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Impact of Perinatal Arsenic Exposure on Amino Acid Neurotransmitters and Bioenergetics Molecules in the Hippocampus of Rats

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Abstract

Developmental neurotoxicity of Arsenic (As) is a major concern worldwide. High level As exposure is associated with several chronic diseases including adverse pregnancy and birth outcomes. However, very a lack of information on its ability to impair neurodevelopment at lower exposure. To date, there are very few animal studies during the perinatal period of As exposure. Although exposure to As induces developmental neurotoxicity, there is a lack of data regarding its specific effects on amino acid neurotransmitters and bioenergetics biomolecules in the hippocampus of developing rats exposed to As during the perinatal period (GD6-PD21). In continuation of previous studies, rats were exposed to As from gestational day (GD 6) through PD 21 with targeted doses of 0, 2.0, and 4.0 mg/kg/day, respectively. HPLC-UV method was used to estimate the level of amino acid neurotransmitters (aspartate, glutamate, homocysteine, glutamine, serine, and glycine) and the level of Adenosine 5'-Triphosphate (ATP), Adenosine Diphosphate (ADP), Adenosine Monophosphate (AMP), Nicotinamide Mononucleotide (NMN), Nicotinamide Adenine Dinucleotide (NAD^{+}), reduced Nicotinamide Adenine Dinucleotide (NADH) in the hippocampus of rats after the exposure of As. Amino acid neurotransmitter levels, a predictive biomarker of As-induced developmental neurotoxicity were found to be altered. ATP, ADP, and AMP were also significantly impaired in the hippocampus of As-exposed rats. We have observed that the hippocampus is susceptible to As toxicity, both because of the high energy depletion and the alterations in the levels of selected amino acid neurotransmitters. Taken together, our results indicate that perinatal As exposure appears to be critical and vulnerable.

Keywords: Amino Acid Neurotransmitters, Arsenic, Bioenergetics Molecules, Developmental Neurotoxicity, High-Performance Liquid Chromatography

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1. Introduction

There is no clear mechanism for the developmental neurotoxicity of As still to be explored. On the other hand, various scientific studies have demonstrated that chronic exposure to As during the developmental period affects the growth, neurobehavioral and neurological functions in the early stage of life, which may either continue or develop neurological diseases in the later stage of life[1-3]. Furthermore, impaired Intelligence Quotient (IQ), learning, and memory have been linked with As-exposed children even below the permissible limit[4,5]. As crosses the placental and blood-brain barrier and accumulates in the organisms, further it engenders neurodevelopmental, and cognitive dysfunctions and other developmental toxic effects[6-9]. The presence of As and its metabolites in the urine of pregnant women and the umbilical cord blood indicates that As easily crosses the placental barrier and reaches the fetus[10,11]. Using a rat model, earlier, we have reported that As reaches the brain and accumulates in the various regions of the brain such as the frontal cortex, hippocampus and corpus striatum in the prenatal and postnatal exposed rats at lower doses of As[12]. Therefore, exploring the mechanisms underlying the developmental neurotoxicity from As contaminated water was of the utmost interest to researchers. Despite this, As induced develop there is very little research on the developmental neurotoxicity caused by As. In animals, long-term exposure to As during critical periods of brain development has been found to contribute to learning and memory impairments by affecting synaptic functional plasticity and long-term potentiation in the hippocampal region, as well as changes in central nervous system neurotransmitters. Amino acid neurotransmitters play specific, but interconnected, roles for excitatory and inhibitory neurotransmission in the central nervous system[13,14]. However, some of the amino acid neurotransmitters are closely linked with motor control cognition and various pathological conditions of the central nervous system[15]. In addition, due to the interconnected network of amino acid transport systems in the central nervous system, alterations in the levels of some amino acid neurotransmitters could affect others as well. Further, amino acid neurotransmitter levels are closely associated with susceptibility to developmental neurological dysfunctions. ATP, a co-transmitter, is released at the same time as a neurotransmitter to the proper action of the neurotransmission process, and its release together with the Gamma-Aminobutyric Acid (GABA) Acetylcholine, norepinephrine, glutamate, and neuropeptide[16]. Other bioenergetics molecules including Adenosine Monophosphate (AMP), Adenosine Diphosphate (ADP), Nicotinamide Mononucleotide (NMN), oxidized and reduced Nicotinamide Adenine Dinucleotides (NAD+ or NADH) have a major role in neuromodulation in the central and peripheral nervous system. In addition, these bioenergetic molecules maintain the homeostasis of the nervous system and work as extracellular messengers to regulate cell function[17,18]. Amino acid neurotransmitters and bioenergetics molecules have a role in the neurotransmission process and maintain the homeostasis of the nervous system during brain development[19-21]. Variations in the levels of glutamine, serine, aspartate, homocysteine, glutamate, glycine, and γ-aminobutyric acid were reported in the various studies performed on the rats[22-24]. Further, various scientific studies have reported alterations in the levels of ATP, ADP, AMP, NAD, NADH, and NMN associated with neurotransmission decline.

To investigate the developmental neurotoxicity during the developing period of the brain, we investigated the variations in the amino acid and bioenergetics molecules in the hippocampus of As-exposed rats. At present, the effect of As at low doses on bioenergetics molecules and amino acid neurotransmitters has not been reported. In this research, alterations in selected amino acid neurotransmitters and bioenergetics molecules were measured by high-performance liquid chromatography in the hippocampus of As-treated rats. Therefore, the present study is very important to understand the mechanism of developmental neurotoxicity induced by As.

2. Materials and Methods

2.1 Animal and Treatment

2.1.1 Perinatal Exposure of Rats to Sodium Arsenite

Rats of the Wistar strain obtained from the central animal breeding colony of CSIR-Indian Institute of Toxicology Research (CSIR-IITR), Lucknow, were used for the study all through. Rats were housed in experimental rooms at 25±2 ºC with a 12-h light/dark cycle under standard hygiene conditions. Animals had free access to pellet diets arranged through the national suppliers and water *ad libitum*. The institutional animal ethics committee of CSIR-IITR, Lucknow (Ref No. ITRC/ IAEC/35/11-41/12)

approved this study and all experiments were carried out according to the guidelines approved by the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forests (Government of India), New Delhi, India.

Adult female rats of proven fertility were kept for mating with the males in a ratio of 1:1. The day of appearance of the vaginal plug was considered as day one (GD-1) of gestation and the pregnant rats were separated into individual cages. Pregnant rats were divided into three groups (6 rats in each group) and treated with sodium arsenite (2 mg/kg or 4 mg/kg p.o.) on day sixth of gestation (GD-6) till Postnatal Day (PD) 21 as follows.

Group I- Pregnant rats treated with sodium arsenite (2 mg/kg body weight, p.o.) dissolved in distilled water once daily from GD6 to PD21.

Group II- Pregnant rats treated with sodium arsenite dissolved in distilled water (4 mg/kg body weight, p.o.) once daily from GD6 to PD21.

Group III- Pregnant rats treated with normal saline (p.o.) identically as in groups I and II and served as controls.

All pregnant rats were allowed to deliver naturally and rear their pups to weaning until PD21. The litters were culled to 8 pups/litter in each case till parturition. Male rats in each group were sacrificed on PD22 to assess the neurochemical alterations. The brain was taken out quickly, washed with ice-cold saline, and dissected out the hippocampus following the standard procedure as described by Glowinski and Iversen[25]. The dissected hippocampus was stored at -80°C for neurochemical assays.

2.2 Determination of Amino Acid Neurotransmitters in the Hippocampus Brain Tissue

The standards of aspartate, glutamate, homocysteine, glutamine, serine, glycine (amino acid neurotransmitters) and dansyl chloride (derivative reagent) were purchased from Sigma Chemical Corporation (SIGMA-USA). Levels of amino acid neurotransmitter (aspartate, glutamate, homocysteine, glutamine, serine, and glycine) in the hippocampus was measured following the procedure as described by Huo *et al.*[26] Briefly, hippocampus tissue samples were weighted and homogenized in acetonitrile (2mL/0.1gm tissue). The homogenized sample was centrifuged at 13,000 rpm for 10 min at 4°C. The supernatant was collected and evaporated to dryness at 40°C under a gentle stream of nitrogen. Na_2CO_3–$NaHCO_3$ buffer (400 µL, pH 9.5) was added to the dried residue. In the next step, dansyl chloride (200 µL, 10 mmol/L) was added and mixed with the help of a vortex and incubated in dark condition for 25 min at 65°C then cooled to room temperature. Amino acid analysis was carried out on a Shimadzu Reverse-Phase-High-Performance Liquid Chromatography (RP-HPLC) system module with system controller (CBM-20A), chromatographic pump (LC-20AD), column oven (CTO-20AC), UV-VIS detector (SPD-20A), and reversed-phase C18 column (250×4.6 mm^2, particle size 5 mm). Na_2HPO_4–NaH_2PO_4 buffer solution (pH 7.2, A) and acetonitrile (B) mobile phase were used in this method with a flow rate of 1.0 mL/min. The mobile phase was filtered by using a Nylon filter (0.22 µm) and degassed under an ultrasound sonicator for 20 min. The column was eluted with a linear gradient of 14–22 % B over 0-12 min, 22–28 % B over 12-30 min and held at 28% B for 13 min. Detection of the amino acid neurotransmitter was monitored at 254 nm and the injection volume was 20 µL. The hippocampus (0.1g) tissue of the brain was spiked with the standard amino acid solution for the calibration graph and the Limit of Quantification (LoQ) of the amino acid standard was defined as follows: aspartate: 43 ppb, glutamate: 50 ppb, homocysteine: 26 ppb, glutamine: 53 ppb, serine: 32 ppb, and glycine: 20 ppb.

2.3 Determination of Energy Biomolecules in the Hippocampus Brain Tissue

All standard solutions were prepared in water from the pure compound (Sigma-USA). ATP, ADP, AMP, NMN, NAD^+ and NADH levels were estimated by using RP-HPLC[27,28]. pre-chilled perchloric acid was added to hippocampus tissue (1:30 ratio, tissue/perchloric acid) and homogenized. Homogenate was kept on ice for 15 minutes and centrifuged (15000 rpm) for 5 min at 4°C. The supernatant was taken in another tube carefully and transferred to a new sample tube. The supernatant was neutralized with 3M potassium carbonate solution (pH 6.0–7.0, 1:10 ratio, potassium carbonate/supernatant). The neutralized extract was centrifuged at 15000 rpm) for 5 min at 4°C. The supernatant was collected and stored at -80°C and used for the determination of ATP, ADP, AMP, NMN, NAD+ and NADH by RP-HPLC. RP-HPLC instrument specifications are the same as those mentioned in the above method. The mobile phase was composed of 0.05 M Phosphate Buffer (pH 7, A) and methanol (B). A nylon filter (0.22 µm) was used to vacuum filter the mobile phase and degassed under an ultrasound sonicator for 20 min. Vials were placed in the

auto-sampler and a 100µL sample injected into the sample loop using a gradient elution system (0.05 M Phosphate Buffer) from 0-2 min, a linear gradient to 95% buffer A/5% buffer B (100% methanol) from 2-4 min, 95% buffer A/5% buffer B from 4-8 min, a linear gradient to 85% buffer A/15% buffer B from 8-10 min, 85% buffer A/15% buffer B from 10-16 min, a linear gradient to 100% buffer A from 16-20) with a flow rate of 1 mL/min. The detection of the peak was monitored at 260 nm.

3. Statistical Analysis

Statistical analysis of the data has been carried out using the Graph Pad Prism -3.02 software. A one-way Analysis of Variance (ANOVA) was performed, followed by the Newman-Keuls test to compare all pairs of columns. The results are presented as mean ± SEM (n=6), and significance was determined at a threshold of $p < 0.05$.

4. Results

4.1 Effect on the Amino Acid Neurotransmitters in the Hippocampus Brain Tissue

The level of selected amino neurotransmitters was evaluated in all groups of rats, where rats were exposed to As at low doses during the developing period of the brain (GD6 to PD21) on PD22. Levels of ASP and GLN were found to reduce in the arsenic-exposed rats on PD22 in comparison to controls. Moreover, the levels of ASP (48.23%, $p<0.05$) were found to significantly decreased at the higher dose of As exposed rats on PD22 as compared to controls. Significant higher levels of hippocampal GLU (57.79, $p<0.05$; 58.28, $p<0.05$) were observed in arsenic-exposed rats as compared to controls on PD22. GLY (17.68%, $p>0.05$; 54.56%, $p<0.05$) was found to increase in the hippocampus of rats at both doses of As. However, alterations in the levels of GLY were significant in rats exposed to arsenic at a higher dose (4.0 mg/kg body weight) on PD22 in comparison to respective controls. Elevated levels of GLU and GLY may cause excitotoxicity in the hippocampus of the developing brain. Further, HCY levels in the hippocampus were analyzed. As observed in Figure 1, levels of HCY (24.37, $p<0.05$; 32.94, $p<0.01$) in the hippocampus in both arsenic-exposed groups were significantly higher than in the control group on PD22. Moreover, no significant difference was observed in the levels of hippocampal SER of As-exposed rats as compared to controls.

4.2 Effect on the Energy Biomolecules in the Hippocampus Brain Tissue

To evaluate the effects of arsenic exposure on the energy biomolecules were assessed in the hippocampus of rats. Exposure to arsenic during the perinatal period (GD6-PD21) caused significant alterations in the levels of energy biomolecules. The hippocampus ATP levels in perinatal As-exposed rats on PD22 were significantly decreased to 8.93 Nano moles/gm tissue (As 2 mg/kg, 28.28%, $p<0.05$) and 6.46 (As 4 mg/kg, %, $p<0.01$) Nano moles/gm tissue from control levels of 12.45 Nano moles/gm tissue. Perinatal (GD6-PD21) exposure to As (4.0 mg/kg body weight) caused a significant decrease in ADP (32.8%,

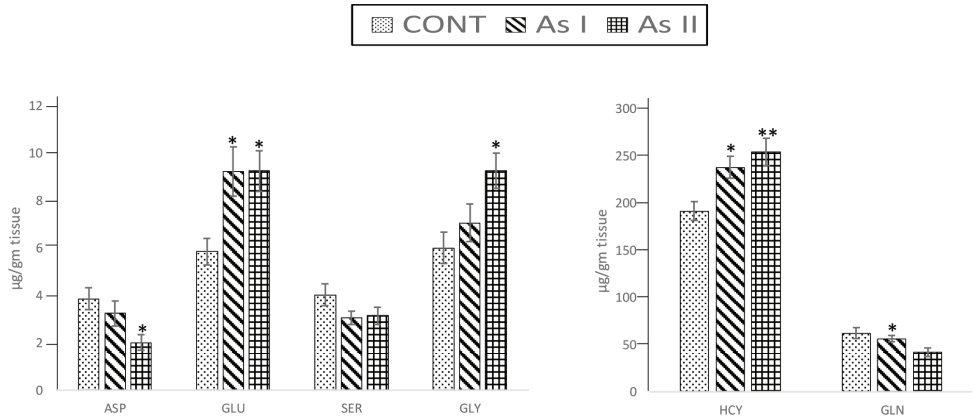


Figure 1. Levels of ASP, GLU, SER, GLY, HCY and GLN in the hippocampus of As-exposed rats on PD22. Values are the mean ± SEM of five animals in each group. Different from the control group, *$p < 0.05$, and **$p < 0.01$ were determined by one ANOVA with the Newman–Keuls test.

p<0.05) levels on PD22 as compared to controls. ADP levels were also reduced at a lower dose of As (As 2 mg/kg, 15.65%, p>0.05) on PD22, albeit reductions were not significant. The exposure of As from GD6 to PD21 resulted in a significant increase in the levels of AMP at a higher dose of As (17.31%, p>0.05; 38.59%, p<0.01) as compared to controls (Figure 2).

The levels of NAD+, NADH and NMN were measured. Further, based on the finding, no significant changes were observed in the levels of NAD+, NADH and NMN in the hippocampus of rats even at a higher dose of As on PD22 as compared to controls.

5. Discussion

Beyond amino acid's role in protein synthesis, amino acids have wide a range of roles in neurotransmission and central nervous system development. Additionally, due to their role in the synthesis of brain neurotransmitters, amino acids are closely linked to the vulnerability of the developing brain to various physiological and pathophysiological conditions of brain and cognitive decline. Therefore, the investigation of amino acids is very important to evaluate the effect of As on central nervous system dysfunction during the developing period of the brain.

Glycine (GLY) is the major inhibitory neurotransmitter in the central nervous system and is involved in cell migration and synaptogenesis in embryonic and postnatal brain development[29-32]. N-Methyl D-Aspartate (NMDA) receptors need GLY as a co-agonist with Glutamate (GLU) to facilitate excitation[33,34]. Elevated levels of GLY in the hippocampus of As exposed rats may indicate excitotoxicity in the developing brain. The glutamate/GABA-glutamine cycle is where Glutamine (GLN) plays an important role in the neurotransmission process. Alterations in the levels of GLN in the hippocampus of As exposed rats indicate a disturbance of the glutamate/GABA-glutamine cycle. The altered glutamate/GABA-glutamine cycle in the brain has also been reported in epilepsy and depression[35,36]. Glutamate (GLU) binds to ionotropic receptors i.e., NMDA, α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionic acid (AMPA), and kainate receptors and metabotropic GLU receptors, which are trigger different signaling cascades.[37-39] In As treated group at low doses (2.0 or 4.0 mg/kg body weight), significantly elevated levels of GLU may produce excitotoxicity and lead to neuronal cell death[40]. Aspartate (ASP), a secondary excitatory neurotransmitter in the central nervous system works as an agonist for NMDA receptors and regulates neurogenesis in the adult brain[41]. ASP also plays a major role in the development of the nervous system and hormone regulation i.e., prolactin and luteinizing hormone[42,43]. Significant levels of ASP in the hippocampus As treated rats at higher doses, which may disturb hormone regulation and NMDA receptor-mediated transmission and functions, indirectly contributed to the alteration in the learning memory of As-treated rats. Homocysteine (HCY) can trigger the secretion of other excitatory amino acids and contribute to excitotoxicity via an NMDA receptor-mediated pathway. Elevated levels of HCY, considered a neurotoxin, may be a risk factor for neurological disease and cerebrovascular disease[44]. Increased levels of HCY were found in the hippocampus of As exposed rats, which may associated with cognitive decline. SER, signalling molecules and neuromodulators have been linked with various neurodevelopment disorders[45].

ATP is a form of chemical energy, which plays a key role in ion transport, nerve impulse propagation and various

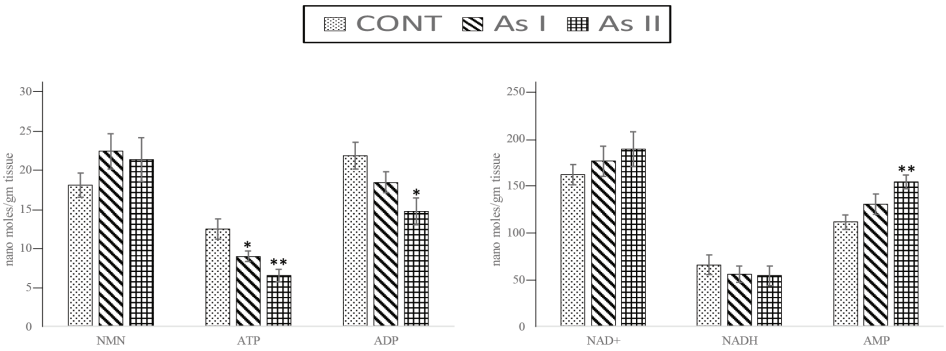


Figure 2. Levels of NMN, ATP, ADP, NAD+, NADH and AMP in the hippocampus of As-exposed rats on PD22. Values are the mean ± SEM of five animals in each group. Different from the control group, *p < 0.05, and **p < 0.01 were determined by one ANOVA with the Newman–Keuls test.

functions of the cells. ATP has a regulatory role in the various functions of the central nervous system. However, decreased levels of ATP in the hippocampus of As exposed rats may disturb the neurotransmission process and also develop pathological conditions including inflammation in the brain[46]. ADP is a bioenergetics molecule, that is combined with the phosphate group to make ATP, and it is also very essential for brain development. AMP, activated by the protein kinase pathways is associated with neurodegeneration and neuronal processes, such as synaptic transmission, neuronal proliferation, and differentiation. Increased levels of AMP may be associated with alterations in the differentiation, neuronal proliferation, and synaptic transmission during the critical period of brain development[47]. It may also be possible to hamper the 5′ AMP-activated protein kinase activity which can lead to the development of neurological disorders[48,49].

6. Conclusion

In conclusion, the present study demonstrated that As can impair energy metabolism, as indicated by the alterations in the ATP, ADP, and AMP levels. Depletion of the energy by ATP and other energetic biomolecule-mediated mechanisms may underline dysfunction in the hippocampus of As-exposed rats, even at low doses. Moreover, it is associated with the neurotransmission process and brain homeostasis in the developing brain. As inhibited the amino acid neurotransmitter in the hippocampus of rats during the developing period of the brain. Furthermore, changes in the levels of amino acid neurotransmitters may have an impact on the neurotransmission process, motor functions, and cognition and may also be linked with various pathological conditions of the central nervous system. We may conclude that the development neurotoxicity of As has been linked with perinatal exposure (GD 6 to PD21) to energy depletion and alteration of the level of amino acid neurotransmitters in the hippocampus of developing rats. As discussed in this research, the results of the present study will help to understand the central nervous system developmental risks of As exposure in pregnancy through weaning.

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