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Research Article
Acetylcholinesterase as a Biomarker of Arsenic Induced Cardiotoxicity in Mammals

[1]Vivek Kumar Gupta, [1]Abhishek Kumar, [1]Sarita H. Yadav, [2]Rashmi Pandey and [1]Bechan Sharma

[1]Department of Biochemistry, Faculty of Science, University of Allahabad, 211002 Allahabad, India
[2]Department of Pulmonary and Critical Care Medicine, KGMU Lucknow, 226001 UP, India

Abstract

Background and Objective: Arsenic is an environmental toxicant and it exerts its toxic effect through the inhibition of various enzymes. The toxicity of arsenic results due to its ability to interact with sulfhydryl groups of proteins and enzymes and to substitute phosphorus in various biochemical reactions. The main objective of this study was to determine the effect of arsenic trioxide on the activity of acetylcholinesterase. In present article, the endeavor has been made in order to assess the arsenic mediated cardiotoxicity *in vitro* by assaying the activity of AChE from the post nuclear supernatant (PNS) of rat heart treated in the presence of different concentrations of arsenic. **Materials and Methods:** The rat homogenate heart tissue (10% w/v) was made in sodium phosphate buffer (50 mM; pH 7.4) containing Triton X-100 (0.2% v/v) and another that did not contain detergent using Potter-Elvehjam homogenizer fitted with teflon coated pestle under the ice-cold condition. The post nuclear supernatant of rat heart tissue was prepared by homogenizing the tissue followed by the centrifugation of tissue homogenate at 9000 rpm for 30 min using Sigma 2-16KL refrigerated centrifuge at 4°C. Statistical analysis of the data was performed using GraphPad Prism and chi-square goodness of fit test. **Results:** The experimental findings indicated that the enzyme was localized in the membrane bound fraction and could be solubilized by using 0.2% Triton X-100. The enzyme was found to be stable up to 30 days when stored at -20°C in phosphate buffer (50 mM, pH 7.4) containing 0.2% Triton X-100. The AChE exhibited K_m, V_{max}, K_{cat}, catalytic efficiency and IC_{50} values computed to be 0.1287 mM, 0.073 mM sec^{-1}, 0.0017 sec^{-1}, 0.0160 $mM^{-1}sec^{-1}$ and 1.14 mM, respectively. **Conclusion:** Taken together, these results indicated that AChE from rat heart could be exploited as a biomarker of arsenic induced cardiotoxicity.

Key words: Arsenic, acetylcholinesterase, biomarkers, cardiotoxicity, mammalian heart

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Corresponding Author: Bechan Sharma, Department of Biochemistry, Faculty of Science, University of Allahabad, 211002 Allahabad, India Tel: +91-9415715639

Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Heavy metals are generally characterized as the inorganic elements having specific gravity 5 times of water's specific gravity. Among heavy metals listed in d-orbital, arsenic (As) is one of them which get accumulated into the liver. The mechanism of heavy metal pathogenesis has been reported that the exposure of these heavy metals may induce production of free radicals i.e. reactive oxygen species (ROS) and reactive nitrogen species (RNS)[1-3]. Free radicals have been reported to damage major biomolecules (oxidative DNA damage, oxidation of proteins and lipid peroxidation)[4,5] and the resultant chemical species may produce toxicity, cellular dysfunction and finally death of the cell. The heavy metals toxicity is largely associated to their reactions with sulfhydryl groups of the enzyme[6].

Arsenic is an environmental pollutant and a natural drinking water contaminant[7,8], exists in trivalent arsenite (As III) and pentavalent arsenate (As V) and the elemental arsenic (nontoxic) in the environment. As(III) is 10 times more toxic than As(V). Arsenic widely utilizes its three different oxidation numbers (+5, +3 and -3) to form chemical complexes with biomolecules present in the body[9]. There are certain arsenical drugs still used in treatment of tropical diseases such as amoebic dysentery, African sleeping sickness and also in veterinary medicine to treat filariasis in dogs and black head in chickens[10]. The inorganic arsenic can be absorbed by gastrointestinal tract of most animals. The analysis of arsenic induced toxicity is very complicated because it's toxicity depends on the oxidation state and many different inorganic and organic forms as well as their solubility[11]. Their accumulation in the body generally occurs through inhalation, sea food and absorption through skin which causes disorders in neurotransmission, gastro-intestinal tract, blood circulation system and respiratory system[2,3]. The chronic exposure of this heavy metal has been reported to be linked with various possible health effects including malignancies of skin and internal organs, hypertension, cardiovascular disease, neurological dysfunctions, reproductive disorder and pulmonary disease[8,9].

The exposure of arsenic is known to generate oxidative stress[12] via production of free radicals causing cytotoxicity and genotoxicity[13,14]. It could be due to generation of NO causing DNA damage and activating poly (ADP-ribose) polymerase (PARP)[14]. It influences the activity levels of enzymatic antioxidant elements such as [superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), heme oxygenase-1 (HO-1)] and the non-enzymatic factors (peptides containing sulfhydryl group and proteins) in human systems[2]. The liver has been well established as a major target organ for arsenic biotransformation[15]. The toxicity of arsenic adversely influences Krebs cycle and oxidative phosphorylation which results into the depletion of energy as well as rapid depletion of thiol group containing critical peptides and proteins[16]. In liver, As(V) is reduced to As(III) in a reaction dependent on glutathione (GSH) or other endogenous reductants. Arsenic has been reported to inhibit the complex I of mitochondrial electron transport chain, which results into the generation of ROS coupled with mitochondrial permeability transition (MPT) and thiol oxidation[17]. The exact molecular mechanism of arsenic induced toxicity is not well documented, but conceivably reflects differential damage to a number of cellular organelle systems and their biochemical functions.

Acetylcholinesterase or acetyl hydrolase (AChE, EC 3.1.1.7) is a serine protease that hydrolyzes the neurotransmitter acetylcholine to be acetyl Co A and choline (Fig. 1). Normally, AChE is found to be at neuromuscular junctions and cholinergic synapses but it has also been reported to be present in the membrane of erythrocytes and other organs (liver and muscles)[18-20] where its activity serves to terminate synaptic transmission. It is synthesized in the endoplasmic reticulum (ER) and exported towards the cellular surface[21]. In mammalian heart, AChE is distributed in purkinje fiber, vena cava, bundle of his, nodal tissue and ganglia. AChE is an enzyme which has been found to be in the brain and nervous tissues with high specific activity and readily detected in the membranes of muscles and erythrocytes[18-20]. The application of detergents, particularly the Triton X-100, a non-ionic detergent, is the most widely adopted solubilization methods for mammalian membrane bound AChE[22]. AChE has been the focus of much attention since it was first suggested that it plays an important role in the rapid destruction of acetylcholine (ACh) in a living organ[23], therefore, widely exploited as a primary target of action by organophosphorus compounds[20,24].

Since arsenic has been found to induce toxicity in mammals on accumulation and influence the functions of brain and other organs, it was imperative to evaluate the impact of arsenic on the biochemical behavior of AChE in the

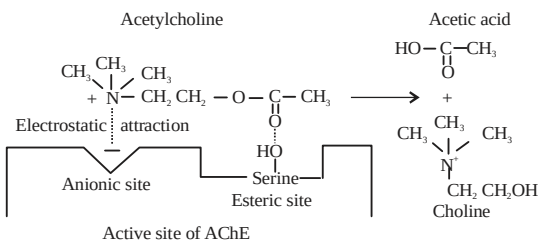

Fig. 1: Hydrolysis of acetylcholine (ACh) by AChE

PNS of rat heart in order to understand its mechanism of action *in vitro*. This study has endeavored to characterize AChE from the rat heart and to monitor its interactions with arsenic under different experimental conditions *in vitro*.

MATERIALS AND METHODS

This study was done at the Department of Biochemistry, University of Allahabad, Allahabad in the month of July, 2016.

Chemicals: S-acetylthiocholine iodide (ATI), Triton X-100 and 5, 5′-Dithio-bis (2- nitrobenzoic acid) (DTNB) were procured from Tokyo Chemical Industry Co. Ltd. Japan, Merck and SRL Pvt. Ltd. Mumbai, India, respectively. Arsenic trioxide (As_2O_3), bovine serum albumin (BSA), sodium dihydrogen orthophosphate and di-sodium hydrogen phosphate were obtained from Fisher Scientific. Folin-Ciocalteu's phenol reagent was purchased from Spectrochem Pvt. Ltd. Mumbai, India and all other chemicals were of analytical grade purity.

Animals: Healthy adult male albino rats of same age group (8 weeks), weighing between 200-210 g were used for experiments. Animals were obtained from CDRI, Lucknow, India. The animals were randomly selected and housed in propylene cages at temperatures of 25±2°C having relative humidity (50-70% humidity) with a 12:12 h dark/light cycle. The animals have free access to pelleted feed and fresh tap water. The animals were supplied with commercially available dry food pellets from (Dayal Industries, Lucknow, India). They were allowed to acclimate for 15 days before experiment. Protocols for care and maintenance of rats were strictly followed and the study had the approval from institutional animal ethical committee.

Excision of rat heart and preparation of post nuclear supernatant (PNS) from homogenate: The healthy rats were sacrificed using mild euthanasia followed cervical dislocation. The rat heart was quickly excised, washed with isotonic ice cold 0.9% (w/v) NaCl solution, blotted to dryness and weighed. The rat homogenate heart tissue (10% w/v) was made in sodium phosphate buffer (50 mM; pH 7.4) containing Triton X-100 (0.2% v/v) and another that did not contain detergent using Potter-Elvehjam homogenizer fitted with a teflon coated pestle under ice-cold condition. The homogenates were centrifuged at 9000 rpm for 30 min using Sigma 2-16KL refrigerated centrifuge at 4°C. The PNS was gently decanted and the pellets were reconstituted in equal volume of homogenizing buffer. Both the supernatants and the pellet's suspensions were used for the determination of AChE activity and protein estimation. Only PNS was used for determination of IC_{50}, effect of substrate, temperature, pH and storage time.

Protein estimation: The protein estimation was done using Folin-Ciocalteu's phenol reagent[25]. The bovine serum albumin (BSA) was used as a standard and the absorbance of blue colored complex was monitored at 620 nm.

Acetylcholinesterase assay: The AChE activity in the rat heart tissue was determined by the method of Ellman *et al.*[26]. The total reaction mixture volume (3 mL) contained 0.50 mM of ATI, 0.5 mM of DTNB and 50 mM phosphate buffer (pH 7.4) in quartz cuvette having 1 cm path length. The change in optical density (OD) was measured at λ_{max} 412 nm at the interval of 30 sec for 3 min. The AChE activity was calculated using extinction coefficient 13.6×10^3 M^{-1} cm^{-1} and expressed as µmoles of ATI hydrolyzed mL^{-1} min^{-1} or units (U). The specific activity of enzyme was expressed in U mg^{-1} of protein. The enzyme assays were performed on UV-Visible double beam spectrophotometer (Thermoscientific Spectroscan UV 2700). The catalytic activity was measured by the increase of yellow anion concentration, 5-Thio-2-nitrobenzoate produced due to reaction of thiocholine with 5, 5′-Dithio-bis-(2-nitrobenzoic acid) (DTNB). The assay system without enzyme/substrate was considered as an enzyme or substrate blank and any change in absorbance min^{-1} recorded in this condition were subtracted from the experimental observations.

Effect of pH on the rat heart AChE activity: The effect of pH on the activity of AChE from rat heart was estimated by assaying the enzyme activity using varying the pH of different buffer system (sodium acetate buffer for pH 3.6 and 5.0 sodium phosphate buffer for pH 6.3 and 7.4, carbonate buffer for pH 9.2 and 10.0) with constant enzyme concentration (109.2 µg) at room temperature (26±2°C). The enzyme was stored in phosphate buffer (50 mM, pH 7.4) containing 0.2% Triton X-100.

Effect of temperature on the activity of rat heart AChE: The effect of temperature was determined by assaying the activity at varying temperature (4, 10, 20, 37, 50 and 60°C) at pH 7.4 of phosphate buffer with constant enzyme protein (109.2 µg).

Effect of storage time at -20° C on the activity of AChE from rat heart: The effect of storage time was determined by assaying the activity of enzyme at the different time intervals at constant pH (50 mM phosphate buffer, pH 7.4), temperature (26±2°C) and the enzyme protein (109.2 µg).

Table 1: Extraction of rat heart acetylcholinesterase in different buffers

Fractions in phosphate buffer (50 mM, pH7.4)	Activity of AChE (μmole min^{-1} mL^{-1})	Protein (mg mL^{-1})	Specific activity (unit mg^{-1} of protein)
Supernatant with Triton-X 100 (0.2% v/v)	0.22	10.92	0.020
Supernatant without Triton-X 100	0.04	08.51	0.005
Pellet with Triton X-100 (0.2% v/v)	0.09	12.19	0.007
Pellet without Triton -X 100	0.05	13.50	0.003

One unit of the activity of AChE has been defined as the micromoles of substrate hydrolyzed min^{-1} mL^{-1}

Table 2: Effect of pH on the rat heart AChE activity

pH	AChE activity (μmole min^{-1} mL^{-1})
3.6	0.07
5.0	0.08
6.3	0.08
7.4	0.14
9.2	0.12
10.0	0.09

Effect of pH at room temperature (26+2°C) on the activity of AChE from rat heart was observed by carrying out the enzyme assay employing 109.2 μg protein at different pH of different buffers (sodium acetate buffer for pH 3.6 and 5.0, sodium phosphate buffer for pH 6.3 and 7.4, carbonate buffer for pH 9.2 and 10.0. The enzyme was stored in phosphate buffer (50 mM, pH 7.4) containing 0.2% Triton X-100

Table 3: Effect of temperature on the rat heart AChE activity

Temperature (°C)	AChE activity (μmole min^{-1} mL^{-1})
4	0.08
10	0.08
37	0.09
40	0.08
50	0.07
60	0.02

Effect of varying temperatures on the activity of AChE from rat heart was observed by carrying out the enzyme assay employing 109.2 μg protein at pH 7.4 of phosphate buffer. The enzyme was stored in phosphate buffer (50 mM, pH 7.4) containing 0.2% Triton X-100

Effect of substrate concentration on the activity of AChE: The kinetic parameters Michaelis-Menten constant (K_m) and maximum velocity (V_{max}), were estimated by assaying the enzyme activity using varying substrates concentrations ATI (from 0.00-1.00 mM) and constant enzyme concentration (109.2 μg) at room temperature (26±2°C).

Determination of IC_{50} value for arsenic: The enzyme (109.2 μg) was assayed in the presence of different concentrations of arsenic trioxide and the residual activity was monitored. The activity recorded in absence of arsenic was considered as 100%. The IC_{50} value was calculated by extrapolating the data taking percent residual activity on Y-axis and the varying arsenic concentrations at X-axis on a graph using GraphPad Prism (version 5.0) for windows.

Statistical analysis: Statistical analysis of the data was performed using GraphPad Prism (version 5.0) for windows. Chi-square goodness of fit test was used to decide the level of significance and considered significant at $p \leq 0.0001$. All values were expressed as mean standard deviation of three observations.

RESULTS

Membrane bound nature of AChE from rat heart tissue: The enzyme from heart tissue was localized into the membrane bound fraction of the heart tissue homogenate. It was solubilized using 0.2% (v/v) of a nonionic detergent, Triton X-100, in phosphate buffer (50 mM, pH 7.4). The extent of enzyme activity was recorded more in detergent solubilized fraction than that of without detergent. The protein contents in soluble fractions of these two preparations were also found significantly different. The Triton X-100 treated fraction contained 10.92 mg mL^{-1} protein as against 8.51 mg mL^{-1} protein present in the fraction without detergent. The pellet with Triton X-100 contained 12.195 mg mL^{-1} protein against 13.50 mg mL^{-1} in the fraction without treatment with Triton X-100, thereby showing solubilization and release of proteins from the pellet in presence of Triton X-100 (Table 1).

Effect of pH on the rat heart AChE activity: Effect of pH on the activity of AChE from rat heart was estimated by assaying the enzyme activity using varying the buffer systems of different pH (sodium acetate buffer for pH 3.6 and 5.0, sodium phosphate buffer for pH 6.3 and 7.4, carbonate buffer for pH 9.2 and 10.0) with constant enzyme concentration (109.2 μg) at room temperature (26±2°C) as mentioned in materials and methods. The enzyme was found to be optimally active at pH 7.4 when assayed using buffers of different pH systems under standard assay conditions (Table 2).

Effect of temperature on the rat heart AChE activity: The effect of temperature was determined by assaying the activity at varying temperature at pH 7.4 in the phosphate buffer with constant enzyme concentration (109.2 μg). The enzyme was stored in phosphate buffer (50 mM, pH 7.4) containing 0.2% Triton X-100. When this enzyme was assayed at varying temperatures, it has maximum activity at 37°C followed by gradual loss in its activity after increasing temperature (Table 3).

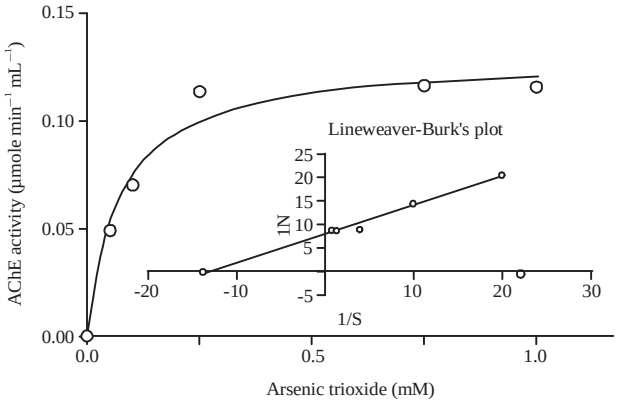

Fig. 2: Effect of substrate (ATI) on the activity of AChE from rat heart was observed by assaying the enzyme at varying substrate concentration ATI at room temperature (26+2°C) employing 109.2 μg protein. The K_m and V_{max} values were calculated using the intersection of the straight line at Y-axis and at the negative abscissa on X-axis, respectively

Table 4: Stability of AChE activity from rat heart

Days	AChE activity (%)
0	100.00
7	100.00
14	99.80
21	99.80
28	99.50
30	99.50

Effect of storage time at -20° C on the activity of AChE from rat heart was observed by carrying out the enzyme assay employing 109.2 μg protein on different days. The enzyme was stored in phosphate buffer (50 mM, pH 7.4) containing 0.2% Triton X-100

Table 5: Effect of arsenic on the activity of AChE rat heart

Arsenic concentration (mM)	AChE activity (μmole min^{-1} mL^{-1})	AChE activity (%)
0.00	0.097	100.00
0.10	0.090	92.36
0.50	0.062	63.62
1.00	0.060	61.57
2.00	0.040	41.05

The effect of varying concentrations of As on the activity of AChE from heart tissue of rat has been determined. The enzyme assay was carried out employing 109.2 μg protein using the procedure. The enzyme without arsenic served as a control and was considered to have 100% activity

Effect of storage time at -20° C on the activity of AChE from rat heart: The effect of storage time at -20°C was determined by assaying the activity of enzyme at the different time intervals by employing the enzyme protein (109.2 μg) in the phosphate buffer containing 0.2% Triton X-100. The results (Table 4) indicated that the enzyme was found to be stable up to 30 days with not much loss in activity.

Effect of substrate concentration on the AChE activity from rat heart: The enzyme protein (109.2 μg) was assayed at varying concentrations of substrate (ATI) at room temperature (26±2°C). The enzyme activity at corresponding substrate concentration displayed a hyperbolic curve and showed direct correlation (Fig. 2). The Lineweaver Burk's double reciprocal plot as shown in Fig. 2, demonstrated a straight line which intersects at Y-axis and negative abscissa of X-axis, from where V_{max} and K_m values could be calculated; the values being 0.192 μmoles mL^{-1} min^{-1} and 0.037 mM, respectively. The turnover number or K_{cat} (the number of substrate molecule each enzyme site converts to product/unit time, in which the enzyme is working at maximum efficiency) was computed to be 0.0017 sec^{-1}. The catalytic efficiency or ratio of k_{cat}/K_m (defines a measure of an enzyme-substrate pair) was computed to be 0.0160 $mM^{-1}sec^{-1}$.

Effect of arsenic (As)on the activity of AChE from rat heart tissue: The enzyme when assayed in presence of varying concentrations of arsenic (0.05-10 mM), displayed consistent decrease in its activity (Table 5). When this data were extrapolated using percent residual activity and the arsenic concentrations on Y and X-axes, respectively, the IC_{50} value of this heavy metal for rat heart AChE could be determined, the value being 1.14 mM (Fig. 3).

DISCUSSION

There is increasing interest within the development of recent strategies for assessing chemical toxicity and therefore the use of data of mechanisms of cytotoxic actions to enhance this assessment. An enzyme should be extracted and solubilized from the selected tissue before its characterization.

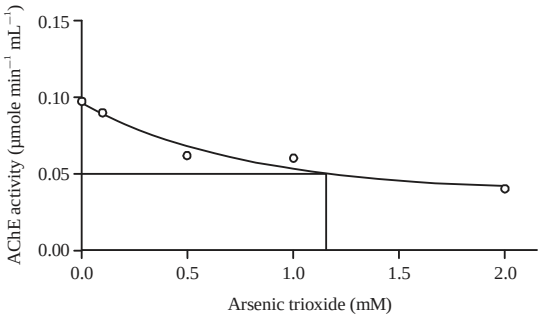

Fig. 3: Determination of IC_{50} value of As for rat heart AChE using the data given in Table 5. The IC_{50} is a concentration of substrate at which the activity of AChE becomes half. This value was calculated by increasing the concentration of inhibitor arsenic trioxide (As_2O_3). By calculating this parameter, the reduced activity of AChE at 50% can be measured. The IC_{50} value of arsenic trioxide for AChE was calculated from the graph and value obtained was 1.14 mM, which means 1.14 mM of As_2O_3 inhibits the 50% activity of AChE

Some enzymes are membrane-bound and their solubilization has been achieved by using organic solvents, detergents[18-20], proteolytic and lipolytic enzymes[27]. The results from the present study demonstrated that the membrane bound form of rat heart AChE which could be easily solubilized using Triton X-100, a non-ionic detergent. The membrane bound nature of AChE has been reported in some other living systems also[18-20,28,29]. However, AChE has been shown to exist in a membrane bound form in the human erythrocytes[17], rat brain[19], rat liver[30], electric eel[31], electric fish[31,32] and certain helminthes[28,33]. The enzyme from these systems has been solubilized by applying both the ionic as well as non-ionic detergents in the phosphate buffer[28]. The blood arsenic is redistributed quickly to the heart and other organs[2,3] and produces ROS mediated generation of oxidative stress[12]. Arsenic induced oxidative stress resulted into alterations in the levels of glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and heme oxygenase-1 (HO-1)[12]. Arsenic has been also known to cause generation of free radicals mediated oxidative stress[34,35].

The inhibition of AChE by neurotoxins such as copper, cadmium and organophosphorus compounds has been established[36]. The specific activity of rat heart AChE was found to be 0.02 U mg^{-1} which is very much closer to the specific activity of AChE reported in mouse heart (0.03-0.05 U mg^{-1})[37] and about equals to the AChE activity reported in skeletal muscle (0.15-0.20 U mg^{-1})[37]. Gomez *et al.*[38] has also been reported that the activity of normal rat heart ranges from 0.035±0.014 U mg^{-1} protein to 0.044±0.010 U mg^{-1} protein.

The K_m value for any enzyme is a measure of its affinity towards substrate. In present study, the K_m value for rat heart AChE was found to be 128 μM which was about much closer to the value reported for the enzyme from fetal bovine serum (120 μM) and human brain (107 μM)[39]. The rat heart AChE displayed K_m about half of that reported for the analogous enzyme from the human erythrocytes (225 μM). The relatively lower K_m value for rat heart AChE indicated its enhanced affinity to the substrate as compared to other mammalian systems.

Exposure of arsenic may lead to decreased nervous coordination with myocytes, thereby affecting functions of the rat heart in diverse ways ranging from perturbed physiology and behavior. The entry of arsenic in mammalian heart may alter heart function. The results from this study displayed that arsenic caused inhibition of rat heart AChE (IC_{50} = 1.14 mM). These results indicate that arsenic may induce toxic stress on cardiac system of rat. The present study showed that arsenic inhibited the activity of rat heart AChE *in vitro*. No such reports are available from other workers to be used for the sake of the comparison. However, the exposure of arsenic has been reported to suppress the activity of AChE in the cardiac tissue in a time dependent manner in *Lamellidens marginalis*[40]. Suppression in the activity of AChE demonstrates the vulnerability to the exposure of arsenic. The global concern over the threats of arsenic contamination to habitats has increased the efforts to monitor and assess the environmental conditions. This study demonstrates the necessity for monitoring of arsenic contamination as a potential biomarker[38].

In the present study, the decrease in the activity of AChE was found to be associated with the dose of arsenic. The inhibition of enzyme by arsenic in the present investigation could be a puzzling development, as AChE does not contain the structural options typically related to its inhibition by arsenic. The inhibition of some other enzymes by this element has been reported to occur by reaction with free sulfhydryl groups and the acute toxicity manifests as a cholinergic crisis in the organ. However, different compounds known to be sulfhydryl reactants equivalent to organomercury compounds failed to inhibit AChE[41]. This is the first study of its kind to assess the inhibitory potential of AChE from rat heart *in vitro*. These results provide new insights on the cardiotoxicity induced by arsenic thereby indicating that acetylcholinesterae activity could be exploited as a potential biomarker of cardiotoxicity in rats and other experimental animals.

CONCLUSION

The results from the present study indicated the presence of membrane bound form of AChE in the rat heart tissues which could be solubilized employing a non-ionic detergent, Triton X-100. The hyperbolic curve obtained shows a positive correlation between the activity and the increase in the substrate concentration. Arsenic sharply inhibited the enzyme activity at low concentration indicating thereby its strong cardiotoxic potential to the mammals. Though the exact mechanism of action of arsenic on heart AChE is not known but this study presented an evidence that this heavy metal may exert negative impact on this enzyme from rat heart. It therefore appears that the rat heart AChE may serve as a potential biomarker of cardiotoxicity induced by arsenic.

SIGNIFICANCE STATEMENTS

This study indicated the presence of membrane bound form of AChE in the rat heart tissues. The analysis of the AChE activity with the increased substrate concentration displaying a hyperbolic curve indicated a positive correlation. This study discovers strong cardiotoxic potential of arsenic to the mammals. The information from this study may be useful in detecting arsenic toxicity as well as in the risk management of those who are occupationally involved in arsenic infested environment.

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