



# Alpha lipoic acid (ALA) modulates expression of apoptosis associated proteins in hippocampus of rats exposed during postnatal period to sodium arsenite ( $\text{NaAsO}_2$ )



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## ABSTRACT

The present study focused on the role of exogenous alpha lipoic acid (ALA) in amelioration of inorganic arsenic (*iAs*) induced effects on apoptosis and apoptosis associated proteins in developing rat hippocampus.  $\text{NaAsO}_2$  (1.5/2.0 mg/kg bw) alone or along with ALA (70 mg/kg bw) was administered to rat pups (experimental groups) by intraperitoneal (i.p.) route from postnatal day (PND) 4–15. Controls received no treatment/distilled water/ALA. On PND 16, the animals were perfusion fixed and the brains were processed for paraffin embedding (CV and TUNEL staining) and cryopreservation (immunohistochemistry). The fresh brain tissue was used for Western blotting. Significant increase was observed in TUNEL positive cells and Bax (pro-apoptotic protein) expression in hippocampal sub-regions of *iAs* alone treated groups, whereas Bcl-2 expression was intensified in animals receiving ALA with *iAs*. Densitometric analysis (Western blots) revealed optimal restoration of Bax and Bcl-2 ratio in animals receiving ALA with *iAs*, thereby suggesting the protective role of ALA in *iAs* induced developmental neurotoxicity.

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## 1. Introduction

Arsenic (As) is present ubiquitously in the environment. The vulnerability of the biological systems to *inorganic arsenic (iAs)* induced toxicity is evident from documented reports of carcinogenic manifestations, hyperkeratosis,

neuropathies, functional deficits of organ systems, etc. following exposure to *iAs* [1–4]. Millions of people worldwide are reported to be at risk of getting exposed to *iAs* by drinking water based on their inhabitation in areas adjacent to industrialization units dealing with *iAs* [5,6]. A number of *iAs* contaminated endemic areas have been identified across the globe, with countries like India, Bangladesh, Inner Mongolia, Taiwan, America (Michigan, Minnesota, South Dakota, Oklahoma, Wisconsin) etc. being toppers in the list [7]. *iAs* induced neurotoxic effects have been reported in human [8] as well as in animal studies [9]. Various reports in literature are suggestive of increased susceptibility of the juvenile central nervous system (CNS) to various environmental contaminants including *iAs* [10]. The severity of chemically induced pathological effects, especially in the developing nervous system (showing prolonged immaturity), vary according to the developmental

**Abbreviations:** ALA, alpha lipoic acid; *iAs*, arsenic;  $\text{Na}_2\text{AsO}_2$ , sodium arsenite; i.p., intraperitoneal; PND, post natal day; CV, cresyl violet; TUNEL, TdT mediated dUTP biotin nick-end labeling; CA1, CA2, CA3, cornu amonis subregions; DG, dentate gyrus.

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stage at the time of exposure [11,12]. The extrinsic as well as intrinsic environmental influences are likely to induce structural alterations and long lasting functional deficits in later life. The ability of arsenic to cross the blood–brain barrier [13] and get accumulated in the brain tissue [14] has previously been reported. This holds relevance since uncertainty prevails with context to efficacy of BBB during developing period. The rapid brain growth period (RBGP) extends along early postnatal period in rodents whereas in humans it extends from third trimester of pregnancy to early infancy [15]. During this period of rapid structural and functional reorganization, trillions of connections per day are established. Accordingly, the nervous system shows increased susceptibility during this period toward various environmental insults especially neurotoxins [2], thereby making the child population even more vulnerable to insult induced adverse effects both before and after birth. The study carried out on Mexican children [16] living in an area contaminated with *iAs* and *Pb*, showed a substantial influence of *iAs* on their verbal abilities whereas the attention process was more sensitive to *Pb* exposure. There are also reports of impaired cognitive function majorly associated with *iAs* exposure in children living in areas contaminated by both *iAs* and *Pb* [17]. The role of plasticity of the developing nervous system in determining the severity of insult induced adverse effects needs to be explored in depth.

Induction of oxidative stress by toxic stimuli assumes an important role in brain damage [18]. Under normal physiological conditions, the increased vulnerability of the nervous system to oxidative damage is associated with its higher rate of oxygen consumption along with rich polyunsaturated fatty acid content of neurolemma on one hand and the provision of lower levels of cellular defense mechanisms on the other [19]. The published data suggests a direct link between insult induced oxidative stress and altered neuronal characteristics (synaptic plasticity, cellular and dendritic morphology, neuronal networking, etc.) whereas supplementation of antioxidants (vitamins A, E and C) has been reported to modulate the redox system under these circumstances [20]. These facts are of paramount importance especially during the developmental periods, when the above mentioned processes are going on in full swing. Among various antioxidants, lipoic acid has specific antioxidant role in the CNS based on its ability to cross the blood–brain barrier and on its equal uptake by the central and the peripheral nervous system [21–23]. In our earlier study [24], the role of lipoic acid in modulation of oxidative stress markers and the subsequent reversal of the functional deficits to a substantial extent, was reported in the hippocampus of rats subjected to early postnatal exposure to NaAsO<sub>2</sub>.

The present study was designed to determine the ameliorating role of antioxidant ( $\alpha$ -lipoic acid, ALA) supplementation on adverse effects induced by *iAs* exposure (PND 4–15) on rat hippocampus. The information available till date regarding the protective role of antioxidants in arsenic induced developmental neurotoxicity continues to be patchy and insufficient. Also, the importance of translation of experimental toxicological studies to human health continues to stay.

## 2. Materials and methods

### 2.1. Animals and treatments

Approval for the experimental protocol was obtained from the Institute Animal Ethical Committee (IAEC-390/07). Timed pregnant Wistar rats, procured from Experimental Animal Facility (AIIMS), were housed in standard laboratory cages maintained in temperature (20–24 °C) and humidity (50–60%) controlled environment in a reverse 12 h light/dark cycle. The animals had free access to standard rodent diet (Ashirwad Industries, India) and water. For assessing the delivery status, the animals were checked daily at 10 am and the day of delivery was considered as postnatal day (PND) 0 for the pups. For random distribution, the pups across litter groups were pooled together (irrespective of sex) on PND 2 and distributed among the lactating mothers [25], ensuring that the litter size did not exceed six/lactating mother.

Group I (control group) – the animals received either no treatment (Nc) or distilled water (Sc) or ALA alone (AO).  
Group II (arsenic group) – the animals received 1.5 (IIa) and 2.0 (IIb) mg/kg bw NaAsO<sub>2</sub>.  
Group III (arsenic+ALA group) – the animals received NaAsO<sub>2</sub> and ALA (1.5 mg and 70 mg/kg bw – IIIa) and NaAsO<sub>2</sub> and ALA (2.0 mg and 70 mg/kg bw – IIIb).

NaAsO<sub>2</sub> (chemical purity of >98.5%) and ALA were procured from Loba Chemicals (India) and Sigma Chemicals (USA) respectively. LD<sub>50</sub> of NaAsO<sub>2</sub> when administered i.p. in adult Wistar rats has been reported as 15.86 mg/kg bw [26]. In the present study, 1.5 mg/kg bw NaAsO<sub>2</sub> (equivalent to 9.4%) was administered i.p., the effective dose (ED) being 1/10th of LD<sub>50</sub>. The other dose (2.0 mg/kg bw) was higher by 0.5 mg than the first dose. For the *iAs* + ALA groups, the dosage of ALA (70 mg/kg bw) was adopted from Shila et al. [18] and the substances were administered with half an hour interval between the two. To ensure the delivery of requisite amount of the substances and their effective uptake in the system, the intraperitoneal (i.p.) route was chosen. The exposure period extended from postnatal day (PND) 4–15, thereby covering the rapid brain growth period (RBGP).

Twenty-four hours after the last injection, the animals were anaesthetized and transcardially perfused fixed (4% paraformaldehyde in 0.1 M PB). The brains were dissected out and processed for paraffin embedding and cryopreservation. A coronal orientation was maintained for serial sectioning (Bregma – 3.2 mm to –3.8 mm) [27]. Paraffin sections (7  $\mu$ m) and cryocut sections (30  $\mu$ m) were subjected to routine CV, TUNEL and immunohistochemical staining respectively. The fresh hippocampal tissue obtained from animals sacrificed by cervical dislocation was processed for immunoblotting.

### 2.2. TUNEL assay

TUNEL (TdT-mediated dUTP biotin nick-end labeling) staining is used for identifying DNA strand breaks by labeling their free 3'-OH termini with dUTP (deoxyuridine

triphosphate nucleotides). TUNEL staining was carried out following manufacturer's protocol (Roche Diagnostics, Germany). The deparaffinized hippocampal sections were hydrated and incubated with proteinase-K (working solution – 30 µg/ml) for 15 min at room temperature (RT) and rinsed thrice with 0.1 M phosphate buffer saline (PBS). The sections were incubated in TUNEL reaction mixture (1:45) (In Situ Cell Death Detection kit, POD, Roche) in humid atmosphere at 37°C for 60 min in dark. After washing, converter POD was added for signal conversion followed by DAB (3,3'-diaminobenzidine) reaction in dark. In the mounted sections, the intensely dark brown stained nuclei were identified as TUNEL positive. Negative and positive controls were processed simultaneously with omission of enzyme in the TUNEL reaction mixture for the former and incubation of sections with DNase (Roche Diagnostics) prior to TUNEL mixture for the later.

TUNEL stained sections showing various subregions of Cornu Ammonis (CA1, CA3) and Dentate gyrus (DG) from either hemisphere were examined under the microscope (Nikon, E-600) fitted with Digital Camera System (DS-Fil-U2) and having attached Nikon Imaging Software (NIS Elements-AR 3.10) [28]. The first section was randomly chosen followed by every seventh section (42 µm) and a total of six sections per animal were analyzed [29]. A measured rectangular frame (250 µm × 40 µm) was superimposed (40×) on selected reference areas (three for CA1, two for CA3 and one each for ectal (DGEC) and endal (DGEN) limb of DG) for counting the neuronal profiles and leaving the cell bodies along the left and the lower margin of the frame (Forbidden line rule [30]). For TUNEL positive cells, dense dark brown nuclei were counted. The mean of positive neurons in the reference areas was taken as mean number of positive cells in that section and the mean of six such sections was taken as mean number of positive cells (mean number of cells/mm<sup>2</sup>) of that specimen [29].

### 2.3. Immunohistochemistry (Bax, Bcl-2)

Free floating immunohistochemical staining technique was adopted for localizing the expression of apoptosis associated proteins (Bax and Bcl-2). Cryocut coronal sections (30 µm) were incubated with mouse monoclonal anti Bcl-2 and anti Bax (Sigma, USA) for 72 h at 4°C (1:50) followed by further incubation with goat-anti mouse secondary antibody (SC 2050, Immuno Cruz TM Staining System, Santa Cruz) at 4°C. The antigen-antibody bound sites were visualized by 3,3'-diaminobenzidine (DAB) reaction (VECTOR-DAB staining kit). The sections were dehydrated, mounted and observed under the microscope. Negative and positive controls were processed simultaneously by carrying out incubation with species-specific normal serum instead of primary serum for the former and staining of cerebellar sections for the later.

### 2.4. Western blot analysis

Western blot analysis was carried out to determine the tissue levels of apoptosis associated proteins (Bax and Bcl-2). Fresh hippocampal tissue was weighed and collected

in lysis buffer (300 µl 1.0 M Tris-HCl; 15 µl 0.5 mM EDTA, 1.5 ml 1.0 mM phenylmethyl-sulphonyl fluoride (PMSF), 15 µl proteases inhibitors cocktail). Following sonication (VCX 500, Sonics, USA) of lysates, centrifugation (R280, Remi) (4°C, 30 min, 10,000 rpm) [31] was carried out. The supernatant was used for protein estimation [32]. For Western blot analysis, equal amount of protein ( $n=6$ /sub group) in buffer was mixed with equal amount of sample buffer (50 mM Tris-HCl with 25% glycerol and 10% SDS) and resolved on 12% SDS polyacrylamide gels using Gel Electrophoresis Apparatus (Mini PROTEAN Tetra Cell, Bio-Rad, USA). After electrophoretically transferring the separated proteins to nitrocellulose membranes (pore size 0.45) (Sigma Chemicals, USA), the gels were stained with Coomassie Blue to ascertain their complete transfer. After washing, the membranes were incubated in 3% BSA (bovine serum albumin) for 2 h at RT for blocking nonspecific binding [31] followed by incubation with specific mouse monoclonal primary antibodies for Bcl-2 and Bax (Santa Cruz, Biotechnology) at a dilution of 1:1000 (overnight, 4°C). Further incubation with HRP – conjugated goat – antimouse IgG (SC-2005, SantaCruz) (1:1000, 2 h, RT) was followed by treating the membranes with reaction mixture (substrate 0.01% H<sub>2</sub>O<sub>2</sub>) and chromogen DAB (SK 4100, Vector lab). Immediate scanning and photography was carried out to eliminate any signal loss and the differences in the background staining. Mouse monoclonal α-tubulin, 1:5000 (Abcam) was used as a loading control. Quantity 1 software of Gel Documentation System (Bio-Rad, USA) was used for densitometric analysis of the blots.

### 2.5. Statistical analysis

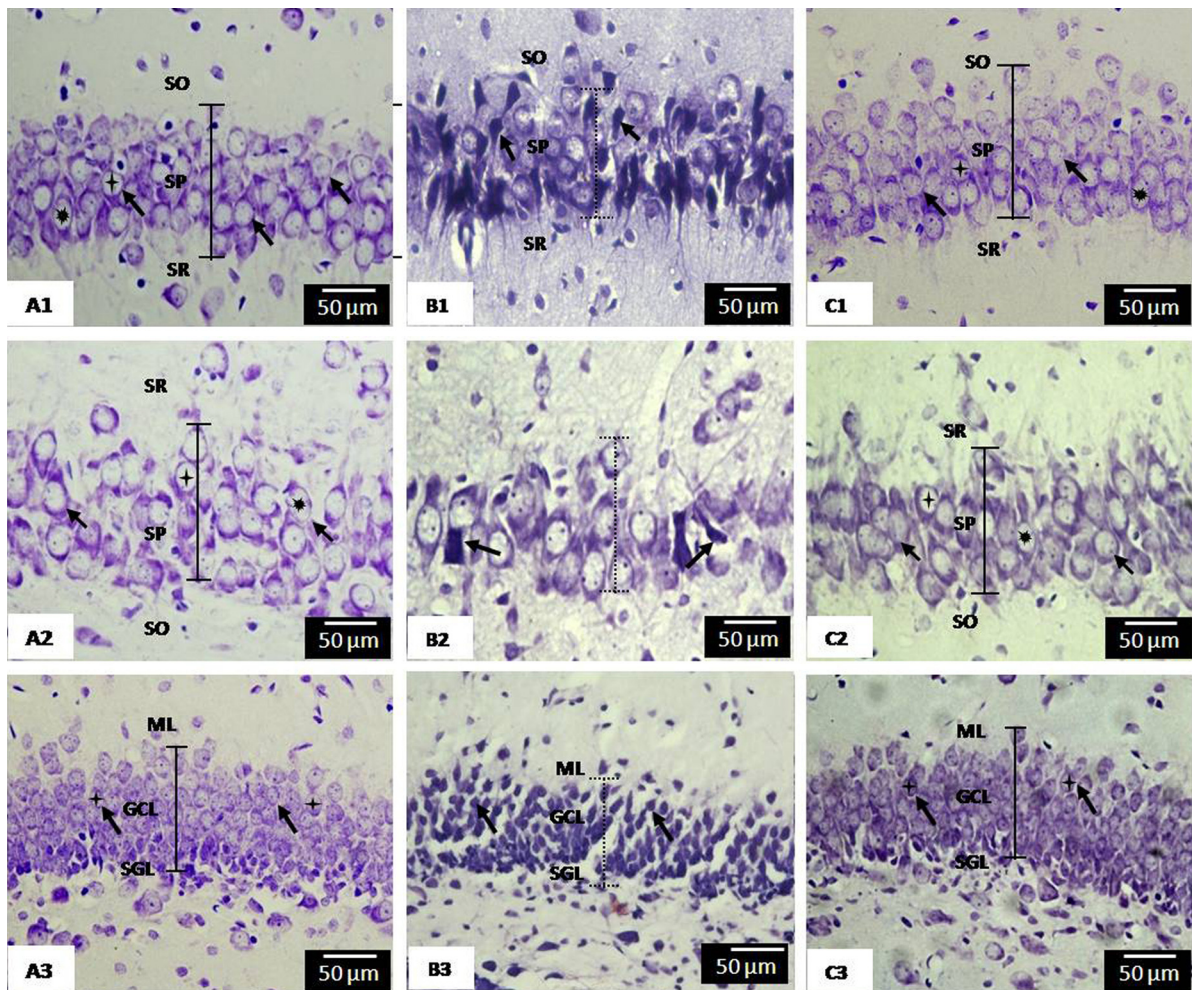
The data presented as mean ± SD was statistically analyzed by One Way Analysis of Variance (ANOVA) followed by Bonferroni correction using the software STATA 9.0 (College Station, TX, USA). *P* value <0.05 was considered significant.

## 3. Results

### 3.1. CV staining

Since the observations pertaining to various parameters were not significantly different across the control groups, the observations only of the normal control group (Nc) were included for comparison. The CV stained sections of hippocampus from control and experimental animals showed intact layering pattern such as stratum oriens (SO), stratum pyramidale (SP), stratum radiatum (SR), granule cell layer (GCL), sub granular zone (SGZ) (Fig. 1). Well organized pyramidal cells in SP of CA1, CA3 (Fig. 1A1, C1, A2 and C2) and granule cells in GCL of Dentate Gyrus Ectal limb (DGEC) (Fig. 1A3 and C3) were observed with preserved structural integrity such as discernable Nissl substance around the pale spherical/oval nuclei and adequate packing density of cells. Disruption in structural integrity (dissolution of Nissl substance and decreased neuronal packing density) of pyramidal cells (Fig. 1B1 and B2) and granule





**Fig. 1.** Photo micrographs of CV stained coronal sections of CA1, CA3 and DG (DGEC) from control (A1–A3); *iAs* alone (2.0 mg/kg bw) (B1–B3) and *iAs* + ALA (2.0 mg/kg bw) + ALA (C1–C3) treated groups showing well arranged 3–5 cell layer thick SP (†) having cells with thin rim of cytoplasm around large (✦)/oval (✧) nuclei and 1–3 small nucleoli. Disruption of pyramidal cell layers in SP (1–3 cell layer thick—.....), loosely packed pyramidal neurons (↘), with shrunken nuclei (↔) (B1 and B2). SO – stratum oriens; SP – stratum pyramidale; SR – stratum radiatum; ML – molecular layer; GCL – granule cell layer; SGZ – sub granule zone.

cells (Fig. 1B3) was evident in *iAs* alone treated groups (Table 1).

### 3.2. TUNEL staining

The intense dark brown staining of nuclei of pyramidal and granule cells was observed in SP (CA1, CA3) and GCL (DGEC) of *iAs* alone treated animals (Fig. 2B1–B3). The staining intensity of pyramidal and granule cell nuclei in SP (CA1, CA3) and GCL (DGEC) of *iAs* + ALA co-treated animals was evidently lighter (Fig. 2C1–C3), being comparable to those of the controls (Fig. 2A1–A3). These qualitative observations were substantiated by quantitative estimation of TUNEL positive cells/mm<sup>2</sup> in hippocampal subregions (CA1, CA3, DG) of *iAs* alone treated groups (Table 2) which was relatively higher than the number of such cells in ALA cotreated groups and the controls.

### 3.3. Bax expression

*iAs* induced Bax (pro-apoptotic factor) upregulation was evident by intense expression in the perikarya and dendritic processes of the pyramidal cells in SP of CA1, CA3 (Fig. 3B1 and B2) and granule cells in GCL of DG (Fig. 3B3). The upregulated Bax immunoreactivity was also evident in neuronal perikarya dispersed through SO, SR and SGZ. In hippocampal subregions (CA1, CA3, DG) of animal groups co-treated with *iAs* and ALA, Bax expression was less intense, being comparable to those of the controls (Fig. 3C1–C3, A1–A3).

### 3.4. Bcl-2 expression

The pyramidal and granule cells of various hippocampal subregions (CA and DG) of *iAs* alone treated groups presented an overall weak expression of BCL-2 (mainly



**Table 1**

Mean neuronal number (number of neurons/mm<sup>2</sup>) of pyramidal neurons in CA1, CA3 and granule cells in DG (DGEC, DGEN), of hippocampus in the control and the experimental groups (PND 16).

Groups	Mean ± SD			
	CA1	CA3	DGEC	DGEN
Sc (n = 6)	6936.5 ± 971.48	2926.16 ± 241.95	9761.33 ± 1009.99	11,969.17 ± 032.46
NaAsO <sub>2</sub> 1.5 (n = 6)	4077.16 ± 828.17	1976.66 ± 571.95	5919.66 ± 1277.48	7463.5 ± 1757.67
	( <i>p</i> < 0.001 <sup>*,b</sup> )	( <i>p</i> < 0.011 <sup>*,b</sup> )	( <i>p</i> < 0.001 <sup>*,b</sup> )	( <i>p</i> < 0.001 <sup>*,b</sup> )
NaAsO <sub>2</sub> 2.0 (n = 6)	3710.66 ± 762.58	1810.33 ± 201.99	5922.83 ± 890.06	7175 ± 1295.94
	( <i>p</i> < 0.001 <sup>*,c</sup> )	( <i>p</i> < 0.002 <sup>*,c</sup> )	( <i>p</i> < 0.001 <sup>*,c</sup> )	( <i>p</i> < 0.001 <sup>*,c</sup> )
NaAsO <sub>2</sub> 1.5 + ALA (n = 6)	6136.5 ± 961.55	2955.5 ± 481.75	9100 ± 1589.01	10,553 ± 1930.60
	( <i>p</i> < 0.009 <sup>*,d</sup> )	( <i>p</i> < 0.008 <sup>*,d</sup> )	( <i>p</i> < 0.003 <sup>*,d</sup> )	( <i>p</i> < 0.020 <sup>*,d</sup> )
NaAsO <sub>2</sub> 2.0 + ALA (n = 6)	6099 ± 1029.96	2833.33 ± 477.60	9363.83 ± 1166.74	10,261 ± 1416.14
	( <i>p</i> < 0.002 <sup>*,e</sup> )	( <i>p</i> < 0.005 <sup>*,e</sup> )	( <i>p</i> < 0.001 <sup>*,e</sup> )	( <i>p</i> < 0.020 <sup>*,e</sup> )
<i>p</i> <sup>a</sup>	0.0001 <sup>*</sup>	0.0001 <sup>*</sup>	0.0001 <sup>*</sup>	0.0001 <sup>*</sup>

<sup>a</sup> Results compared using one-way analysis of variance (ANOVA) followed by Bonferroni correction, CA1, CA3 and DG (pairwise comparison).

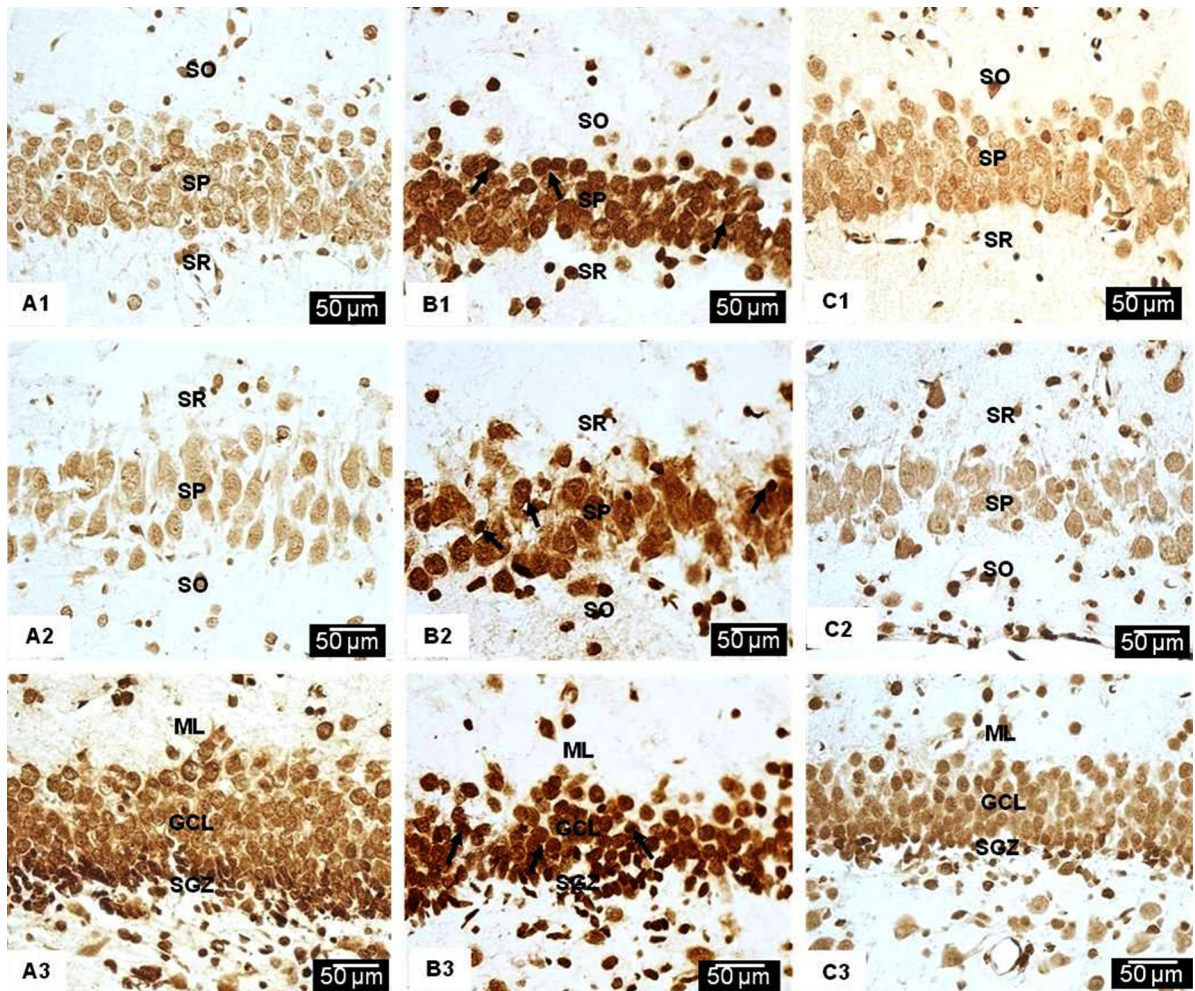
<sup>b</sup> Sc vs NaAsO<sub>2</sub> 1.5.

<sup>c</sup> Sc vs NaAsO<sub>2</sub> 2.0.

<sup>d</sup> NaAsO<sub>2</sub> 1.5 vs NaAsO<sub>2</sub> 1.5 + ALA.

<sup>e</sup> NaAsO<sub>2</sub> 2.0 vs NaAsO<sub>2</sub> 2.0 + ALA.

<sup>\*</sup> *p* < 0.05, statistically significant.



**Fig. 2.** Photo micrographs of TUNEL stained coronal sections of CA1, CA3 and DGEC from control (A1–A3); iAs alone (2.0 mg/kg bw) (B1–B3) and iAs + ALA treated groups (C1–C3). Intensely stained dark brown TUNEL positive pyramidal neurons (↑) in SP (B1 and B2) and granule cells (→) in GCL (B3). Light brown staining in C1–C3 is comparable to A1–A3.



**Table 2**  
Number of TUNEL +ve cells/mm<sup>2</sup> in CA1, CA3 and DG (DGEC, DGEN) of the control and the experimental groups (PND 16).

Groups	Mean ± SD			
	CA1	CA3	DGEC	DGEN
Sc (n = 5)	131.25 ± 11.13	123.69 ± 16.92	106.16 ± 16.97	111.33 ± 9.60
NaAsO <sub>2</sub> 1.5 (n = 5)	57.78 ± 4.97 ( <i>p</i> < 0.001 <sup>*,b</sup> )	58.80 ± 10.42 ( <i>p</i> < 0.001 <sup>*,b</sup> )	52.12 ± 6.34 ( <i>p</i> < 0.001 <sup>*,b</sup> )	57.81 ± 5.05 ( <i>p</i> < 0.001 <sup>*,b</sup> )
NaAsO <sub>2</sub> 2.0 (n = 5)	53.54 ± 3.75 ( <i>p</i> < 0.001 <sup>*,c</sup> )	55.66 ± 9.20 ( <i>p</i> < 0.001 <sup>*,c</sup> )	46.38 ± 5.32 ( <i>p</i> < 0.001 <sup>*,c</sup> )	49.81 ± 8.76 ( <i>p</i> < 0.001 <sup>*,c</sup> )
NaAsO <sub>2</sub> 1.5 + ALA (n = 5)	109.31 ± 11.61 ( <i>p</i> < 0.001 <sup>*,d</sup> )	110.59 ± 12.93 ( <i>p</i> < 0.001 <sup>*,d</sup> )	98.93 ± 5.47 ( <i>p</i> < 0.001 <sup>*,d</sup> )	102.5 ± 6.32 ( <i>p</i> < 0.001 <sup>*,d</sup> )
NaAsO <sub>2</sub> 2.0 + ALA (n = 5)	108.26 ± 19.65 ( <i>p</i> < 0.001 <sup>*,e</sup> )	105.96 ± 19.60 ( <i>p</i> < 0.001 <sup>*,e</sup> )	101.56 ± 11.37 ( <i>p</i> < 0.001 <sup>*,e</sup> )	103.84 ± 14.66 ( <i>p</i> < 0.001 <sup>*,e</sup> )
<i>p</i> <sup>a</sup>	0.0001 <sup>*</sup>	0.0001 <sup>*</sup>	0.0001 <sup>*</sup>	0.0001 <sup>*</sup>

<sup>a</sup> Results compared using one-way analysis of variance (ANOVA) followed by Bonferroni correction, CA1, CA3 and DG (pairwise comparison).

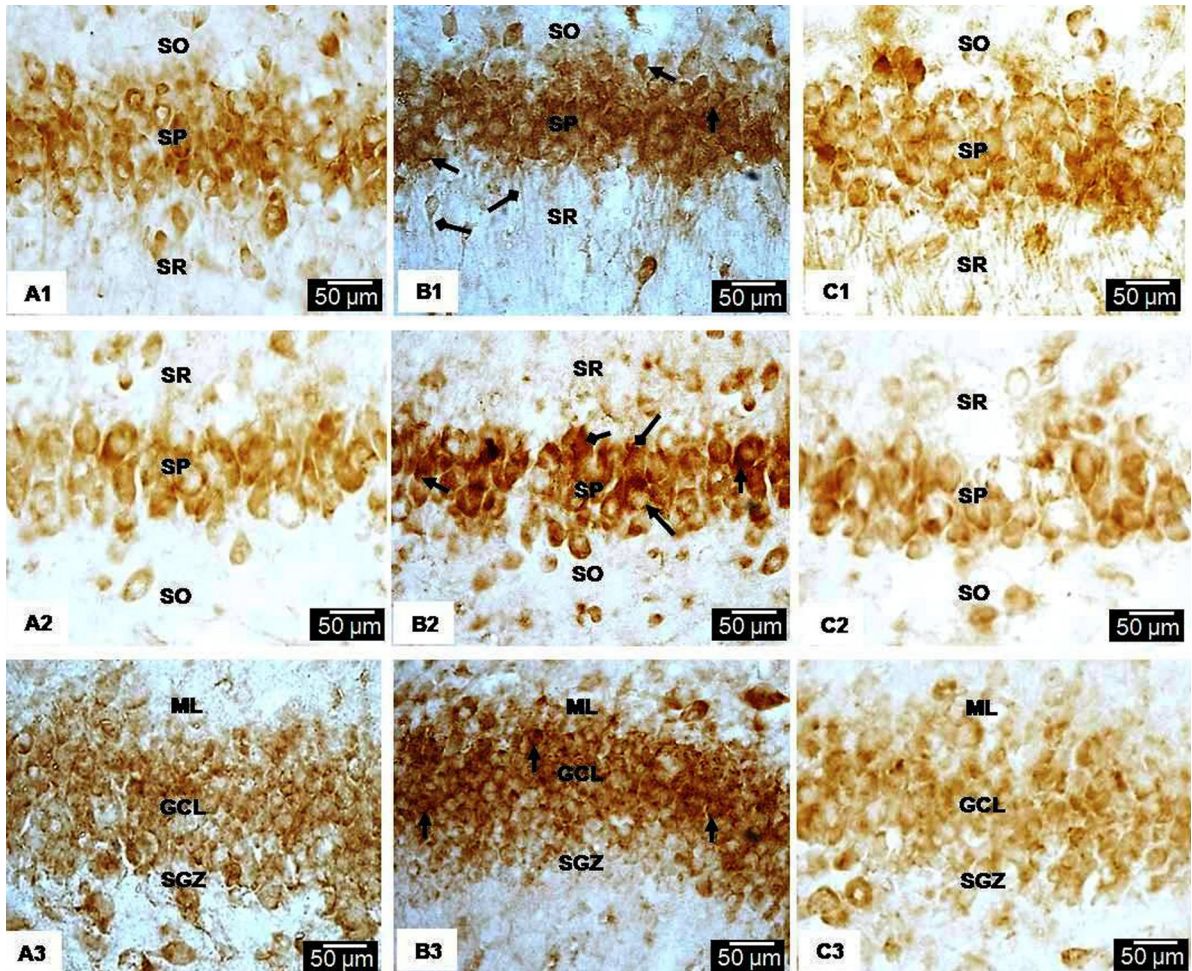
<sup>b</sup> Sc vs NaAsO<sub>2</sub> 1.5.

<sup>c</sup> Sc vs NaAsO<sub>2</sub> 2.0.

<sup>d</sup> NaAsO<sub>2</sub> 1.5 vs NaAsO<sub>2</sub> 1.5 + ALA.

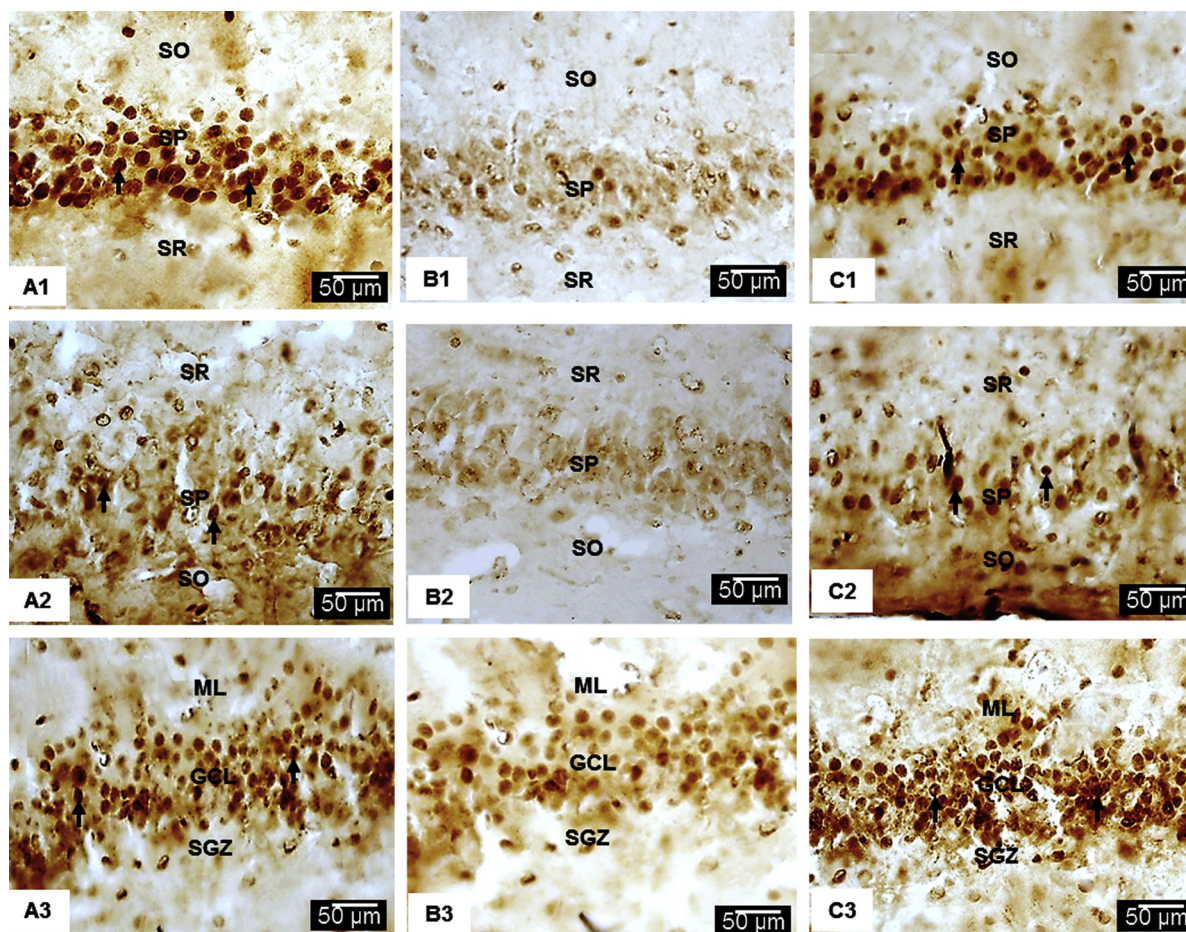
<sup>e</sup> NaAsO<sub>2</sub> 2.0 vs NaAsO<sub>2</sub> 2.0 + ALA.

\* *p* < 0.05, statistically significant.



**Fig. 3.** Coronal sections of CA1, CA3 and DGEC from control (A1–A3); *i*As alone (2.0 mg/kg bw) (B1–B3) and *i*As + ALA treated groups (C1–C3) showing intense expression of Bax in neuronal perikarya (→) and processes (↗) of pyramidal and granule cells in experimental groups (B1–B3). The intensity of Bax expression in C1–C3 is comparable to A1–A3.





**Fig. 4.** Coronal sections of CA1, CA3 and DGEC from control (A1–A3); *iAs* alone (2.0 mg/kg bw) (B1–B3) and *iAs*+ALA treated groups (C1–C3) showing intense expression of Bcl-2 in pyramidal cell nuclei (→) (C1 and C2) and granule cell nuclei (→) (C3), the intensity being comparable to A1–A3. Bcl-2 is faintly expressed in pyramidal cell nuclei (B1 and B2) and granule cell nuclei (B3).

confined to the nuclear region) with some predominance in the SP (CA1) and a somewhat uniform distribution in SR, SP, SO (CA3) (Fig. 4B1 and B2). Weak Bcl-2 expression was also evident in SGZ and GCL (DGEC). However, Bcl-2 expression was more intense across hippocampal subregions of animals co-treated with ALA and *iAs*, the intensity being comparable to that of the controls (Fig. 4C1–C3, A1–A3).

### 3.5. Western blotting

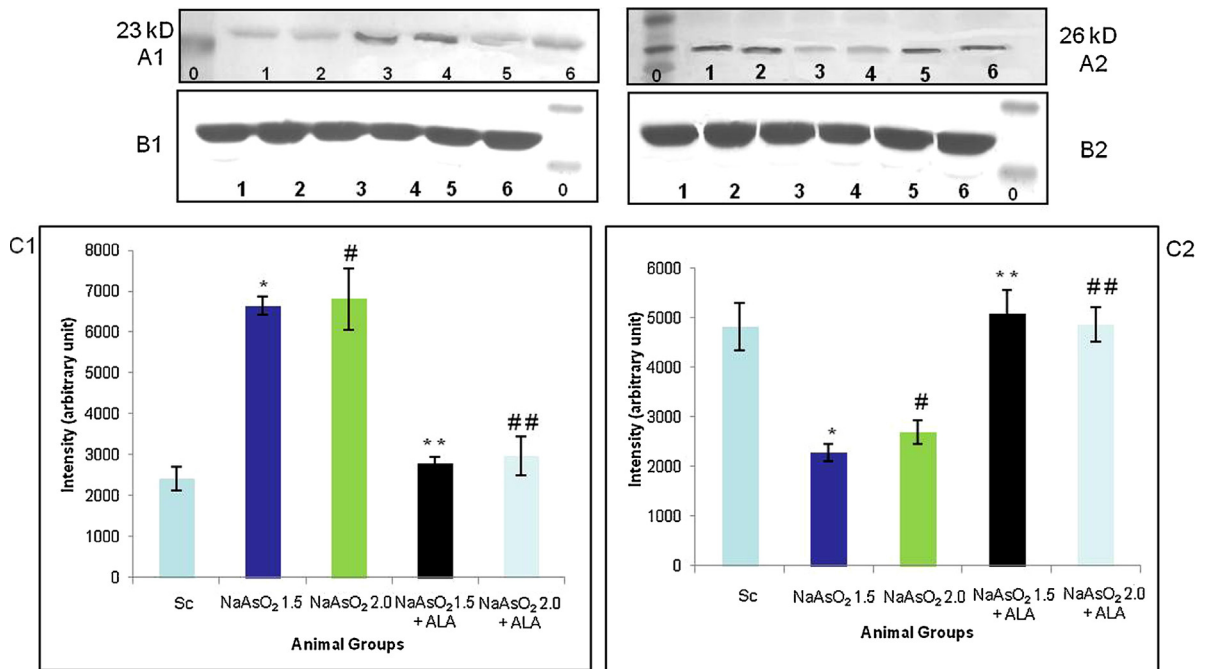
The immunoreactive bands for Bax and Bcl-2 were seen corresponding to 23 and 29 kDa proteins respectively. In *iAs* alone treated groups, the Bax expression was more intense whereas in *iAs* and ALA co-treated groups, the intense banding corresponded to BCL-2 expression. The densitometric analysis in the respective group bands showed a significant and marginally dose dependent increase in Bax expression in *iAs* alone treated groups whereas enhanced Bcl-2 expression was evident in the animals receiving ALA with *iAs* (Fig. 5A2 and C2).

The Bax:Bcl-2 ratio based on densitometric values for all bands presented a shift toward Bax (Bax ~ 75%, Bcl-2 ~ 25%) in *iAs* alone treated groups, thus substantiating *iAs* induced

up-regulation of Bax. The shift in the anti and pro apoptotic protein ratio toward Bcl-2 (Bcl-2 ~ 66%, Bax ~ 34%) in animals co-treated with *iAs* and ALA suggested ALA induced upregulation of Bcl-2.

## 4. Discussion

While there are reports of *iAs* induced neuronal cell death *in vitro* [33], the present study focused on *iAs* induced adverse effects on apoptosis associated proteins and evaluated the role of simultaneous administration of ALA on these proteins *in vivo*. Our results showed that cotreatment with ALA decreased the number of TUNEL positive cells and induced modulatory effects on apoptosis associated proteins (Bax, Bcl-2) in hippocampus of rats exposed to *iAs* from PND 4 to 15. This modulatory influence of ALA on apoptosis associated proteins as judged by the upregulation of Bcl-2 and downregulation of Bax, suggests positive influence of ALA on cell survival which could be attributed to its ability to activate various neuroprotective and repair pathways. One of the accepted phenomenon underlying ALA induced amelioration is linked to its role in neutralizing insult induced ROS and maintaining cortical and



**Fig. 5.** Immunoblots showing increased intensity of Bax expression in lanes 3 and 4 (A1) and Bcl-2 expression in lanes 5 and 6 (A2); Molecular weight marker lane 0. Band of  $\beta$ -tubulin as loading control (B1 and B2). Nc and Sc (lanes 1 and 2); *iAs* 1.5 and 2.0 (lanes 3 and 4); *iAs* 1.5 + ALA and 2.0 + ALA (lanes 5 and 6). Comparison by Kurskal–Wallis test followed by Bonferroni correction (pairwise comparison). \* Sc vs *iAs* 1.5 ( $p < .001$ ), # Sc vs *iAs* 2.0 ( $p < .001$ ), \*\* *iAs* 1.5 vs *iAs* 1.5 + ALA ( $p < .001$ ), ## *iAs* 2.0 vs *iAs* + ALA ( $p < .001$ ).

hippocampal GSH levels in compromised situations such as ischemia and exposure to toxic insults such as *iAs* [24,34]. A number of reports do suggest the role of antioxidant supplementation in slowing and/or stopping the cells from dying though the existent neuronal loss might persist [28].

The mitochondrial membrane potential is largely regulated by the balancing equation of pro- and anti-apoptotic proteins of BCL-2 family [35] and in turn, these proteins are fairly sensitive to toxic insults [36,37]. Upregulation of Bax mRNA and proteins has been reported to precede cell death in selectively vulnerable neurons of hippocampus and substantia nigra following ischemic insults and neurotoxin (MPTP) administration [38,39]. A number of investigators [40] have proposed insult induced changes in Bax-BCL-2 genes, leading to distorted balance between activators and suppressants of cell death. The optimal balance of these molecules in individual neurons has been reported to act as the deciding factors in determining cell fate [41].

In the present study, ALA induced Bcl-2 upregulation in association with decreased number of TUNEL positive cells was observed in hippocampal subregions of animals cotreated with *iAs* and ALA. These observations were supported by the results of immunoblotting which showed substantial increase in the intensity of Bcl-2 bands in the ALA cotreated animals, the intense expression of Bcl-2 could be linked to ALA induced enhancement of Bcl-2 gene expression.

A number of studies have suggested the role of antioxidants in modulation of Bax:Bcl-2 ratio by targeting diverse signaling pathways [42–44]. The earlier investigations,

focusing on the effects of traumatic brain injury especially ischemic insults [37,45,46] have reported altered Bax:Bcl-2 ratio as the underlying factor for increased apoptosis. Das et al. [47] reported taurine induced significant reversal of Bcl-2 and Bax gene in liver of Swiss albino male rats exposed to As (2 mg/kg bw) for 6 months. Das and coworkers associated the ability of taurine to target ROS production at mRNA level via JNK MAP K signaling pathway. Singh et al. [48] noted *iAs* (1, 10, 100  $\mu$ M, 48 h) induced increase and decrease in Bax and Bcl-2 expression respectively in murine embryonic maxillary mesenchyme (MEMM) cultures obtained from mice embryos (gd 13) exposed on gd 7 and 8 to sodium arsenate (20 mg/kg i.p.). Singh and coworkers further observed reversal of arsenate mediated oxidative injury by pretreatment with n-acetyl cystine (NAC). Lin et al. [43] studied the effect of melatonin supplementation on *iAs* induced apoptosis in CNS and noted upregulation of Bcl-2, active caspase-3 and cytosolic cytochrome-c in the infused substantia nigra and downregulation of dopamine in the nigrostriatal dopaminergic system. Based on these observations, Lin and coworkers suggested the therapeutic potential of melatonin in reversing *iAs* induced inhibition of mitochondrial and ER pathways. Pugazhenthii and coworkers [42] evaluated the role of antioxidant administration in oxidative stress induced downregulation of CREB dependent Bcl-2 expression and they arrived at the conclusion that antioxidant mediated over-expression of CREB could result in upregulation of Bcl-2, in turn laying the basis for cell survival. These above mentioned reports are suggestive of



beneficial effects of antioxidant supplementation based on their ability to modulate expression of apoptosis associated proteins under compromised situations.

The observations of our present study substantiate the potential of ALA (antioxidant) in upregulation of Bcl-2 expression and influencing the status of cell loss under conditions of *i*As induced neurotoxicity, thus providing protection to hippocampal neurons. ALA induced restoration of the optimal level of Bax:Bcl-2 ratio (1:2) presumably is the key factor in pushing the cells toward survival, thus strongly supporting the view of earlier investigators [41] pertaining to importance of maintained optimal balance of Bcl-2 family proteins as an important step in ensuring cell survival.

### Conflict of interest

The authors declare that there are no conflicts of interest.

### Transparency document

The [Transparency document](#) associated with this article can be found in the online version.

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