Journal of Pharmacology and Toxicology

ISSN 1816-496X

Academic Journals Inc.

www.academicjournals.com

Journal of Pharmacology and Toxicology 3 (6): 471-477, 2008
ISSN 1816-496X

Effects of Folic Acid and Vitamin C on Arsenic Induced Mice

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Abstract: In this study, 5 weeks old mice weighing 22±2 g were grouped in four, each group consisting of six animals. Group-I, II, III and IV of the animals were fed by a standard diet quantity sufficient, normal diet with dissolved arsenic, arsenic with folic acid mixed and arsenic with vitamin C mixed, respectively. Blood was collected from the sacrificed animals and the blood glucose levels were determined by spectrophotometrically and glucometer. The average blood glucose level of arsenic induced animals was 9.37 mmol L^{-1} compared to 6.53 mmol L^{-1} in control animals whereas the blood glucose level of Group-III and Group-IV were 6.73 and 7.03 mmol L^{-1}, respectively. Weight gain of the arsenic induced animals was lower compared to that of the animals fed with normal diet, folic acid mixed diet or vitamin C mixed diet. After sacrifice, the weight of kidney, heart and lung of arsenic induced animals were less than that of the Group-III and Group-IV. The reduction of arsenic induced higher blood glucose level by folic acid and vitamin C demonstrates that folic acid and vitamin C has significant effect in preventing arsenic induced disease.

Key words: Blood glucose level, internal organ, lethal dose, prevention

INTRODUCTION

Arsenic is the 20th most abundant element in the Earth's crust 14th in the seawater and 12th in the human body (Woolson, 1975). In Bangladesh, an estimated 57 million people have been exposed to arsenic through contaminated wells. This incident serves as an unfortunate reminder of the toxic consequences of arsenic mobilization and underscores the need to understand the factors controlling the mobility and solubility of arsenic in aquatic systems (Newman *et al*., 1997). Chronic exposure to arsenic in drinking water is associated with a greater risk of cancers of the skin, bladder, lung and liver and of stroke (Chiou *et al*., 1997), ischemic heart disease (Tseng *et al*., 2003) and neurologic consequences in adults (Feldman *et al*., 1979) and of neurologic consequences in children (Wasserman *et al*., 2004). Furthermore, inorganic arsenic has long been considered to be a teratogen, causing neural tube defects in many mammalian species (Wlodarczyk *et al*., 2001). We have estimated the cancer burden to be doubling in Bangladesh (Chen and Ahsan, 2004). Clinical manifestations of arsenic toxicity vary widely between persons and populations. Several observational and biochemical studies have led to a prevalent supposition that nutritional status may account for a substantial portion of this variability.

Moreover, drinking water in various regions in the world contains this arsenic poison. It is four times more poisonous than mercury (Rashid and Mridha, 2007). For reducing or solving this problem, many researches are going on to arsenic issue. A recent randomized control trial has been conducted on Bangladeshi people regarding folic acid effect on arsenic imposed people and placebo group. That

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epidemiological study shows that the participants had low blood foliate levels at the start of the study. In that study, the researchers report that, as a result of folic acid supplementation total blood arsenic levels were reduced by 13.6 and by 2.5% in the placebo group (Gamble *et al*., 2007). But the effect of folic acid on the specific organ of the body and the quantitative measurement of blood glucose level should be understood.

Therefore, in present study an attempt was made to study the effect of arsenic on the internal organ e.g., spleen, liver, kidney, heart and lung of the mice; to study the effect of folic acid and vitamin C on arsenic induced change of internal organs; to study the quantitative effect of arsenic on the mice; to study the quantitative effect of folic acid and vitamin C on arsenicosis.

MATERIALS AND METHODS

Mice of white albino strain (collected from International Centre for Diarrhoeal Disease Research, Lab shed, Dhaka) were used throughout the study. The mouse, bearing the scientific name *Mus musculus* is thought to have originated from Asia. The experiment was conducted at two laboratories in Khulna University such as Microbiology laboratory, Pharmacy Discipline and Animal laboratory, Biotechnology and Genetic Engineering Discipline. Experimental animal diets were prepared in the laboratory. Ingredients such as wheat flour, gram, oil-cake, soyabean oil were mixed. The water was added quantity sufficiently. Moreover, chemicals such as sodium arsenite, dimethyl ether were used. Commercial pharmaceutical products such as FOLCID (Folic acid 5 mg, Manufacturer: Pharmic Laboratories Ltd. Dhaka; Manufacturing License No. 371 and 174) and CEEVIT (Vitamin C 250 mg, manufacturer: Square Pharmaceuticals Limited, Bangladesh, Manufacturing License No. 33 and 114) was used.

The experiment was carried out through April 2007- January 2008. The given methods involved were the followings:

Handling and Restraint of Experimental Animal in the Laboratory

It is customary to pick up a pet mouse by gently lifting it up by the tail and placing it into a cupped hand. If a more secure hold is necessary (giving medications or food orally), the hander may grasp or pinch as much as skin as possible over the neck, just behind the head. Then the mouse can be picked up and turned over on its back by rotating the wrist. The tail can be restrained by gently grasping it with other hand, or between the fourth (ring) and fifth (pinkie) fingers of the same hand.

Mice can be lifted by their tails but great caution must be exercised in doing so. The skin of a mouse's tail can easily tear, so it is best to grasp only the base of the tail. Furthermore, suspending the entire weight of a mouse by its tail is, no doubt, painful for the mouse. Therefore, the practice should be only momentary. Tail-lifting a mouse that is grasping a fabric (wire mesh, etc.) may injure the tail and may also break or tear the toenails. The best way to pick up a pet mouse is to place one hand over the back, just behind the head, gently grasp it around the rib case and lift it upward. Then the mouse can be gently cradled against the handler's body, using minimal restraint.

Housing of Experimental Animal in the Laboratory

The experimental mice were housed within enclosures made of wire and durable plastics. The construction and design of the enclosure was ensured that the resident(s) could not escape. Furthermore, the enclosure was free of sharp edges and other potential hazards. The enclosure was roomy enough to allow the mice to pursue normal movement. Enclosures should be easy to clean, well lighted and adequately ventilated.

Determination of Median Lethal Dose (LD_{50}) of Arsenic by using its Various Doses of Arsenic on Mice

In this experiment, mice were fed with normal diet and normal physiological condition was maintained. During experiment, the drug preparations (Na arsenite) were administered intraperitonially. First, mice from a selected group were taken and appropriate condition for injection was made by glove-weared hand. Then drug preparations in syringe were taken and injected intraperitonially with a selected dose. Then rest of the mice in that group treated with the same dose. Accordingly, rest of the group was treated with other dose selectively. Mainly 1 mL from stock solution for each mouse was used. The cages were marked and labeled for proper identification. Their condition was monitored for time to time. More specifically 12 h and then after 24 h, the results were observed and marked. The injected mice were observed for 2 h and then occasionally for further 4 h. Finally overweight and mortality was recorded. Determination of Median Lethal Dose (LD_{50}) of Arsenic by using its various doses of arsenic on mice shown in Table 1.

Collection of Blood

Blood from the experimental animals collected by syringe in selected small glass tubes containing heparin solution and shook the tube to mix the blood with heparin so that coagulation is inhibited. After sacrificing the animal, blood was collected by heart puncture.

Collection of Serum

For preparing serum, blood was collected in glass tube and allowed to clot at 37°C for 1 h and centrifuged. The clear supernatant was removed with Pasteur pipette and stored in vials at -20°C.

Estimation of Blood Glucose Level in Mice

At first seven test tubes were labeled as 20, 40, 60, 80 and 100 μg mL^{-1}; one for blank and another for blood. Then blood was collected by syringe in a small glass tube containing heparin solution and shook the tube to mix the blood with heparin so that coagulation is inhibited. In the blood marked tube 0.5 mL of blood and 8.5 mL distilled water were taken. Added 0.5 mL of 10% Na tungstate and 0.5 mL of 2/3 N H_2SO_4 and it is mixed well for complete precipitation of protein. After that to sediment the precipitated proteins and to obtain supernatant fluid, the mixture were centrifuged at a maximum speed for 5 min. Then 1 mL of supernatant liquid were taken by 1 mL pipette into a test tube containing 1 mL Fehling's solution (Fehling solution were prepared *in situ* by mixing 25 mL solution A and 1 mL of B) and then heated in a water bath for 30 min. After cooling, 1 mL of arsenic molybdate and 22 mL of distilled water were added and mixed well. Finally the absorbance at 520 nm is measured and the concentration of glucose has been determined using standard curve.

Collection and Weight of Internal Organs of Animal

After collection of blood, the abdomen and thorax of the animals was opened (cut) and spleen, liver, kidney, heart and lung were collected and weighed individually by electronic balance.

Table 1: Determination of LD_{50} of arsenic by using its various doses of arsenic on mice

Na arsenite (g/100 mL)	No. of animals	No. of survivals	No. of death	LD_{50} (g/100 mL)
0.2500	6	0	6	0.015
0.2000	6	0	6	
0.1500	6	0	6	
0.1000	6	1	5	
0.0500	6	2	4	
0.0150	6	3	3	
0.0125	6	4	2	
0.0100	6	5	1	
0.0090	6	6	0	
0.0050	6	6	0	

RESULTS

Change of Blood Glucose Level

The experimental animals were grouped in four. One group of the animals was fed by a standard diet quantity sufficient and designated as Group-I. The second group (Group-II) was fed with normal diet and arsenic. Arsenic and folic acid mixed diet were given to the third group (Group-III). The fourth group (Group-IV) was fed by vitamin C mixed diet and arsenic. The animals were sacrificed after 12 weeks of experimental feeding.

At the end of the feeding schedule, respective blood glucose levels were determined after sacrificing them. The average blood glucose level of Group-I, Group-II, Group-III and Group-IV are shown in Table 2.

Growth of the Animals

The growth pattern of the controls, arsenic group, folic acid and vitamin C groups were significantly different. The relative changes in body weight during the first one weeks of the feeding period were almost similar, but after then the change among the four groups appeared to be markedly different. The control and group-III and IV continued a pattern of steady weight gain whereas the arsenic induced group showed decreased growth pattern compared to others.

Weight Change of Internal Organs

After collection of blood, the abdomen and thorax of the animals were opened and spleen, liver, kidney, heart and lung were collected and weighs individually by electronic balance. The weights of different organs are given in Table 3-7.

After sacrifice, the average blood glucose level of control group (Group-I), arsenic induced group Group-II), folic acid group (Group-III) and vitamin C group (Group-IV) are 6.53, 9.37, 6.73 and 7.03 mmol L^{-1}, respectively. Blood glucose level of Group-III was close to that of the Group-IV.

Table 2: Concentration of blood glucose for different group of animals

Animals[a]	Glucose concentration (mmol L^{-1})	p-value
Group-I	6.53±0.15[b]	vs Group-II Sig[d]
	(6.40-6.70)[c]	vs Group-III NS[e]
		vs Group-IV Sig[d]
Group-II	9.37±0.21[b]	vs Group-III Sig[d]
	(9.20-9.60)[c]	vs Group-IV Sig[d]
Group- III	6.73+0.15[b]	vs Group-IV NS[e]
	(6.60-6.90)[c]	
Group-IV	7.03±0.15[b]	
	(6.90-7.20)[c]	

[a]Six animals were used; [b]Values are Mean±SD; [c]Range of individual value; [d]Significant (The mean difference is significant at the 0.05 level); [e]Not significant

Table 3: Weight of spleen and the ratio of the spleen to body weight of mice for different groups

Animals[a]	Weight of spleen (mg)	Weight of spleen/Animal wt. (mg g^{-1})	p-value
Group-I	74.82±0.423[b]	1.8 1±0.16	vs Group-II Sig[d] Sig[d]
	(74.33-75.10)[c]	(1.62-1.92)	vs Group-III Sig[d] Sig[d]
			vs Group-IV Sig[d] Sig[d]
Group-II	101.05±1.63[b]	2.74±0.19	vs Group-III Sig[d] NS[e]
	(99.20-102.25)[c]	(2.52-2.92)	vs Group-IV Sig[d] NS[e]
Group-III	125.08±2.80[b]	3.11±0.45	vs Group-IV Sig[d] NS[e]
	(122.20-127.80)[c]	(2.66-3.55)	
Group-IV	119.30±0.66[b]	3.11±0.13	
	(118.70-120.20)[c]	(2.98-3.24)	

[a]Six animals were used; [b]Values are Mean±SD; [c]Range of individual value; [d]Significant (The mean difference is significant at the 0.05 level); [e]Not significant

Table 4: Weight of liver and the ratio of the liver to body weight of mice for different groups

Animals[a]	Weight of liver (mg)	Weight of liver/Animal wt. (mg g^{-1})	p-value
Group-I	1171.97±2.15[b]	28.27±2.40	vs Group-II Sig[d] Sig[d]
	(1170.30-1174.40)[c]	(25.53-30.01)	vs Group-III Sig[d] Sig[d]
			vs Group-IV Sig[d] Sig[d]
Group-II	1566.29±6.91[b]	42.41±2.34	vs Group-III Sig[d] NS[e]
	(1560.64-1574.20)[c]	(40.01-44.69)	vs Group-IV Sig[d] Sig[d]
Group-III	1915.89±3.15[b]	47.58±5.72	vs Group-IV Sig[d] Sig[d]
	(1912.30-1918.20)[c]	(41.68-53.11)	
Group-IV	2241.87±3.21[b]	58.54±2.24	
	(2238.18-2244.00)[c]	(56.10-60.49)	

[a]Six animals were used; [b]Values are Mean±SD; [c]Range of individual value; [d]Significant (The mean difference is significant at the 0.05 level); [e]Not significant

Table 5: Weight of kidney and the ratio of the kidney to body weight of mice for different groups

Animals[a]	Weight of kidney (mg)	Weight of Kidney/Animal wt. (mg g^{-1})	p-value
Group -I	361.77±2.94[b]	8.72±0.69	vs Group-II Sig[d] Sig[d]
	(358.60-364.40)[c]	(7.92-9.19)	vs Group-III Sig[d] NS[e]
			vs Group-IV Sig[d] Sig[d]
Group-II	426.26±5.15[b]	11.54±0.53	vs Group-III Sig[d] Sig[d]
	(420.46-430.32)[c]	(10.97-12.01)	vs Group-IV Sig[d] NS[e]
Group-III	385.23±4.07[b]	9.57±1.23	vs Group-IV Ns[e] NS[e]
	(381.55-389.60)[c]	(8.36-10.82)	
Group-IV	392.63±2.13[b]	10.25±0.45	
	(390.24-394.33)[c]	(9.76-10.62)	

[a]Six animals were used; [b]Values are Mean±SD; [c]Range of individual value; [d]Significant (The mean difference is significant at the 0.05 level); [e]Not significant

Table 6: Weight of heart and the ratio of the heart to body weight of mice for different groups

Animals[a]	Weight of heart (mg)	Weight of heart/Animal wt. (mg g^{-1})	p-value
Group-I	139.35±0.97[b]	3.36±0.30	vs Group-II Sig[d] NS[e]
	(138.60-140.44)[c]	(3.02-3.60)	vs Group-III Sig[d] Sig[d]
			vs Group-IV Sig[d] Sig[d]
Group-II	146.69±1.54[b]	3.97±0.22	vs Group-III Sig[d] Sig[d]
	(145.42-148.40)[c]	(3.73-4.17)	vs Group-IV Sig[d] Sig[d]
Group-III	194.78±1.32[b]	4.84±60	vs Group-IV Sig[d] NS[e]
	(193.33-195.90)[c]	(4.24-5.44)	
Group-IV	201.89±4.32[b]	5.27±0.24	
	(198.72-206.81)[c]	(5.00-5.44)	

[a]Six animals were used; [b]Values are Mean±SD; [c]Range of individual value ; [d]Significant (The mean difference is significant at the 0.05 level); [e]Not significant

Table 7: Weight of lung and the ratio of the lung to body weight of mice for different groups

Animals[a]	Weight of lung (mg)	Weight of lung/Animal wt. (mg g^{-1})	p-value
Group-I	218.95±1.81[b]	5.28±0.49	vs Group-II Sig[d] Sig[d]
	(217.18-220.80)[c]	(4.72-5.66)	vs Group-III Sig[d] Sig[d]
			vs Group-IV Sig[d] Sig[d]
Group-II	136.39±1.21[b]	3.69±0.23	vs Group-III Sig[d] Sig[d]
	(135.00-137.22)[c]	(3.46-3.91)	vs Group-IV Sig[d] Sig[d]
Group-III	279.72±1.61[b]	6.95±0.87	vs Group-IV Sig[d] NS[e]
	(278.00-281.20)[c]	(6.08-7.81)	
Group-IV	242.36±2.19[b]	6.32±0.24	
	(240.22-244.60)[c]	(6.05-6.49)	

[a]Six animals were used; [b]Values are Mean±SD; [c]Range of individual value ; [d]Significant (The mean difference is significant at the 0.05 level); [e]Not significant

DISCUSSION

The internal organs such as spleen, liver, kidney, heart of arsenic group appeared increased in weight compared to those in control group animals. But in case of lung, lung of arsenic group animals appeared decreased in weight compared to those in control groups. The weight of spleen, liver, heart

and lung of folic acid and vitamin C group increased compared to that of the control and arsenic induced group. It is highly mentionable that the weight of kidney of arsenic group is higher than that of the control, folic acid and vitamin C groups.

In case of control group, the average weight of spleen, liver, kidney, heart and lung were 74.81, 1171.97, 361.77, 139.35 and 218.95 mg, respectively. For arsenic induced group, the average weight of spleen, liver, kidney, heart and lung were 101.05, 1566.29, 426.26, 146.69 and 136.39 mg, respectively. For folic acid group, the average weight of spleen, liver, kidney, heart and lung were 125.08, 1915.89, 385.23, 194.78 and 279.72 mg, respectively. Similarly for vitamin C group, the average weight of spleen, liver, kidney, heart and lung were 119.30, 2241.87, 392.63, 201.89 and 242.36 mg, respectively.

Urinary creatinine increased after 12 weeks of folic acid supplementation was somewhat surprising but it is consistent with the requirement of folate for creatine biosynthesis (Gamble *et al*., 2006). This investigation shows the similar result, because vitamin C lowered the arsenic induced higher blood glucose level. Similarly folic acid also lowers the arsenic induced higher blood glucose level in mice.

In a study of folate-depleted elderly women, genomic DNA hypomethylation began to respond to a similar regimen of folic acid repletion only after 7 weeks (Rampersaud *et al*., 2000), which is consistent with kinetic estimates of a very slow turnover of whole-body folate pools (Steinmaus *et al*., 2005). In the current study suggest that folic acid supplementation may help to reduce the overall body burden of arsenic. We speculate that the schedule for urine collections missed a more substantial early peak of arsenic excretion in response to folic acid. What effect may we expect folic acid supplementation to have on arsenic-related health outcomes? Studies in Taiwan reported that persons who have a low secondary methylation index (i.e., SMI≤5) and who are exposed to high concentrations of arsenic are at greater risk of skin and bladder cancers than are persons with an SMI >5 (Chen *et al*., 2003a, b; Hsueh *et al*., 1997; Yu *et al*., 2000; Tseng *et al*., 2005).

A case-control study in West Bengal, India, found a modest increase in the risk of arsenic-induced skin lesions in persons who fell within the lowest quintiles for dietary intake of animal protein, folate, calcium and fiber (Mitra *et al*., 2004), which is supported to this study. It is now established that folic acid and vitamin C are essential to lower the risk and incidence of various types cancer caused by arsenic.

As evident from the above discussion, folic acid and vitamin C have their capacity, individually, for reducing arsenic induced higher blood glucose level. So, the folic acid and vitamin C have anti-diabetogenic effects. In addition, they have remarkable positive effect in changing the weight of arsenic induced internal organs. Apart from folic acid and vitamin C, a lot of vitamins such as vitamin A, vitamin E, vitamin B etc., may have effect like folic acid and vitamin C on arsenic induced diseases. Further researches are essential to know the effect of other vitamins whether they may be used as dietary additives in arsenicosis. Future studies of the folic acid and vitamin C on arsenic induced disease may give a clear picture of the role of folic acid and vitamin C for the prevention of arsenic induced diseases.

ACKNOWLEDGMENTS

We thank Professor Mohammed Rahmatullah, Former Head, Biotechnology and Genetic Engineering (BGE) Discipline, Khulna University, Khulna, Bangladesh for providing important instructions for this experiment and kind consent for using Animal Laboratory, BGE Discipline, Khulna University. We also thank M.T.H. Khan, Ph.D School of Molecular and Structural Biology and Department of Pharmacology, University of Tromsø, Norway, for critically revising the manuscript.

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