcumin showed an increase by 33.84% and 33.59% in the MBP expression (Fig. 5F) being evident by decreased gray values.

3.5. Western blotting (MBP)

A single band of 21.5 kD was visualized for MBP in the immunoblots. A significant ($p < 0.05$) dose dependent decrease was observed in the intensity of the MBP bands of *iAs* alone

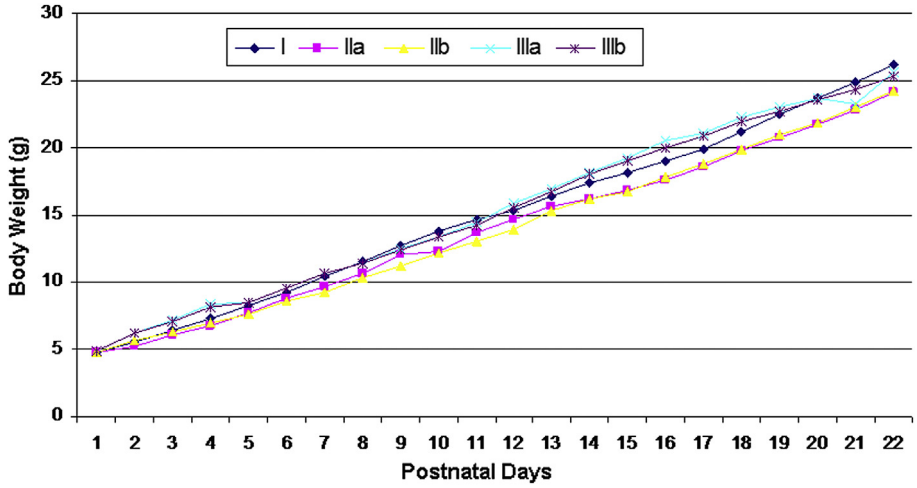

Fig. 3 – Line diagram showing the gain in body weight of the control and the experimental animals during the experimental period.

treated animals (compared to controls). The immunoblots of animals receiving *i*As and Curcumin showed enhanced expression of MBP compared to *i*As alone treated group (Fig. 6A), these observations being reinforced by densitometric analysis of the blots (Fig. 6C).

4. Discussion

The present study was designed to evaluate the cerebellar function and the associated MBP status following postnatal exposure of rat pups to $NaAsO_2$ and their modulation by co-administration of Curcumin. The observations of the present study are suggestive of the potential role of Curcumin in reducing *i*As induced toxicity. The ameliorative role of Curcumin in *i*As induced neurotoxicity in adult animal model has previously been suggested by various investigators.[12,24,25]

The involvement of cerebellar networks has been suggested to play a crucial role in execution of procedural components of spatial event processing along with strategies of navigational system involved in object finding.[20,26] Although, observation of a task as an effective way of learning that task has been reported in humans and animals based on the tendency to imitate a large range of actions, yet, the actual performance during execution of the task is dependent on additional factors such as inertia, friction, proprioceptive feedback etc., so that, for path finding system, the sensory information has to be closely integrated with movement generation.[27–29] The observation of impaired exploratory behavior in *i*As alone treated animals in the present study is in corroboration with earlier studies,[1] reporting decreased spontaneous locomotor activity and alterations in a spatial learning task of animals exposed to *i*As. The decreased exploratory performance of *i*As alone treated animals in the present study could be associated with *i*As induced down-regulation of MBP as myelination process has been reported to play a crucial role in cognitive developments.[30] Some investigators have reported decreased levels of Acetyl cholinesterase (AChE) in brain and plasma of rats along with alteration of motor activity (rota-rod test) following exposure to *i*As, thereby drawing an association between *i*As induced altered behavioral tasks and decreased AChE levels.[12,24,25] These workers further observed increased AChE levels following co-administration of Curcumin with *i*As which led them to opine regarding the neuroprotective role of Curcumin. In the present study, the protective role of Curcumin was evident from the improvement in the exploratory ability (increased usage of clue), in the motoric ability (increased swimming speed) and in motivation of the animals receiving Curcumin with *i*As. These observations were further

Table 1 – Morris Water Maze testing in various animal groups.

Animal groups	% Of animals using the clue to reach the platform			Average swimming speed (cm/sec)	Time to reach the platform (sec)	Thigmotaxis (sec)
	Grade I	Grade II	Grade III			
I	41.67	33.33	25	20.07 ± 3.12	26.19 ± 8.40	4.31 ± 1.45
IIa	14.28	42.85	42.85	12.41 ± 1.63	36.84 ± 9.14	5.732 ± 1.99
IIb	14.28	28.57	50	11.39 ± 2.98*	43.50 ± 16.70	8.78 ± 3.05*
IIIa	40	40	20	17.14 ± 6.39	31.74 ± 11.22	5.164 ± 1.10
IIIb	40	40	20	15.33 ± 6.7	39.80 ± 17.20	7.02 ± 2.67

I – Control; IIa & IIb – 1.5 & 2.5 mg/kg bw $NaAsO_2$; IIIa & IIIb – 1.5 & 2.5 mg/kg bw $NaAsO_2$ + 150 mg/kg bw Curcumin. *P* value <0.05 significant * vs control.

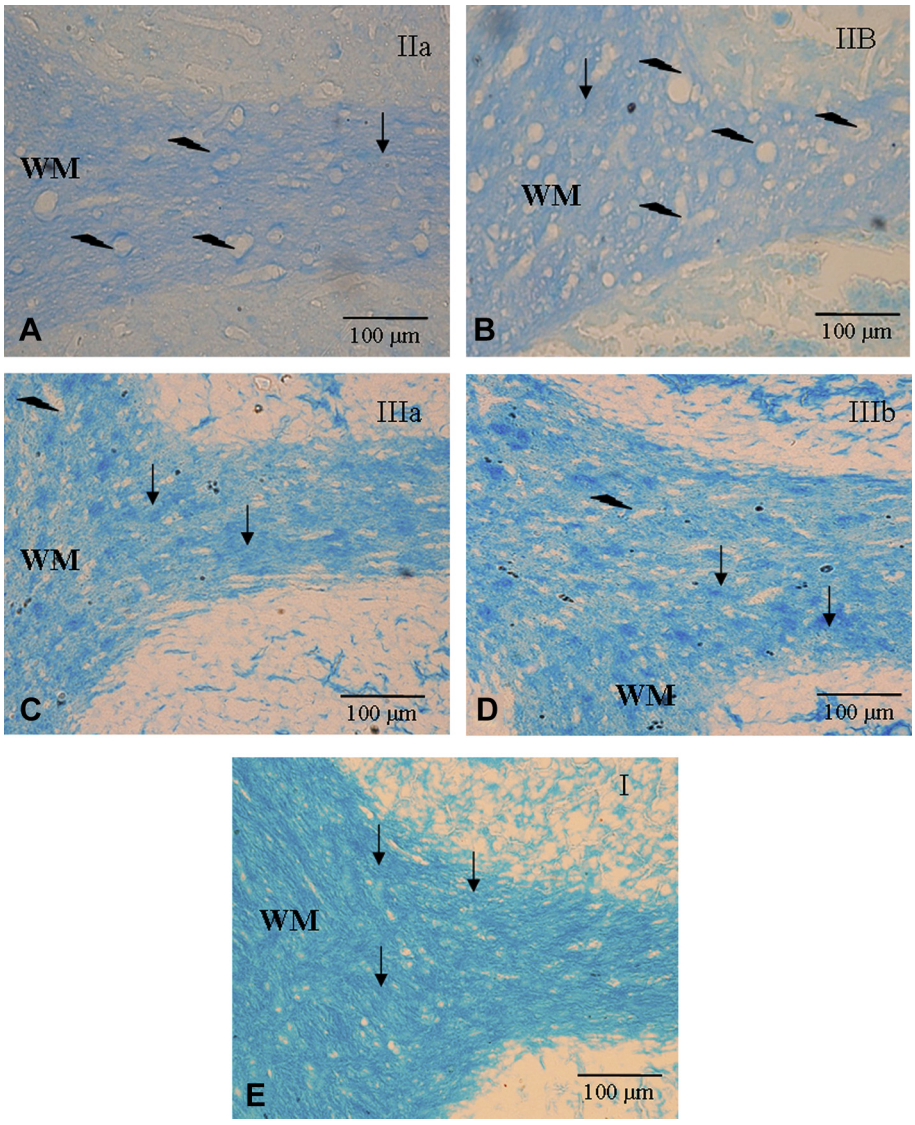

Fig. 4 – Photomicrographs of LFB stained cerebellar sections of control (E) and experimental animal groups (A–D). Note: Diffuse vacuolization and empty spaces (↖) in the tracts in the *i*As alone treated (A, B) group as compared to the control (E) and the Curcumin co-treated groups (C, D).

substantiated by the observed decrease in thigmotaxis, indicative of improvement in problem solving ability of animals subjected to combined administrations of Curcumin and *i*As.

Morphological observations of disintegrated fiber tracts and the presence of vacuolization in the cerebellar white matter of *i*As alone treated animals is in congruence with findings of earlier workers.[11,31,32] Santoyo and co-workers noted vacuole formation in the myelin sheath of striatum of rats exposed to *i*As (4 mg/kg bw) for four months.[32] Also, Zarazua and co-workers (2010), reported somewhat similar findings in rats exposed to 36 ppm *i*As in drinking water (gestational, lacational and postnatal exposure for 1, 2,3 and 4 months). These workers hypothesized myelin to be one of the major targets of *i*As toxicity based on *i*As induced interference with arginine methylation reactions.[11]

Myelin, a dielectric material, comprised of lipids (70–80%) and proteins (15–30%) is essential for the proper transmission of neuronal impulses along the axons. Besides myelin oligodendrocyte glycoprotein and proteolipid protein with galactocerebroside in the myelin sheath, Myelin Basic Protein (MBP) remains as one of the major proteins of myelin. Being located in the major dense lines corresponding to cytoplasmic surfaces of the oligodendrocytes, MBP plays an important role in holding these lines together and imparting compactness to the myelin.[33] Quantification of MBP has been reported to be useful for monitoring the myelin content of the central nervous tissue.[34] The synthesis of MBP involves the process of methylation (at arginine 107 residue), utilizing S-adenosylmethionine (SAM) as the methyl group donor, the reaction being catalyzed by protein-arginine N-methyltransferase.[35] The nexus of interaction between *i*As toxicity and SAM lies

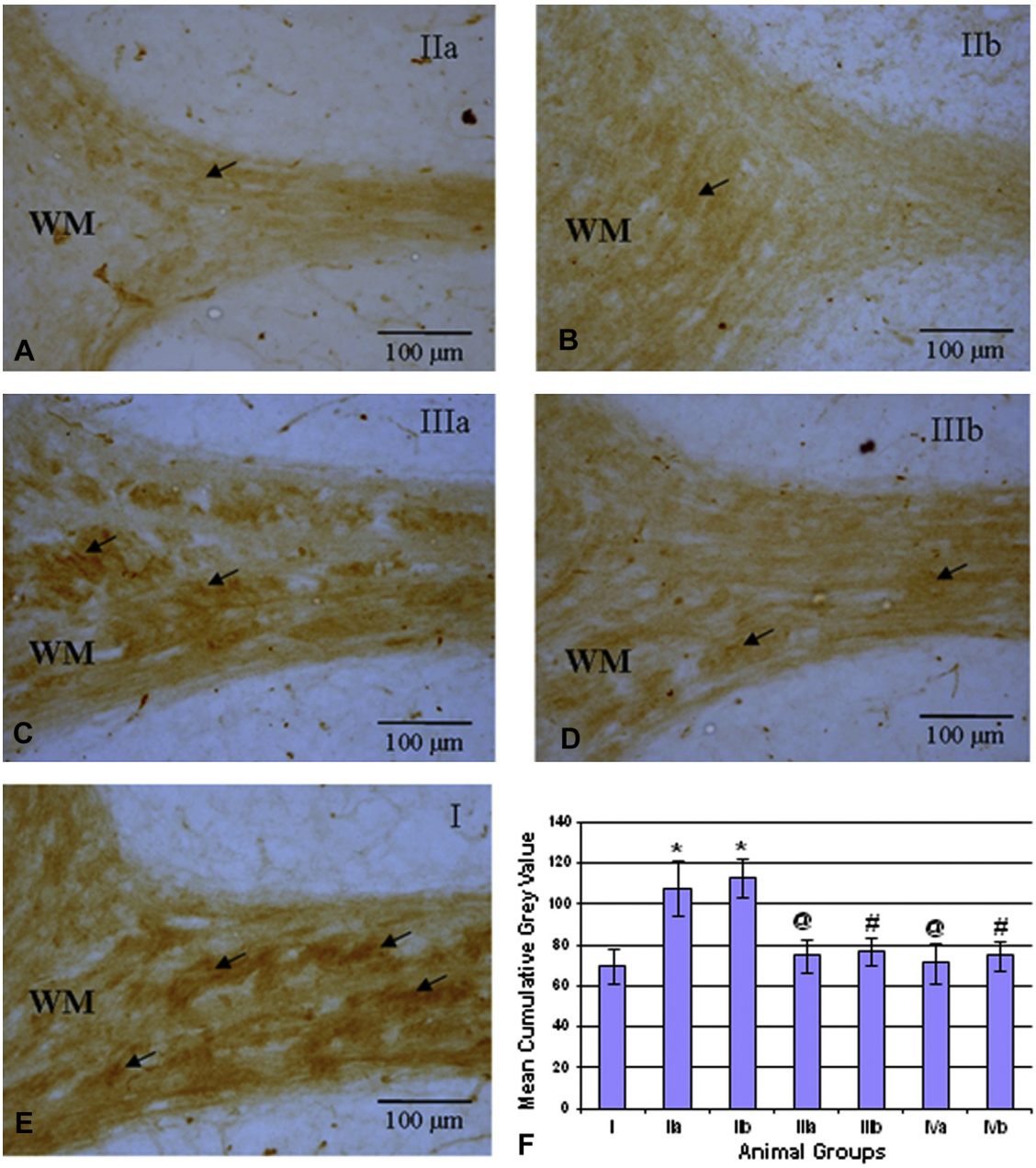

Fig. 5 – Photomicrographs of MBP immunostained cerebellar sections obtained from the control (E) and the experimental animals (A–D) along with their semiquantitative analysis (F). Note: Decreased MBP immunostaining in the animals treated with *i*As alone (A, B, F) as compared to the controls (E, F) and the curcumin co-treated animals (C, D, F).

in the fact that the process of detoxification of *i*As also involves methylation, being catalyzed by methyltransferases and using SAM as the methyl group donor to form monomethyl arsenic acid (MMA^V) which is reduced to MMA^{III} by GSH. Methylation of MMA^{III} in turn forms DMA^V which gets reduced to DMA^{III} by GSH. SAM deficiency and methylation status are closely associated, so that methylation processes of various pathways such as synthesis of MBP, neurotransmitters, phospholipids, DNA methylation etc. could be targeted by deficiency of SAM and get manifested as alterations in the behavioral tasks. Thus, it could be proposed that deficiency of SAM and GSH induced by *i*As exposure might play pivotal role in down-regulation of MBP expression in *i*As alone treated animals. Besides, the MBP down-regulation has been correlated with morphological distortions and increased vacuolization following exposure to metal mixture containing *i*As, lead and cadmium.[36] Alteration in the potentials and excitatory properties of nerve fibers following deficits in myelination could form the basis of deranged behavior observed in *i*As alone treated animals.

The properties of Curcumin as an antioxidant, anti-inflammatory and scavenger of ROS[37] could be considered as the basis for its protective action against *i*As induced toxicity. Ambegaokar and co-workers, (2003), reported the positive role

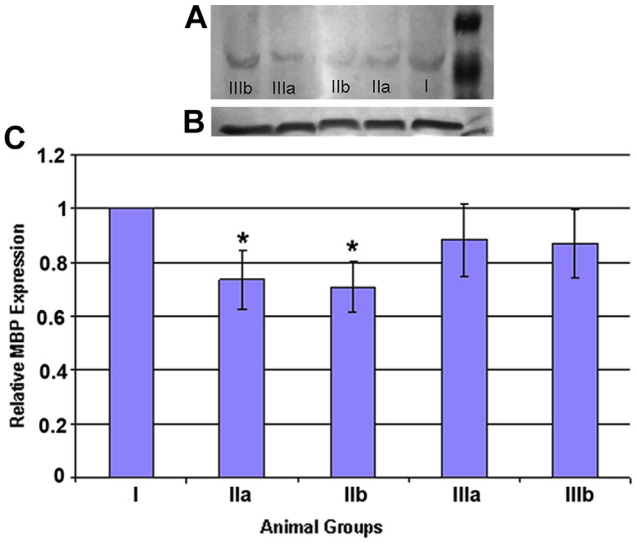

Fig. 6 – Immunoblots showing bands for MBP (A) and beta actin (B). Bar diagram showing the change in MBP expression with respect to (wrt) control (c).

of Curcumin in promoting oligodendrocyte differentiation and improving myelinogenesis in C-6 rat glioma 2B – clone cells.[38] The role of Curcumin in maintaining the cellular structure and tissue morphology is also reported.[39] These investigators reported Curcumin induced preservation of dendritic morphology of CA3 pyramidal neurons in the hippocampus of rats subjected to chronic restraint stress.[39]

Oxidative stress has been suggested as one of the important underlying factors in iAs induced toxicity.[10,40] Accumulation of iAs at tissue level results in generation of reactive oxygen species (ROS), predisposing the tissue to oxidative damage[10,12] on one hand and decreasing the antioxidant machinery on the other.[9] Besides, binding of iAs to the crucial thiol groups of various important proteins has also been reported.[41] Thus, it could be anticipated that accumulated cerebellar iAs could act in a multi-pronged manner by targeting the thiol groups of the proteins, utilizing antioxidant machinery and SAM for its metabolism, in turn generating oxidative stress and interfering with various other signaling pathways.[42]

Although it is difficult to trace the precise mechanism underlying Curcumin induced neuro-protection, yet the observations of the present study pertaining to up-regulation of MBP could be considered as one of the mechanisms underlying Curcumin induced beneficial effects on $NaAsO_2$ induced neurotoxicity in rats. It can thus be proposed that, the therapeutic potential of Curcumin can be utilized in the endemic areas where it could be administered as a dietary adjuvant based on its easy availability, cost efficiency and no reported toxic effects at the dose level used in the present study.

Conflicts of interest

All authors have none to declare.

Acknowledgment

This study was partially funded by Department of Science and Technology (DST INSPIRE Fellowship IF10053 – Parul Kaushal) and Department of Anatomy, AIIMS. Technical support of Mr. Kirpal Singh is acknowledged.

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