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Combined arsenic and di-(2-ethylhexyl) phthalate exposure elicits responses in brain ATPases different from hepatic and renal activities in rats

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Arsenic and di-(2-ethylhexyl) phthalate (DEHP) are environmentally ubiquitous and epidemiologically important toxic agents that millions of people are currently exposed to, worldwide. Although the adverse impact due to exposure to either arsenic or DEHP are documented, the toxicological effects of co-exposure to these agents are largely unknown. In this study, exposure to these chemicals was investigated for their effects on ATPase activities in the brain, liver and kidney of rats. Male Wistar rats were exposed daily to 100 mg L^{-1} arsenic via drinking water and to 100 mg DEHP kg^{-1} body weight in corn oil either individually or concurrently for 30 days. Toxicity was assessed by evaluating changes in body and organ weights, as well as, Na^+/K^+-, Ca^{2+}-, Mg^{2+}- and total ATPase activities in the brain, liver and kidney. Exposure to either arsenic or DEHP resulted in drastic reduction in activities of the enzymes in the compartments investigated, except in the brain where Na^+/K^+- and Mg^{2+}- ATPases had their activities significantly increased. Also, DEHP displayed no effect on the total ATPase and Ca^{2+} ATPase in the kidney and brain, respectively. Interestingly, co-exposure to these toxicants significantly stimulated the activities of all these enzymes in the brain. In this compartment, combined treatment resulted in an additive interaction between the toxicants and a potentiation effect of arsenic on DEHP with regards to the Na^+/K^+- ATPase activity and Ca^{2+}- ATPase activity, respectively. Our findings demonstrate tissue specific response to combined arsenic and DEHP exposure in rats with the effect on the brain significantly different from other compartments.

Key words: Arsenic, di-(2-ethylhexyl) phthalate, Na^+/K^+-ATPase, Ca^{2+}-ATPase, co-exposure.

INTRODUCTION

Arsenic is an important environmental contaminant that is poisoning millions of people globally (Hughes et al., 2011). Exposure to the metalloid may occur as a result of natural or anthropogenic activities. It is a natural

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contaminant of groundwater in certain parts of the world (Kwok, 2007; Chowdhury et al., 2000; Hughes et al., 2011) as a result of its leaching into aquifers from surrounding arsenic-rich geological formation (World Health Organization (WHO), 2001). Anthropogenically, it majorly finds its way into the environment as a result of metal smelting operations (Ng, 2005). Exposure to this metalloid has been linked to an increased incidence of a wide range of adverse health effects resulting from both its cancer and non-cancer effects. Studies have shown that exposure to arsenic at high levels can lead to development of skin, bladder, liver and lung cancers (Hughes et al., 2011; Smeester et al., 2011). Severe metabolic disorders such as diabetes and other non-cancer effects such as, cardiovascular disorders, goiter, hepatomegaly, bronchitis have also been associated with arsenic exposure (Hughes et al., 2011; Mazumder and Dasgupta, 2011).

A class of chemicals that have grown over the years to be a major public health concern is the phthalates. Phthalate esters, due to their high production volume and use have attracted global attention. They are used as plasticizers, additives and solvents in many consumer products like cosmetics, food packaging products, building materials and biomedical devices (Horn et al., 2004; Shea, 2003). The potential to leach out from plastics, a result of the weak linkages formed with the polymer, makes this class of chemicals significant environmental agents (Fromme et al., 2012). Phthalates have been reported to modify several cellular processes in humans. Exposure to this class of chemicals has been associated with endocrine disruption, metabolic disorders, reproductive and developmental defects (Andrade et al., 2006; Stahlhut et al., 2007; Grün, 2010; Olsen et al., 2012; Engel et al., 2010; Miodovnik et al., 2011). Di-(2-ethylhexyl) phthalate (DEHP) is one of the most commonly used of the phthalates and the most abundant of its class in the environment (Kamrin, 2009).

Arsenic and phthalates are both ubiquitous environmental toxicants with high exposure potential to man (Hughes et al., 2011; Mankidy et al., 2013). They are known to induce oxidative stress (Jomova et al., 2011; Erkekoglu et al., 2010) and are reported to be endocrine disruptors (Davey et al., 2007; Li et al., 2012). Both environmental agents are found in the air, soil, drinking water and food (Du et al., 2010; Rudel et al., 2003; Adeyemi et al., 2009; Kamrin, 2009). Their presence in the same environmental compartment could result in contamination of food or water and hence, a simultaneous exposure to these agents. This tendency to be found in the same environment, as well as their shared modes of toxicity makes them of interest. Previously, we have been able to establish that co-exposure to arsenite and di-(2-ethylhexyl) phthalate (DEHP) result in the modulation of oxidative degeneration in tissues of rats different from individual effects (Afolabi et al., 2015). Accumulation of protein oxidative products in tissues indicated in the report may result from oxidative modification of proteins involved in transport systems and signal mechanisms, among many others. The present study was therefore undertaken to investigate the changes in ATPase activities in the membrane of brain, kidney and liver of rats co-exposed to arsenite and DEHP.

MATERIALS AND METHODS

Chemicals

Sodium arsenite and DEHP were procured from Sigma-Aldrich, Munich, Germany. Every other chemical used was of analytical grade and purchased from Qualigens Fine Chemicals (Mumbai, India) and Sigma-Aldrich (Munich, Germany).

Experimental design

Wistar albino male rats weighing between 80 and 100 g (7 to 8 weeks old), obtained from the Faculty of Basic Medical Sciences Animal House, LAUTECH, were acclimatized for two weeks prior to the experiment and were maintained on a standard pellet diet and distilled water *ad libitum.* At the end of the acclimatization, the animals were weighed and then randomly distributed into four groups of six animals each. Group I was administered with corn oil and served as the Control. Group II received 100 mg L^{-1} arsenic in the form of sodium arsenite in drinking water while Group III was administered a homologous mixture of 100 mg DEHP kg^{-1} body weight in corn oil, by gastric intubation. Group IV (As + DEHP) received both 100 mg L^{-1} arsenite in drinking water and 100 mg DEHP kg^{-1} body weight in corn oil. These treatments were maintained for thirty days. At the end of the treatment, the animals were fasted overnight and blood collected by cardiac puncture, under light diethyl ether anesthesia into heparinized tubes. The blood was centrifuged at 3,000 *g* for 10 min to obtain plasma. The liver, kidney and brain were removed and homogenized in ice-cold KCl (150 mmol L^{-1}). The obtained 10% homogenate was then centrifuged at 15,000 g for 15 min at 4°C and the supernatant stored at -20°C until biochemical analysis. Tissue protein levels were determined using Bradford method (Bradford, 1976). The doses used for arsenic and DEHP were selected based on previous studies (Kurata et al., 2012; Akingbemi et al., 2001; Samuel et al., 2005). The experiment was carried out in accordance with the LAUTECH Department of Biochemistry guidelines for the care and use of laboratory animals.

Biochemical analyses

Total ATPase activity in tissue homogenate was measured by the method of Evans (1969). The ATPase activity in 0.1 ml aliquots of the homogenates were measured in a final volume of 2 ml containing 0.1 ml each of 0.1 M Tris-HCl (pH 7.4), 0.1 M NaCl, 0.1 M $MgCl_2$, 1 mM EDTA, 0.01 M ATP and 1.5 ml of 0.1 M KCl. The reaction was stopped at 20 min by adding of 1 ml 10% TCA and then centrifuged (1500 × *g* for 10 min). 160 µl of 0.14 mM of ammonium molybdate and 100 µl of 2% vitamin C were added to the supernatant. The reaction mixture was allowed to stand for 10 min and the inorganic phosphorus (Pi) liberated was then estimated in the supernatant by the method described by Fiske and Subbarow (1953).

The assay of the Na^+/K^+-ATPase activities followed the procedure of Hesketh et al. (1978) and the inorganic phosphate

Table 1. Body weight, relative organ weight of control and experimental rats. Values are expressed as mean ± S.D. (n=6). Means in column with the same letter are not significantly different from each other at $p < 0.05$.

Groups	Body weight			Relative brain weight (g/100 g b.w)	Relative liver weight (g/100 g b.w)	Relative kidney weight (g/100 g b.w)
	Initial (g)	Final (g)	Change (%)			
Control	132.38 ± 3.32	168.65 ± 4.78	27.40 ± 0.81[a]	0.68 ± 0.06[a]	3.34 ± 0.19[a]	0.62 ± 0.03[a]
Arsenic	131.17 ± 2.74	150.34 ± 4.66	14.12 ± 0.79[b]	0.92 ± 0.06[b]	3.63 ± 0.11[a]	0.77 ± 0.03[b]
DEHP	135.60 ± 3.51	154.81 ± 4.81	14.17 ± 0.96[b]	0.70 ± 0.03[a]	3.59 ± 0.12[a]	0.69 ± 0.02[c]
Arsenic + DEHP	133.15 ± 2.91	146.88 ± 5.78	10.31 ± 0.63[c]	0.98 ± 0.05[b]	3.97 ± 0.11[b]	0.63 ± 0.04[a]

released from ATP was monitored. The reaction mixture contained 0.5 ml each of 0.35 M NaCl, 17.5 mM KCl, 21.0 mM $MgCl_2$, 10 Mm Tris-HCl (pH 7.4), and 8.0 mM ATP-Na_2. The reaction was initiated with the addition of 0.2 ml of tissue homogenate and the mixture incubated at 37°C for 1 h. The reaction was terminated by adding 0.8 ml of ice-cold 10% (w/v) trichloroacetic acid and the resultant mixture was allowed to stand for 20 min at 4°C before it was centrifuged at 4,000 g for 5 min. The concentration of phosphate in 1 ml of the supernatant was measured as described already.

The activity of Ca^{2+}-ATPase was assayed according to the method of Hjerten and Pan (1983). In brief, 0.1 ml of tissue homogenate was added to a mixture containing 0.1 ml each of 125 mM Tris-HCl buffer (pH 8), 50 mM $CaCl_2$ and 10 mM ATP. The contents were incubated at 37°C for 15 min. The reaction was then arrested by the addition of 0.5 ml of ice-cold 10% TCA and centrifuged. Pi was then determined following the technique as described already. The activity of Mg^{2+}-ATPase was assayed according to the method of Ohnishi et al. (1982). Briefly, 0.1 ml of tissue homogenate was added to a mixture containing equal volume of 375 mM Tris-HCl buffer (pH 7.6), 25 mM $MgCl_2$, distilled water, and 10 mM ATP. The contents were incubated at 37°C for 15 min. The reaction was then stopped with the addition of 0.5 ml of 10% TCA and centrifuged. Pi was then determined following the technique as described already.

Statistical analysis

Results are expressed as mean ± SD. The statistical significance was evaluated by one-way analysis of variance (ANOVA) followed by Tukey using GraphPad Prism version 5.00 for Windows (GraphPad Software, Inc; CA, USA). A value of $p<0.05$ was considered statistically significant between groups.

RESULTS

There were no noticeable changes in behavior of the rats and no mortality was recorded throughout the period of the experiment. The animals in the treated groups increased in weight at the end of the exposure period, even though the body weight gained were significantly lower than that of the control group (Table 1). Treatment with either arsenic or DEHP alone resulted in a weight gain that was 48% less than that of control. However, co-treatment with the two agents yielded the lowest gain in body weight, producing 62% less than that of the control and about 27% less than either arsenic or DEHP treated group. Exposure to arsenic alone induced significant increase in weights of the brain and liver of the rats, as indicated by the gain in their relative weights, while such increase was only observed in the kidney in the case of DEHP alone. In the animals co-treated with arsenic and DEHP, arsenic induced significant increase in relative weight gained in brain while interacting with DEHP to increase the relative weight gained in the liver of the animals.

Figures 1 to 4 depict ATPase activities in brain, kidney and liver of control and experimental animals. Arsenic exposure resulted in significant reduction of total ATPase activity in all the three organs (Figure 1). The metal lowered the total ATPase activity by 20, 46 and 41% in the brain, kidney and liver, respectively, when compared with the control. Hepatic activity of the total ATPase activity was significantly reduced by DEHP (41%) but increased in the brain by 55%, while it displayed no effect on the renal activity. The mixture of these agents, however, depressed total ATPase activity by 20 and 25% in the kidney and liver, respectively, while increasing the same activity by more than 74% in the brain. The three treatment regimens exhibited similar trend in their effects on the Na^+/K^+ ATPase activity in the three organs (Figure 2). Exposure to arsenite, DEHP and their mixture inhibited both hepatic and renal Na^+/K^+ ATPase activity. In the kidney, the inhibition amounted to 42, 44 and 47%, respectively when at the same time 30, 25 and 28% reduction were observed, respectively, in the liver. In the brain, the toxicants brought about a stimulation of the enzyme, with the co-exposure producing a significant 2.3 fold increase in the activity of the protein.

Activities of Ca^{2+} ATPase in the organs of the rats are presented in Figure 3. After thirty days, arsenic provoked similar lowering effect on the enzyme activity in the organs. Its inhibitory effect amounted to 48, 47 and 43% in the brain, kidney and liver, respectively. DEHP on the other hand displayed no effect on Ca^{2+} ATPase brain activity. However, when arsenite and DEHP were co-administered to the rats, they induced a 35% stimulation of the enzyme in the same compartment. In the kidney and liver, DEHP alone reduced the enzyme's activity by 44 and 26%, respectively and the co-exposure treatment yielded more than 50% inhibition of the protein.

The data in Figure 4 represent Mg^{2+} ATPase activities in the three organs. Arsenite, DEHP and the co-exposure treatment all resulted in significant stimulation of the Mg^{2+}

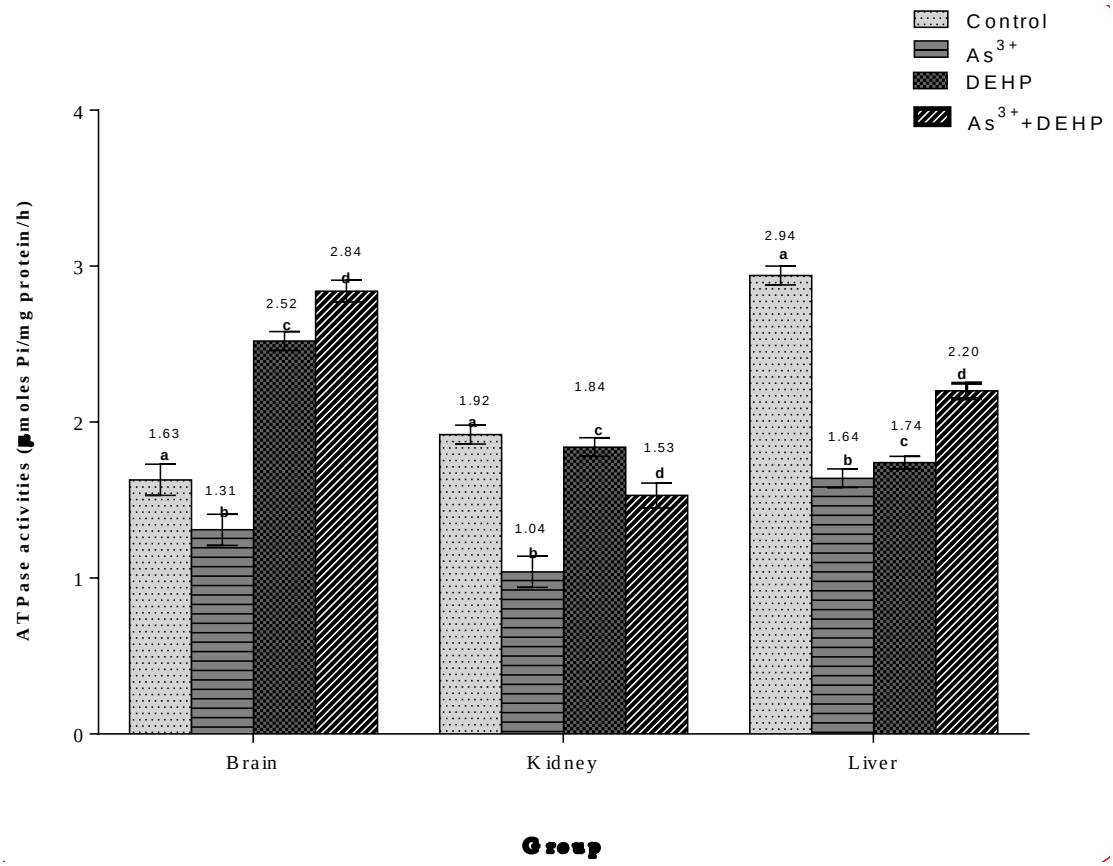

Figure 1. Total ATPase activities in brain, kidney and liver of rats exposed to As^{3+}, DEHP and As^{3+} + DEHP compared with control. Bars of the same compartment carrying different letters of the alphabet are significantly different from each other ($p < 0.05$).

ATPase in the brain of the rats. The respective subsequent effect was 103, 169 and 97% increases in the activity of the enzyme in the brain. The kidney and liver, however, experienced significant depression in Mg^{2+} ATPase activity upon exposure to these three toxic agents. The kidney demonstrated significantly lower Mg^{2+} ATPase activity than the liver, with arsenite, DEHP and their co-exposure reducing the activity by 44, 65 and 47%, respectively. The decreases were 27, 21 and 24%, respectively, in the liver.

DISCUSSION

In this study, ATPase activities in the organs of rats co-treated with arsenic in the form of sodium arsenite and DEHP implied the ability of the combination of these toxicants to modulate these enzymes differently from single exposures. More significantly, the combined treatment displayed tissue specificity in its effect on ATPase activities. Specifically, the ATPase activities in the brain were markedly stimulated by combined exposure to arsenic and the phthalate, DEHP compared to what was obtained in liver and kidney of the rats where the proteins were generally inhibited in their activities.

Na^+/K^+ ATPase is implicated in active transport across cell plasma membrane, keeping potassium concentration relatively high but that of sodium low. The maintenance of the homeostasis of these ions is essential for proper conformation and functioning of proteins, as well as, signaling processes. Either of arsenite or DEHP alone increased the activity of Na^+/K^+ ATPase in the brain, a sharp contrast to earlier studies where they depressed the activity of the enzyme (Dhanya et al., 2003; Bhuvaneswari et al., 2014). The differences in DEHP concentration administered in this present study, which was about 2 orders greater than that stated by Dhanya et al. (2003), and the route of arsenic administration which was oral in contrast to the intraperitoneal injection used by Bhuvaneswari et al. (2014) could be responsible for the dissimilarities observed. In addition, upregulation of the ATPase synthesis by the toxicants could account for the increased protein activities observed. Co-treatment with these agents also significantly enhanced the protein activity. The data is suggestive of an additive effect resulting from interaction between the arsenic and DEHP

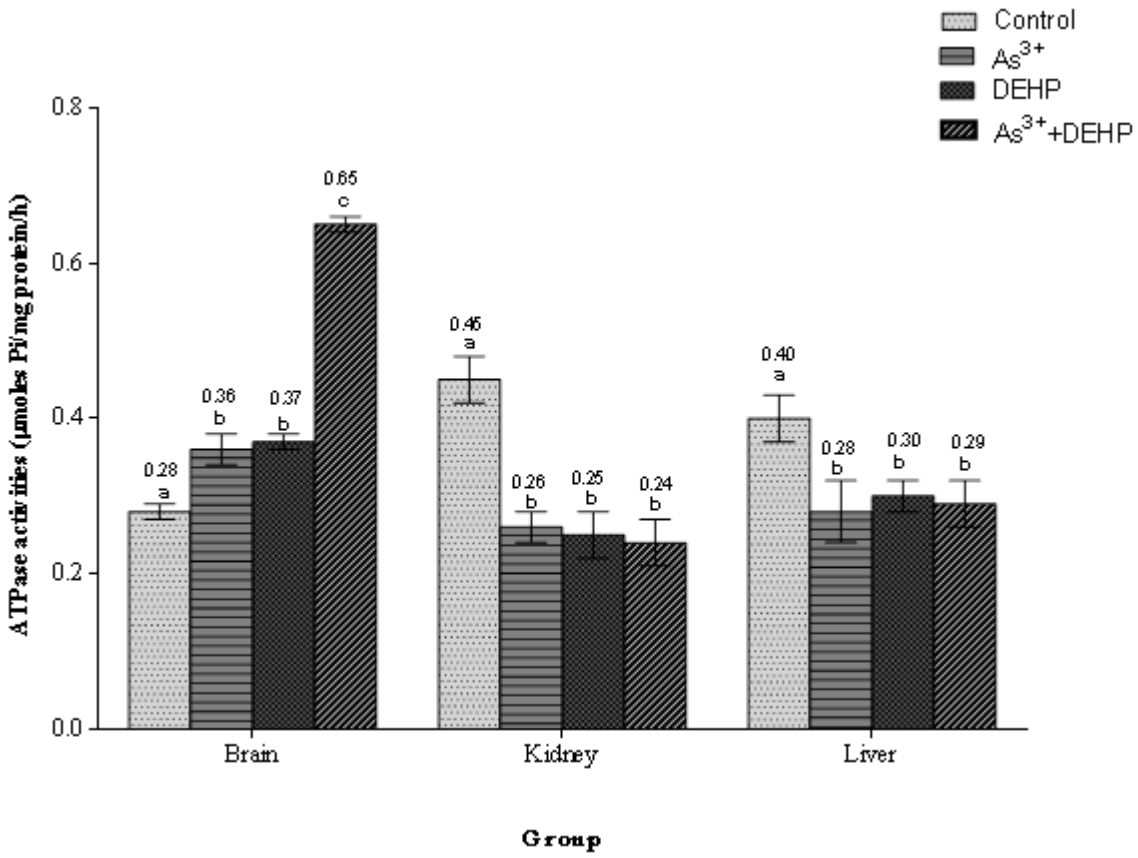

Figure 2. Na^+/K^+ ATPase activities in brain, kidney and liver of rats exposed to As^{3+}, DEHP and As^{3+} + DEHP compared with control. Bars of the same compartment carrying different letters of the alphabet are significantly different from each other ($p < 0.05$).

in the brain of the rats. Na^+/K^+ ATPase is central to the maintenance of the ionic gradient required for neuronal excitability in the brain, where the hydrolysis of ATP is coupled with the transport of Na^+ and K^+ across the cell membrane, creating a transmembranous Na^+-K^+ gradient (Ji et al., 2009). With alteration in the protein's activity been proposed to represent an important neurotoxic mechanism for neurons (Lees, 1993), the increase in activity is therefore of high significance. The upregulation of the enzyme's activity by the co-exposure may disrupt nerve impulse transmission, resulting from depolarization of neuronal terminals, as well as neurotransmitters release, depolarization of neurons and discharge of action potential (Somjen, 2002).

The brain Na^+/K^+ ATPase has been reported to have several isoforms of its subunits which are different from those present in kidney, and possibly other tissues (Blanco and Mercer, 1998; Blanco, 2005; Ray, 2013). The differences in these ATPase-isoforms and the other organs could make the brain more susceptible to modulation by both intrinsic and extrinsic factors, and could account for the brain's distinct response to the toxicants. The increased activity of Na^+/K^+ ATPase in the brain of the animals induced by these treatments could, therefore, be a result of one or a combination of the following: (i) inhibition of the regulatory factor protein kinase C, resulting from an increase in intracellular sodium concentration, leading to dephosphorylation of Na^+/K^+ ATPase and increasing its activity (Mallick et al., 2000); (ii) upregulation of the enzyme by increased cAMP (Burnier, 2008); (iii) enhanced expression of the β1 subunit of Na^+/K^+ ATPase, the rate limiting subunit, which has been suggested to increase the enzyme activity (Sennoune et al., 2000); and (iv) hormonal modulation (McDonough et al., 1988).

In contrast, there were significant reductions in the hepatic and renal activities of Na^+/K^+ ATPase in all the three treatment groups. The inhibition of this enzyme may be a result of direct and/or indirect stimulation of the isoprenoid pathway of which digoxin, an inhibitor of the enzyme, is a product (Dhanya et al., 2003). In addition, alteration by these toxicants of the enzyme's lipid microenvironment could result in moderation of its activity. This is because the functions of membrane-associated proteins are largely dependent on their immediate lipid milieu, the perturbation of which could

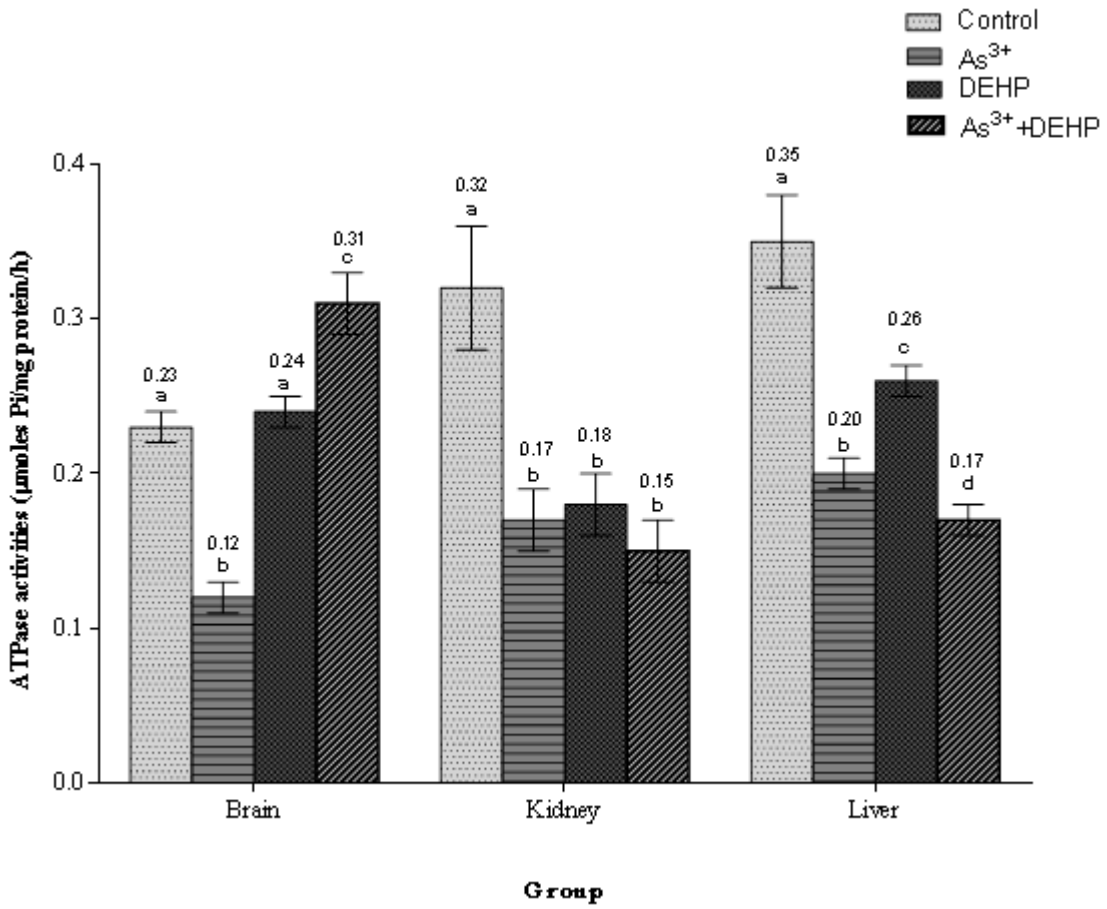

Figure 3. Ca^{2+} ATPase activities in brain, kidney and liver of rats exposed to As^{3+}, DEHP and As^{3+} + DEHP compared with control. Bars of the same compartment carrying different letters of the alphabet are significantly different from each other ($p < 0.05$).

result in derangement of activity. Oxidative stress is one of the factors known to cause such changes. In our previous study, co-treatment of rats with arsenite and DEHP resulted in significant increase in some oxidative indices in these organs (Afolabi et al., 2015). Increased lipid peroxidation in the membrane could have thus, contributed to the decreased enzyme activity observed. Inhibition of Na^+/K^+ ATPase can generally lead to increased intracellular Na^+ and Ca^{2+} concentrations. The disruption of ion homeostasis can trigger cell death involving both apoptotic and necrotic components mediated through the accumulation of Na^+ and Ca^{2+} (Xiao et al., 2002).

Ca^{2+} ATPase functions in the transfer of Ca^{2+} ion which mediates diverse array of physiological processes, including gene expression and regulation in biological system (Mata and Sepulveda, 2010). Both hepatic and renal Ca^{2+} ATPase activities displayed similar trend in their responses to the toxic challenges. Arsenic treatment produced greater levels of inhibition than did DEHP, while their combined treatment led to a more significant inhibition of the enzyme than either toxicants alone. Ca^{2+} ATPase is known to be sensitive to its lipid milieu, and both arsenic and DEHP have been reported to perturb lipid metabolism (Bhattacharjee and Pal, 2014; Muthumani and Milton, 2013; Jia et al., 2015; Xu et al., 2009). The changes in lipid contents caused by these agents may thus, explain the alterations in the enzyme activity. Similar to their effect on brain Na^+/K^+ ATPase activity, combined arsenite and DEHP treatment drastically stimulated brain Ca^{2+} ATPase activity. In this instance, however, the data reflected a potentiation effect of the arsenite on DEHP. The arsenic alone caused a decline in the protein activity, while DEHP though producing a slight increase did not statistically affect the enzyme activity. Perturbations to the membrane caused by the toxicants could enhance the influx of calcium into the cell via ion channels. The increased Ca^{2+} ATPase activity could be a compensatory response to this, as well as, to specific signals generated by an action potential or secondary messenger mediated process such as cAMP-triggered increase in intracellular Ca^{2+}, provoked by the

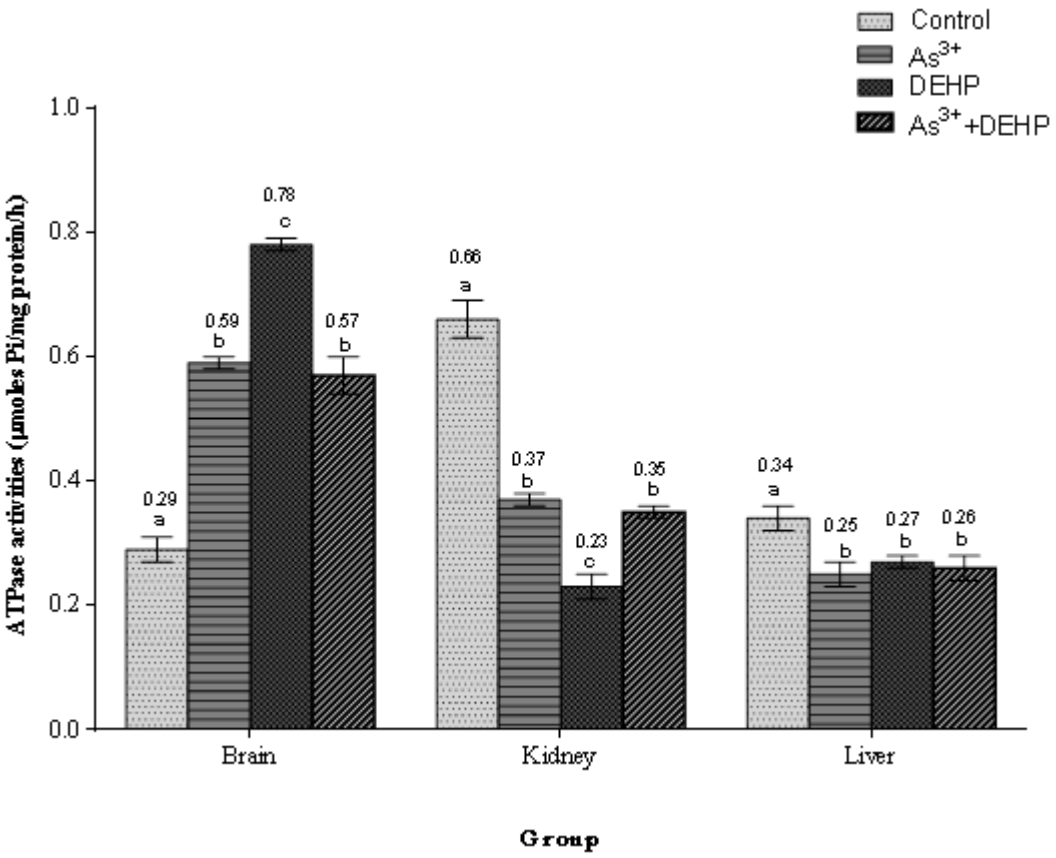

Figure 4. Mg^{2+} ATPase activities in brain, kidney and liver of rats exposed to As^{3+}, DEHP and As^{3+} + DEHP compared with control. Bars of the same compartment carrying different letters of the alphabet are significantly different from each other ($p < 0.05$).

opening of Ca^{2+} channels in the plasma membrane (Ji et al., 2009; Heguilen et al., 2009). Protein kinase C, for example, has been suggested to be involved in the elevation of brain microsomal Ca^{2+} ATPase activity in rats (Hanahisa and Yamaguchi, 1998).

The total ATPase and Mg^{2+} ATPase activities in this study further buttressed the organ specific action of arsenic and DEHP co-treatment in rats. The data showed that there were significant increases in the total ATPase and Mg^{2+} ATPase activities induced by the mixture in the brain, but were inhibited in both the liver and kidney of the animals. The present findings of arsenic and DEHP mixture disrupting ATPase activities in the brain may thus, indicate alteration in membrane and neuro-transmitter functions.

Environmental contaminants like arsenic and DEHP have been implicated in some neuropathological conditions (Testa et al., 2012; Siniscalco et al., 2013; Akyuzlu et al., 2014; Manivannan et al., 2015). There are also growing concerns about the adverse effects of toxicants on neurodevelopment (Kim et al., 2009; Xiu-Juan et al., 2013). The fact that arsenic together with DEHP can increase the activities of ATPases in the brain is, therefore, of great import in explaining the pathogenesis of environmental contaminants induced neurodisorders.

Increased ATPase activities have been associated with a number of pathological states including autism, Alzheimer's disease, Parkinson syndrome, Cushing's syndrome (Berrocal et al., 2009; Ji et al., 2000; Wambach et al., 1980). Brain-region specific increases in the activities of Na^{+}/K^{+} ATPase and $Ca^{2+}Mg^{2+}$ ATPase have also been reported in neurodisorders such as autism and Alzheimer disease. The altered activities of these enzymes may contribute to abnormal neuronal circuit functioning in such neuropathological conditions (Ji et al., 2009). Impairment in Ca^{2+} homeostasis is a common feature in Alzheimer's disease and other neuro-degenerative disorders involving damage and death of neurons (Mattson, 2007; Bezprozvanny and Mattson, 2008; Thibault et al., 2012). Intracellular Ca^{2+} are utilized by neurons in the control of various processes. Disturbances in Ca^{2+} homeostasis caused by malfunctioning of Ca^{2+}/Mg^{2+} ATPase or Ca channels, can result in neuronal dysfunction and eventual neuronal death (Gargus, 2009).

Conclusion

Our findings demonstrate tissue specific responses of ATPases to arsenic and DEHP coexposure in rat, with the effects on the brain different from the hepatic and renal compartments. More research is needed to evaluate the potential role of these contaminants on the pathogenesis of neurodegenerative disorders.

Conflict of interest

The authors have not declared any conflict of interest

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