

Sub-chronic arsenic exposure aggravates nephrotoxicity in experimental diabetic rats

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Received 16 December 2009

The present experiment was planned to study nephrotoxicity in experimental diabetic rats under sub-chronic exposure to arsenic. Alloxan induced diabetic and control rats were exposed to sodium arsenite (0 and 5.5 mg/kg, orally) for 30 days. More pronounced nephrotoxic effects were noted in arsenic exposed diabetic group as evidenced by increased blood urea nitrogen, serum creatinine and relative kidney weight and decreased level of reduced glutathione and glutathione peroxidase activity compared to non arsenic exposed diabetic group. Increased level of lipid peroxidation, protein oxidation, superoxide dismutase and catalase activities under diabetic condition remained unchanged in arsenic exposed diabetic group compared to unexposed diabetic group.

Keywords: Arsenic, Diabetes mellitus, Oxidative stress, Renal tissue

Arsenic, one of the most toxic metalloids, is a ubiquitous in the environment. Globally, millions of people are being exposed to inorganic arsenic through consumption of contaminated drinking water and food¹. Inorganic forms of arsenic are more toxic than the organic forms. Arsenic and arsenic containing compounds are considered as a potent human carcinogen, associated with cancers of skin, lung, bladder, liver and kidney². Additionally, a variety of non-cancerous conditions such as diabetes mellitus, hypertension, neurological effects and cardiovascular disease have been associated with chronic exposure of high levels of arsenic in drinking water³. Recently, various experimental and epidemiological studies have been shown an association between chronic arsenic exposure and development of diabetes mellitus⁴. More over people exposed to arsenic are reported to have an increased risk of diabetes mellitus and its related micro vascular disease including renal diseases. Additionally, long term exposure to arsenic is capable of causing renal dysfunction including nephritis, nephrotic syndrome and nephrosis⁵.

Diabetes mellitus is a group of metabolic diseases characterized by hyperglycemia resulting from decrease insulin secretion, insulin resistance, or both. This condition leads to various pathological changes in

many tissues which can be suspected of increasing susceptibility to toxic hazards. It has been well known that renal dysfunction in diabetic condition eventually goes on to develop significant nephropathy⁶. The significant portion from diabetic population is also being exposed to arsenic. Arsenic toxicity to internal organs including lung dysfunction, neuropathy and nephrotoxicity are clinically evident⁷. Kidney is the most susceptible organ to arsenic exposure as it is involve with arsenic accumulation or excretion⁸. Toxic effects of arsenic are attributed to its ability to induce oxidative stress leading to enhanced production of reactive oxygen species (ROS), that results in alter antioxidant defense system, increased oxidative stress and cell death⁹. On the other hand, recent studies suggest that arsenic induced nephrotoxicity by disturbing antioxidant defense system, protein oxidation and lipid peroxidation products which ultimately produce oxidative stress in kidney.

Currently, susceptibility to toxic hazards in diabetic population, such high-risk group is of an increasing concern. Diabetic rats are more susceptible to cadmium nephrotoxicity than normal rats¹⁰. It is of interest to investigate the susceptibility of experimental diabetic animals to arsenic induced nephrotoxicity. So, the present experiment was performed to study nephrotoxic and oxidative stress response of sub-chronic arsenic exposure on alloxan induced diabetic animals which are more susceptible to adverse effect of toxic hazards.

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Materials and Methods

Chemicals—Sodium arsenite was purchased from Loba Chemie (Bombay, India). 5,5'-Dithiobis(2-nitrobenzoic acid) (DTNB), reduced glutathione (GSH), 2-thiobarbituric acid (TBA), phenazine methosulphate (PMS), nitro blue tetrazolium (NBT), 2,4-dinitrophenyl hydrazine (DNPH), guanidine hydrochlorides and alloxan monohydrate were procured from Hi-Media Chemicals (Bombay, India). All other reagents and chemicals were used of higher purity and analytical grade.

Animal maintenance—The present experiment was performed on an adult male and female Wistar albino rats ($n = 26$) weighing about 110 ± 10 g with an approval from Animal Research Advisory Committee of Sardar Patel University. All animals were obtained from inbred colony maintained in the air-conditioned animal house of Sardar Patel University, Vallabh Vidyanagar, India. Animals were housed in polypropylene cages under environmentally controlled conditions at 25° - 30° C under 12 h dark/light cycles. The animals were fed with standard pellet diet (Amrut Feeds Pranav Agro, Pune) and water *ad libitum*. All the animal experiments were performed according to the guidelines laid by Institutional Animal Ethical Committee (IAEC).

Induction of experimental diabetes—The animals were fasted overnight and diabetes was induced with single intraperitoneal injection of freshly prepared solution of alloxan monohydrate (150 mg kg^{-1} body wt.) in normal sterile saline just before use¹¹. Two days after alloxan administration, blood glucose was measured using glucose oxidase enzymatic kit (Eve's Diagnostics, Baroda). Rats with blood glucose level above 200 mg/dl were considered as diabetic rats and used for further studies.

Animal treatment—The diabetic and normal animals were divided into four groups comprising six animals in each group. Group I (Control): rats received normal saline orally; Group II (Arsenic exposed): rats administered with sodium arsenite (5.5 mg kg^{-1} body weight day⁻¹) orally; Group III (Diabetic): Alloxan (150 mg/kg body weight; single ip injection) induced diabetic rats; and Group IV (Diabetic+ Arsenic exposed): Diabetic rats administered with sodium arsenite (5.5 mg kg^{-1} body weight day⁻¹) orally.

Arsenic treatment was given for 30 days. For sub-chronic oral exposure to arsenic, a dose of 5.5 mg kg^{-1} body wt day⁻¹ was selected which was

lesser than one thirteenth of LD₅₀ value of rats (40 mg/kg body weight)¹². At the end of the experimental period, all animals were fasted overnight and sacrificed by light ether anaesthesia. Blood samples were collected by cardiac puncture and serum was separated by centrifugation at $600 \times g$ for 15 min. Both kidneys were carefully removed, rinsed in cold saline, blotted and weighed on a digital balance. One kidney was kept at -20° C for wet digestion and arsenic content analysis. Other kidney tissue was homogenized using potter and elvehjem type of glass homogenizer with Teflon pestle. Homogenate (10%) was prepared in 50 mM of phosphate buffer (pH 7.4) and centrifuged at 10,000 rpm for 10 min. Supernatant was used for various biochemical assays. Protein concentration was estimated according to the method of Lowry *et al.*¹³ using bovine serum albumin (BSA) as a standard.

Assessment of serum markers for kidney dysfunction—Creatinine and blood urea nitrogen (BUN), serum markers for kidney dysfunction were estimated following Jaff's method and Berthelot method with modification by Fawcett and Scott, respectively using commercially available kit. (Eve's diagnostics ltd., Baroda)

Biochemical markers of oxidative stress—The renal lipid peroxidation was measured as malondialdehyde (MDA) according to the method described previously¹⁴ and expressed as nmole/mg of protein. The protein carbonyl (PCO) content of the renal tissue was assayed by the method of Reznic and Packer.¹⁵ The carbonyl content was expressed in terms of nmole/mg protein using the molar extinction coefficient of DNPH ($\epsilon = 2.2 \times 10^4 \text{ cm}^{-1} \text{ M}^{-1}$). Advanced oxidation protein products (AOPP) level¹⁶ (expressed in nmole/mg of protein); super oxide dismutase (SOD) activity¹⁷ (expressed as unit/min/mg of protein); catalase activity¹⁸ (expressed as unit/min/mg protein; One unit represents $1 \mu\text{mole}$ of H_2O_2 consumed/min/mg protein) were estimated as described elsewhere. The total activity of glutathione peroxidase (GPx)¹⁹ was also determined and expressed as unit/min/mg protein. One unit can be defined as $1 \mu\text{mole}$ GSH oxidized/min/mg of protein. The reduced glutathione (GSH) was determined by the method of Ellman modified by Jollow *et al.*²⁰ and expressed as mg of GSH/100 g tissue using reduced GSH as standard.

Tissue arsenic estimation—Kidney tissue was digested with acid mixture (H_2SO_4 : HNO_3 : HClO_4 in ratio 1:6:1) using a microwave pressure digestion

unit. (Berghof speed wave MWS -3+, Germany) The arsenic content of digested products was determined using atomic absorbance spectrophotometer (WinASS novAA400, Analytik Jena AG, Germany) using sodium arsenite (99%) as standard and results were expressed as $\mu\text{g/g}$ tissue.

Statistical analysis—All the values are represented as mean \pm S.D. ($n = 6$). Data was analyzed using SPSS 11.0 for windows. The significance was calculated using one-way analysis of variance (ANOVA) followed by LSD (Least Significance Difference) test. Values were considered statistically significant at $P < 0.05$ or less.

Results

Effect of arsenic on body weight, organ weight and blood glucose—Per cent change in body weight, kidney weight and relative kidney weight (RKW) of control and experimental groups has been summarized in Table 1. There was progressive increase in body weight of arsenic exposed group, but it was significantly ($P < 0.05$) lower as compared to control group at the end of the experiment. Further, diabetic rats showed a significant reduction in body weight ($P < 0.001$) as compared to control which did not decline further by arsenic exposure.

Kidney weight/body weight ratio, an index of renal hypertrophy increased significantly ($P < 0.001$) in all experimental groups compared to control group. The kidney weight/body weight ratio was significantly elevated in arsenic exposed diabetic group as compared to unexposed diabetic group indicating increased nephrotoxicity in arsenic exposed diabetic group.

The blood glucose level was elevated by 44.14% and 195% in arsenic exposed group and diabetic group, respectively compared to control group. The hyperglycemic condition was more pronounced in arsenic exposed diabetic rats as indicated by significant increased blood glucose level compared to unexposed diabetic rats (Table 2).

Effect of arsenic on serum markers for kidney function—Effects of arsenic on kidney function were assessed by serum creatinine and blood urea nitrogen content (Fig. 1). The serum creatinine and BUN level were significantly elevated in arsenic exposed rats and to greater extent in unexposed diabetic group compared to control. The diabetic group exposed to arsenic showed significant increase in serum creatinine and BUN level compared to unexposed diabetic rats. These results demonstrated that arsenic accelerated renal dysfunction in diabetic animals.

Table 1—Effect of arsenic on body weight, kidney weight and relative kidney weight of control and experimental rats
[Values are mean \pm SD of 6 animals]

Groups	Body wt			Absolute kidney wt (g)	Relative kidney wt (g/100 g body wt)
	Initial (g)	Final (g)	Change (%)		
Control	113 \pm 5.45	171.2 \pm 4.96	50.4 \pm 5.51	1.2 \pm 0.12	0.7 \pm 0.066
Arsenic	115 \pm 4.69	151.8 \pm 5.63*	32 \pm 5.29*	1.27 \pm 0.025	0.84 \pm 0.031**
Diabetic	111 \pm 5.43	101.8 \pm 7.79**	-8.2 \pm 2.87**	1.35 \pm 0.05*	1.3 \pm 0.11**
Diabetic + Arsenic	113.4 \pm 5.5	102.6 \pm 6.91** a	-9.95 \pm 4.54** a	1.41 \pm 0.045** a	1.45 \pm 0.06** †

* $P < 0.05$ and ** $P < 0.001$ denotes value significantly different from control

Arsenic treated diabetic group was compared with untreated diabetic group and the value was significantly different at † $P < 0.05$,
a non-significant

Table 2—Effect of chronic arsenic exposure on blood glucose, lipid peroxidation and protein oxidation level in kidney tissue of control and experimental rats
[Values are mean \pm SD of 6 animals]

Parameters	Treatment			
	Control	Arsenic	Diabetic	Diabetic + Arsenic
MDA (nmol/mg of protein)	0.81 \pm 0.087	1.21 \pm 0.078*	1.97 \pm 0.21**	2.20 \pm 0.38** a
AOPP (nmol/mg of protein)	0.21 \pm 0.011	0.29 \pm 0.032**	0.4 \pm 0.024**	0.42 \pm 0.007** a
PCO (nmol/mg of protein)	1.33 \pm 0.058	2.68 \pm 0.33**	2.91 \pm 0.37**	3.05 \pm 0.064** a
Blood glucose (mg/dl)	92.5 \pm 5.4	137.6 \pm 4.82**	281.9 \pm 5.56**	298.5 \pm 8.46** †

* $P < 0.05$ and ** $P < 0.001$ denotes value significantly different from control

† Denotes a significant ($P < 0.05$) difference; a non-significant between arsenic treated diabetic group and untreated diabetic group

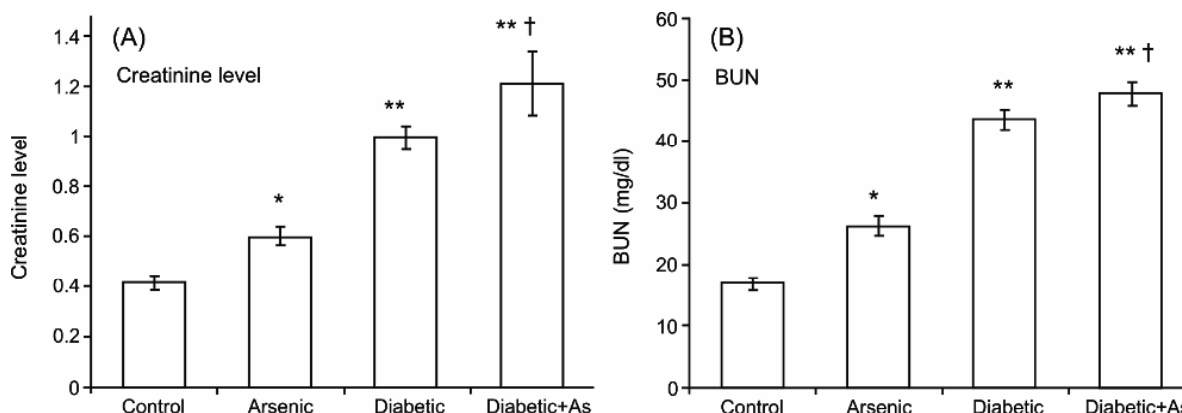


Fig. 1—Effect of arsenic on (A) Serum creatinine; and (B) BUN level of control and experimental groups (n=6). [value differs significantly at *P < 0.05 and **P < 0.001 as compared to control group. † Denotes a significant (P < 0.05) difference between untreated diabetic and arsenic treated diabetic groups]

Table 3—Effect of arsenic administration on antioxidative enzymes activities, reduced GSH level and arsenic content in kidney tissue of control and experimental rats [Values are mean ± SD of 6 animal]

Parameters	Treatment			
	Control	Arsenic	Diabetic	Diabetic +Arsenic
SOD (U/mg of protein)	1.3 ± 0.11	1.85 ± 0.05**	2.0 ± 0.23**	2.1 ± 0.07** a
CAT (U/mg of protein)	26.96 ± 2.89	39.5 ± 4.81**	50.36 ± 5.98**	54.71 ± 4.03** a
GPx (U/mg of protein)	9.84 ± 0.95	7.07 ± 1.21**	5.66 ± 0.35**	4.22 ± 0.16** †
GSH (mg/100g tissue)	29.87 ± 1.86	18.14 ± 1.93**	14.68 ± 0.86**	12.51 ± 0.73** †
Arsenic content (µg/g tissue)	0.12 ± 0.08	27.31 ± 4.89**	0.13 ± 0.09	25.85 ± 5.23** a

*P < 0.05 and **P < 0.001 denotes value significantly different from control rats

Arsenic treated diabetic group was compared with untreated diabetic group and the value was significantly different at † P<0.05;

^anon-significant

Effect of arsenic on antioxidants status—Table 2 depicts the level of MDA as an index of lipid peroxidation, levels of AOPP and PCO indices of protein oxidation in the kidney tissue of control and experimental groups. There was significant elevation of renal MDA, AOPP and PCO levels in diabetic and arsenic exposed group as compared to control, but this trend was higher in the unexposed diabetic group than the arsenic exposed group. Further, arsenic exposure to diabetic group exhibited non-significant increase in kidney MDA, AOPP and PCO levels as compared to its corresponding unexposed diabetic group.

Table 3 demonstrates the level of reduced GSH, GPx, SOD and CAT activities, indicative of the anti-oxidant status and generation of oxidative stress in the kidney tissue of control and experimental animals. SOD and CAT activities in the kidney tissue were significantly elevated in arsenic exposed group as well as in unexposed diabetic group compared to control. In contrast, these antioxidant enzymes activities were non-significantly increased in arsenic

exposed diabetic rats as compared to unexposed diabetic group. GPx activity, a major antioxidant enzymatic system and reduced glutathione (GSH) were significantly decline in kidney tissue of arsenic exposed and unexposed diabetic rats compared to control rats, whereas, GPx activities (by 25.44%) and reduced glutathione (by 14.8%) level were declined significantly in arsenic exposed diabetic group compared to unexposed diabetic group. A significant amount of arsenic was observed in kidney tissue after arsenic exposure in arsenic exposed group, which was not much altered in arsenic exposed diabetic group (Table 3).

Discussion

The present study showed that sub-chronic exposure to arsenic and alloxan induced diabetic rats exhibited nephrotoxicity and oxidative stress in kidney tissue as indicated by increased relative kidney weight, BUN, creatinine, altered oxidant products and anti-oxidative enzymes activities, but these trends were more

pronounced in alloxan induced diabetic rats than arsenic exposed rats. The present study also demonstrated the exaggerated effect of arsenic induced nephrotoxicity in diabetic animals, but the exact mechanisms underlying this effect remained unclear.

There are relatively few clinical reports available on arsenic induced nephrotoxicity in comparison with those of lead and cadmium. Exposure to arsenic or its various forms like AsH₃ can lead to induce nephrotoxicity in experimental animals²¹⁻²². High concentration of arsenic has been accumulated in the kidney tissue than other tissue through various exposure routes²³. Kidney is the major route for the excretion of arsenic and its metabolites from the body and a major site for the biotransformation of arsenic that ultimately makes more sensitive organ to arsenic exposure^{2,24}. Experimental diabetic rats are characterized by severe loss in body weight, elevated blood glucose level and increased relative kidney weight,²⁵ which was also observed in the present study. In the present study, arsenic exposure to diabetic animals led to significant increase in relative kidney weight (RKW) and blood glucose which might be due to the diabetogenic effect of arsenic and protein wasting²⁶. Nephrotoxicity was evaluated from increased BUN level, marker of proximal tubule injury and creatinine level, marker of glomerular injury. Degree of nephrotoxicity was more pronounced in diabetic rats than arsenic exposed rats as evident from higher level of BUN and creatinine. However, arsenic exposure to diabetic rats led to further increase in BUN and creatinine level indicated more pronounced nephrotoxic response (renal infliction) in arsenic exposed diabetic condition due to persistence of arsenic exposure⁸.

Increased SOD and CAT activities in arsenic exposed group and unexposed diabetic group are in agreement with previous findings whereby alloxan induced diabetic rats²⁷ and arsenic exposed rats⁸ have shown marked increased SOD and CAT activities in the kidney and liver. Lipid peroxidation was significantly increased in kidney tissue of diabetic rat and arsenic exposed rats as evinced by increased level of MDA. Results of this study also suggested that rat kidney was associated with protein oxidation as evinced by increased PCO and AOPP level in arsenic exposed rats and unexposed diabetic rats. Li *et al.*²⁸ have reported elevated level of AOPP and PCO, markers of oxidative injury of protein in chronic kidney disease (CKD). Accumulation of these

oxidized proteins (AOPP and PCO) in kidney tissue might contribute to inflammatory process, promotes renal fibrosis and renal dysfunction²⁹. However, arsenic exposure to diabetic rats could not further induce a significant change in some of these markers (SOD, CAT, MDA, AOPP and PCO) of oxidative stress in the present study.

In accordance with the earlier findings, present experiment also revealed the reduction of renal GSH content and GPx activity due to arsenic administration. Decrease GSH content due to arsenic toxicity simultaneously decreased the activities of GPx³⁰. In a previous studies, GSH level has been reported to reduce in diabetic patients and in experimental diabetic models³¹. The renal glutathione redox status was greatly impaired on arsenic exposure to diabetic animals as indicated by a significantly reduced GSH level and GPx activity compared to unexposed diabetic animals. Reaction with protein thiols group and overproduction of reactive oxygen species (ROS) are the major mechanisms by which arsenic exerts its toxicity⁹. Arsenic binds with sulfhydryl groups of proteins and various enzymes and also interferes with metabolism of GSH that serves as an essential antioxidant molecule responsible for metabolism and detoxification of xenobiotic³⁰. Supplementation and maintenance of GSH pool in the kidney is an important for normal kidney function and also in determining susceptibility to chemically induced cytotoxicity. The significant decline in renal GPx activity in arsenic exposed diabetic rats might be results from gradual damage of an active part of nephron which is responsible for biosynthesis of this enzyme. A negative correlation was established between GPx activity and plasma creatinine level as well as between GPx activity and urea nitrogen level in chronic kidney disease (CKD) patients³². High-order thiol cell system like metallothionein, thioredoxine and other redox regulator proteins are ultimately regulate by GSH level.

Hyperglycemic condition was observed more profoundly increased in arsenic exposed diabetic rats compared to unexposed diabetic rats. The persistence of hyperglycemic condition might be stimulating formation of AGEs (Advanced glycation end products) and other metabolic mediator which can cause significant damage in endothelium of kidney tissue³³. Arsenic exposure leads to accumulation of copper in kidney tissue³⁴ that may have accelerated glycation and AGEs formation in the kidney. Additionally, chronic exposure to arsenic leads to

increased renal hexokinase II (HK-II) expression²⁴ which might be playing an important role in arsenic induced pathological changes in the kidney tissue under diabetic condition. Arsenic exposure produced hemolytic product in the rat kidney shows cytotoxic to the cortical epithelial cell²².

It has been known that reactive oxygen species and oxidative damage of biological macromolecules play an important role in arsenic induced nephrotoxicity³⁵. Recently, studies carried out by Wang *et al.*³⁶ have been reported higher level of urinary N-acetyl- β -glucosaminidase (NAG), an early indication of kidney dysfunction, in arsenic exposed diabetic individuals than the control diabetes subjects. The results of this study suggested that kidney function was affected by arsenic in individual with diabetic condition. Inorganic arsenic may increase the risk of development of renal disease, especially in a diabetic subpopulation that is known to be more susceptible to the adverse effects of arsenic³⁷. Increased nephrotoxicity in arsenic exposed diabetic group did not seem to depend on arsenic accumulation as there was no further increase in arsenic accumulation was observed in arsenic exposed diabetic group as compared with arsenic exposure alone.

In conclusion, the present results suggested that sub-chronic arsenic exposure further accelerated the nephrotoxicity in diabetic group, which seemed to depend on compromised oxidative stress. However detailed studies are needed to confirm these observations.

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