13. Akagi, H., Yokozeki, Y., Inagaki, A. and Fujimura, T., *Theor. Appl. Genet.*, 1996, **93**, 1071–1077.
14. Akagi, H., Yokozeki, Y., Inagaki, A. and Fujimura, T., *Theor. Appl. Genet.*, 1997, **94**, 61–67.
15. Rholf, R. J., NTSYS-PC Version 2.0, Exeter Software, Setauket, New York, 1998.
16. Tanksley, S. and McCouch, S. R., *Science*, 1997, **277**, 1063–1066.
17. Das, S. R., Mukherjee, B. K. and Siddiq, E. A., Proc. Natl. Symp. Rice Research for 21st century: Challenges; Priorities and Strategies, Central Rice Research Institute, Cuttack, 1998.
18. Bhatt, G. M. and Derera, N. F., Proc. 4th Int. Wheat Genet. Symp., University of Missouri, Columbia, 1973, pp. 489–493.

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Arsenic trioxide generates oxidative stress and islet cell toxicity in rabbit

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An oral exposure of rabbits to arsenic trioxide, 1.5 mg/kg body wt/day in a single dose for 30 consecutive days changed the normal features of oral glucose tolerance test (OGTT) to a diabetic form. Estimated glycated haemoglobin values supported OGTT results. Light microscopic studies have revealed significant reduction in the number of cells in the islets of treated animals compared to control. Also, increased serum amylase activity and production of nitrite and malondialdehyde were observed in the pancreas of treated animals. It appears that an alteration of the normal features of OGTT to diabetic form by an oral exposure to arsenic possibly could be a reason for islet cell damage caused by an enhanced activity of oxidative stress producing enzymes and/or by their products.

CHEN *et al.*[1], through their human survey study, have provided an initial clue that arsenic might cause diabetes mellitus. A dose–response relationship between cumulative arsenic exposure and prevalence of diabetes mellitus was documented among the residents in the endemic arseniasis in Taiwan[2]. Similar dose-related increased risk of dying from diabetes mellitus with increasing arsenic exposure has been reported among exposed copper-smelter workers[3] and art-glass workers[4]. In a community-based survey of diabetes mellitus in Bangladesh, similar dose–response trend between the prevalence of diabetes mellitus and arsenic level in drinking water has also been reported[5]. In a more recent study, Tseng *et al.*[6] reported prevalence of diabetes in arseniasis-hyperendemic villages in Taiwan, which was significantly higher than that in the general population.

Arsenic has been suggested to be involved in cytotoxicity and genotoxicity by generation of nitric oxide (NO)[7] and lipid peroxide[8]. A relationship in between oxidative stress and development of diabetes mellitus has been proposed by many researchers[9,10]. Indeed, intraperitoneal injection of arsenic plus hydroxylamine has been reported to cause degeneration and necrosis of beta cells in mice[11].

In view of the above-cited literature and human survey reports, the present study was undertaken to test whether or not chronic oral exposure of rabbits to arsenic could develop toxic changes in the pancreatic islets to cause diabetes mellitus, and its possible mechanism of action.

Male albino rabbits weighing 1.4–1.5 kg were used for the experiments. They were divided into two groups: A (control) and B (experimental), consisting of five animals in each group. Animals of both groups were maintained in an environmentally controlled animal house (temperature 22 ± 3°C) on a standard laboratory diet and in a 12 h light/dark schedule with free access to water supply. During the treatment period, animals of group A were pair-fed with those of group B, so as to overcome the impact of any altered food intake in the experimental group (B). All animal experiments were carried out according to the ethical guidelines suggested by the Institutional Animal Ethics Committee. Animals of group B were orally treated with arsenic trioxide at a dose of 1.5 mg/kg body wt/day for 30 days. This dose is well within the range for the human lethal dose (1–4 mg/kg body wt) reported for arsenic[12].

After the experimental period was over (30 days), the animals were kept in overnight fasting. The next morning, blood samples were collected from prominent ear veins. After collection of blood, the animals of both groups were orally administered with 1 g of glucose/kg body wt. Thereafter, blood samples were collected at every 30 min interval for 2 h and 30 min. Glucose oxidase enzyme kit (E. Merck, India) was used for estimation of blood glucose from all these samples. Estimation of serum amylase was performed following the modified method of Somogyi, as described elsewhere[13]. The method utilizes the estimation of glucose liberated by the action of amylase on starch. Blood samples collected were mixed with EDTA (as per specification of the kit) and were used for estimation of glycated haemoglobin (HBA_{1c}) following the ion exchange resin-based method[14].

The animals were sacrificed and a small portion from the gastro-splenic part of the pancreas was quickly removed, weighed and placed in ice-cold Tris-HCl buffer (pH 7.4). It was kept on a small ice-slab and cut into small pieces with scissors and homogenized immediately, according to the method of Koyama *et al.*[15]. The homogenate was centrifuged at 15000 *g* for 20 min. The resulting

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supernatant of the first homogenate thereafter was retained for biochemical estimations.

The role of nitric oxide synthase was indirectly assessed by estimating the amount of nitric oxide produced by Griess reaction[16], which was expressed in the form of nitrite accumulation. In brief, 100 µl of tissue extract was mixed with 100 µl of Griess reagent [equal volumes of 1% (w/v) sulphanilamide in 5% (v/v) phosphoric acid and 0.1% (w/v) napthylethylenediamine HCl] and incubated at room temperature for 10 min, and then the product formed was assayed at 550 nm in a UV–Double Beam Spectrophotometer (Shimadzu 160A). The amount of nitrite in the samples was calculated in µM unit from a sodium nitrite standard curve.

The role of lipid peroxidase was assessed by studying the level of formation of malondialdehyde, an indicator of lipid peroxidation, by following the method as described elsewhere[17]. In brief, to 2 ml of tissue extract was added 1 ml of 20% trichloroacetic acid and 2 ml of 0.67% (w/v) thiobarbituric acid and the contents were heated in a lightly-stoppered tube for 10 min in a boiling water bath. It was cooled and then centrifuged to precipitate out the protein. The colour developed in the supernatant was assayed at 530 nm in a UV–Double Beam Spectrophotometer.

Pancreatic tissue both from control and experimental animals was selectively taken from the gastro-splenic portion and formol-fixed. Paraffin blocks were prepared, 4–5 µm thin sections were cut with a high-precision microtome (IEC Minotome, USA) and stained with haematoxylene–eosine[18]. Stained slides were light-microscopically examined.

Data are expressed as mean ± SE. Significance was determined by Student's *t*-test. Analysis was performed using PSI-PLOT, version 2.0 (Poly Software International; 1992, 1993) with a significance level of $P < 0.05$.

The effects of arsenic-treatment on glycemic response of rabbits are shown in Figure 1. Results indicate that after oral glucose administration, there was a marked difference in peak blood glucose level for control and arsenic-treated rabbits. They were found to be respectively, 163.2 ± 1.87 and 207.5 ± 2.44 mg glucose per dl blood. The time required for peak blood glucose rise in case of arsenic-treated group was 60 min, while in the control group it was more rapid and required only 30 min. In the control group, maximal percentile rise in blood glucose, compared to fasting level, was 107 at 30 min and thereafter the blood glucose level recovered gradually. When recovery glycemic responses were compared against maximal glycemic response, percentile recovery responses were 13, 43 and 71 respectively, at 60, 90 and 120 min, and at 150 min the blood sugar level almost returned to its pre-glucose administration level (94.5% recovery). In arsenic-treated animals, compared to fasting level, maximal percentile rise in blood glucose level was 188.6 at 60 min and thereafter, compared against maximal glycemic response, the percentile recovery was only 26, 33 and 38 respectively, at 90, 120 and 150 min. Results of glycated haemoglobin estimation are presented in Table 1. Results show that, compared to control, glycated haemoglobin level was significantly increased ($P < 0.001$) in all arsenic-treated animals. In the control it was 6.64 ± 0.34, which was within the expected values for non-diabetic condition, according to the kit specification. In arsenic-treated animals the level was 11.44 ± 0.48, which according to kit specifications showed poor glycemic control. Table 1 also shows the results of experiments for serum amylase activity of both control and arsenic-treated animals. Serum amylase activity is expressed in terms of Somogyi amylase unit/dl and, compared to control, in the arsenic-treated group it was found to increase significantly from 292 ± 31 to 421 ± 26 ($P < 0.001$).

Results of islet cell studies of both control and arsenic-treated groups of animals are shown in Figure 2 *a* and *b*. Compared to the control, the number of cells in the islets of arsenic-exposed group were markedly low.

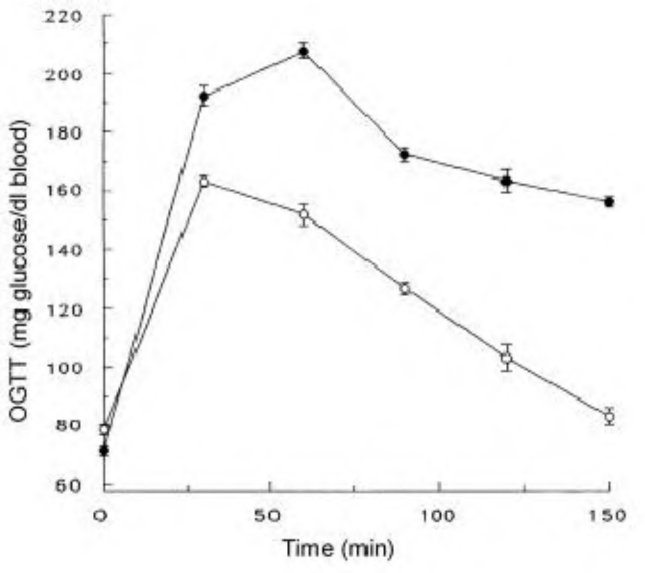

Figure 1. Oral glucose tolerance test (OGTT) in control and arsenic-treated (1.5 mg/kg body wt/day for 30 days) rabbits, expressed in terms of mg glucose per dl blood. Number of animals used in both control and arsenic-treated groups was 5. Open circles denote control and filled circles denote arsenic-treated animals.

Table 1. Effect of oral exposure of arsenic trioxide (As_2O_3; 1.5 mg/kg body wt/day for 30 days) on HbA_1 and serum amylase activity of rabbits

Parameter studied	Control	Arsenic-treated animals	Per cent increase
HbA_1 (%)	6.64 ± 0.34	11.44 ± 0.48*	72
Serum amylase (Somogyi amylase unit/dl)	292 ± 31	421 ± 26*	44

Values are expressed as mean ± SE ($n = 5$); *$P < 0.001$.

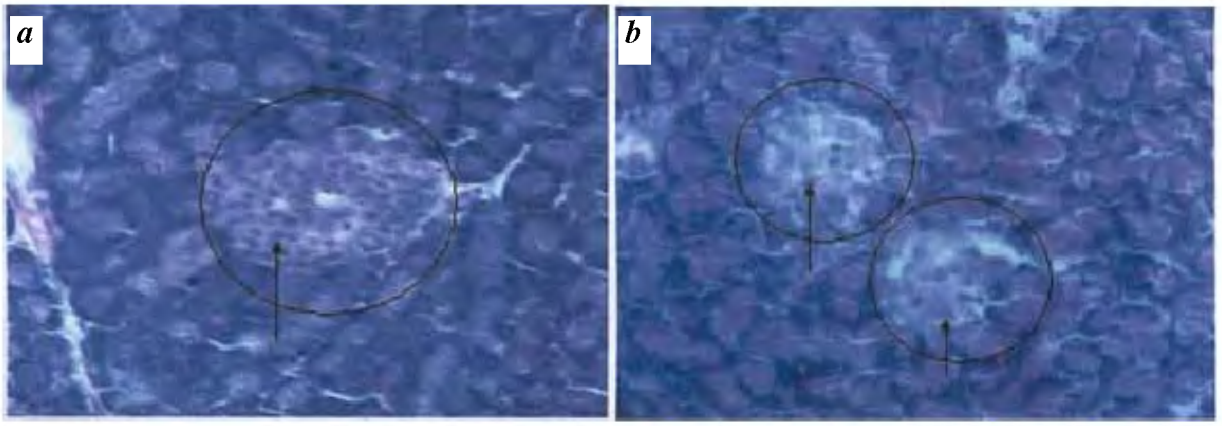

Figure 2. Haematoxylene–eosine stained section (× 50) showing morphology and population of cells in pancreatic islet of (*a*) control rabbits and (*b*) arsenic-treated (1.5 mg/kg body wt/day for 30 days) rabbits. *a*, Control: No pathologic changes were seen. Normal architecture of pancreatic islet and surrounding acini was vivid. Islet size and cell population were normal. Capillary number in islet seemed normal. *b*, Arsenic-treated: Typical characteristics of islet cell injury are present. Islets were shrunken, cell numbers were reduced, capillary number and diameter were increased.

Table 2. Effect of oral exposure of arsenic trioxide (As_2O_3; 1.5 mg/kg body wt/day for 30 days) on NO and MDA production in pancreatic tissue extract of rabbits

Parameter studied	Control	Arsenic-treated animals	Per cent increase
NO (μmol/mg wet tissue)	0.677 ± 0.03	1.212 ± 0.09*	79
MDA (nmol/mg wet tissue)	1.18 ± 0.102	1.83 ± 0.07*	55

Values are expressed as mean ± SE ($n = 5$); *$P < 0.05$.

Table 2 shows the results of experiments for nitrite accumulation in the pancreatic tissue of both control and arsenic-treated animals. Nitrite accumulation, an indicator of nitric oxide synthesis, was expressed in terms of μM/mg of tissue and, compared to control, in the arsenic-treated group it was found to increase significantly from 0.677 ± 0.03 to 1.212 ± 0.09 ($P < 0.01$). Table 2 also shows the results of lipid peroxidation in pancreatic tissue of both control and arsenic-treated animals. Alteration in malondialdehyde (MDA) formation, an indicator of lipid peroxidation, was expressed in terms of nM/mg of tissue and, compared to control, in the arsenic-treated group it was found to increase significantly from 1.18 ± 0.102 to 1.83 ± 0.07 ($P < 0.01$).

The present study revealed that oral exposure of arsenic trioxide possibly produces diabetes mellitus in rabbits, manifested by alteration of normal features of OGTT, viz. changes in peak glycemic response and a typical delay in recovery of normal blood glucose level (Figure 1). These changes thus corroborated well with the findings of earlier survey reports that arsenic might induce diabetes mellitus[1–5]. Results of glycated haemoglobin (Table 1) further substantiated our observations of OGTT, as glycated haemoglobin reflects on a relatively more precise index of the degree of diabetes than glucose itself[13]. This study further provided the experimental demonstration that in rabbits, chronic oral exposure to arsenic trioxide *in situ* caused significant decrease in population of pancreatic islet cells (Figure 2 *a* and *b*), a typical histological feature which may be compared with development of insulin-dependent diabetes mellitus caused by beta-cell injury from specific toxic substances bypassing an autoimmune requirement[19]. Our results of serum amylase activity (Table 1) further indicate that arsenic administration caused severe pancreatic damage[20], including possibly the damage of islet cells. Oxidative stress has been recognized as a major component in the chain of pathogenic events that cause late complications in diabetes mellitus[10]. It is considered as a major contributor to vascular and neurological complications of patients with diabetes mellitus[21–26]. It has also been reported that oxidative stress is involved in the cytotoxicity and genotoxicity of arsenic[8] and such arsenic-induced oxidative stress has been suggested to be due to the generation of NO, which can cause DNA damage and activate poly(ADP-ribose) polymerase[8], a major cause of islet cell damage in diabetes[27]. In our study, the increase in production of NO in the arsenic-treated group compared to control was 79% (Table 2), suggesting that such high overproduction of NO possibly was pathological, as has been manifested by significant reduction in islet cell number.

Lipid peroxidation, compared to control, significantly increased in arsenic-treated animals in our study (Table 2), thus corroborating well with the earlier speculations that arsenite induces oxygen free radicals or promotes formation of lipid peroxides[9], and high levels of such lipid peroxidation products have been indicated in the development of diabetes[28–30]. Thus, it may be proposed that chronic oral arsenic exposure possibly causes significant

damage to pancreas, particularly endocrine cellular components of pancreatic islets and thus may be responsible for transforming the normal physiological features of the OGTT curve to diabetic form.

In summary, we have demonstrated that rabbits on chronic oral exposure to arsenic, possibly develop an oxidative stress to cause deleterious effects on the endocrine pancreas. These effects could be, at least in part, the mechanism of action by which arsenic causes diabetes mellitus.

1. Chen, C. J., Lai, M. and Hsu, M., Dose–response relationship between ingested inorganic arsenic and diabetes mellitus, 4th Annual Meeting of International Society of Environmental Epidemiology, Abstr. Cuernavaca, Morelos, 1992.
2. Lai, M. S. *et al.*, Ingested inorganic arsenic and prevalence of diabetes mellitus. *Am. J. Epidemiol.*, 1994, **139**, 484–492.
3. Rahman, M. and Axelson, O., Diabetes mellitus and arsenic exposure: a second look at case-control data from a Swedish copper smelter. *Occup. Environ. Med.*, 1995, **52**, 773–774.
4. Rahman, M., Wingreen, G. and Axelson, O., Diabetes mellitus among Swedish art glass workers – an effect of arsenic exposure. *Scand. J. Work Environ. Health*, 1996, **22**, 146–149.
5. Rahman, M., Tondel, M., Ahmed, S. A. and Axelson, O., Diabetes mellitus associated with arsenic exposure in Bangladesh. *Am. J. Epidemiol.*, 1998, **148**, 198–203.
6. Tseng, C. H. *et al.*, Long term arsenic exposure and incidence of non insulin dependant diabetes mellitus: A cohort study in arseniasis-hyperendemic villages in Taiwan. *Environ. Health Perspect.*, 2000, **108**, 847–851.
7. Lynn, S., Shiung, J. N., Gurr, J. R. and Jan, K. Y., Arsenic stimulates poly(ADP-ribosylation) by generation of nitric oxide. *Free Radic. Biol. Med.*, 1998, **24**, 442–449.
8. Brenda, M. S., Peter, L. G. and Kenneth, J., The role of general metal specific cellular responses in protection and repair of metal-induced damage: Stress proteins and metallothioneins. *Toxicology of Metals* (ed. Chang, L. W.), Lewis Publishers, 1996, chapter 10, p. 167.
9. Borcea, V. *et al.*, αLipoic acid decreases oxidative stress even in diabetic patients with poor glycemic control and albuminuria. *Free Radic. Biol. Med.*, 1999, **22**, 1495–1500.
10. Kolb, H. and Kolb-Bachofen, V., Type I insulin dependent diabetes mellitus and nitric oxide. *Diabetologia*, 1992, **35**, 796–797.
11. Boquist, L., Boquist, S. and Ericsson, I., Structural beta-cell changes and transient hyperglycemia in mice treated with compounds inducing inhibited citric acid cycle enzyme activity. *Diabetes*, 1988, **37**, 89–98.
12. North, D. W., Gibb, H. J. and Abernathy, C. O., Arsenic: Past, present and future considerations, *Arsenic – Exposure and Health Effects* (eds Abernathy, C. O., Calderon, R. L. and Chappell, W. R.), Chapman and Hall, London, 1997, pp. 406–423.
13. Nath, R. L. and Nath, R. K., *Practical Biochemistry in Clinical Medicine*, Academic Publishers, Kolkata, 1990, 2nd edn, pp. 144–146.
14. Wienner, K., Tests in disorders of glucose metabolism. *Varley's Practical Clinical Biochemistry* (ed. Gowenlock, A. H.), CBS Publishers and Distributors, New Delhi, 1996, 6th edn, Chapter 16, pp. 333–349.
15. Koyama, I., Tsugikazu, K., Yoshikatsu, S. and Munetsugu, K., A possible mechanism for the changes in hepatic and alkaline phosphatase activities in bile duct ligated rats and guinea pigs. *Biochim. Biophys. Acta*, 1983, **760**, 169–174.
16. Giuseppina, M. R., Rosaria, M., Oreste, G., Maria, P. and Raffaele, D. C., Prolactin induction of nitric oxide synthase in rat C6 Glioma cells. *J. Neurochem.*, 1999, **73**, 2272–2277.
17. Wills, E. D., Evaluation of lipid peroxidation in lipids and biological membranes. *Biochemical Toxicology: A Practical Approach* (eds Snell, K. and Mullock, B.), IRL Press, Oxford, 1987, pp. 138–140.
18. Bancroft, J. D., Stevens, A. and Turner, D. R., *Theory and Practice of Histological Techniques*, Churchill Livingstone, New York, 1996, p. 104.
19. Eisenbarth, G. S., Polonsky, K. S. and Buse, J. E., Type I diabetes mellitus, *Williams Textbook of Endocrinology* (eds Larsen, P. R., Kronenberg, H. M. and Melmed, S.), Saunders, 2003, 10th edn, p. 1485.
20. Hotaling, M., Enzymes. *Saunders Manual of Clinical Laboratory Science* (ed. Lehaman, C. A.), W.B. Saunders Company, 1998, chapter 5, p. 82.
21. Baynes, J. W., Role of oxidative stress in development of complications in diabetes. *Diabetes*, 1991, **40**, 405–412.
22. Giugliano, D., Ceriello, A. and Paolisso, G., Oxidative stress and diabetic complications in diabetes. *Diabetes Care*, 1996, **19**, 257–267.
23. Van Dam, P. S., Van Asbeck, B. S., Erkelens, D. W., Marx, J. J. M., Gispen, W. H. and Bravenboet, B., The role of oxidative stress in neuropathy and other diabetic complications. *Diabetes*, 1995, **11**, 181–192.
24. Parthiban, A., Vijayalingam, S., Shanmugasundaram, K. R. and Mohan, R., Oxidative stress and the development of diabetic complications: Antioxidants and lipid peroxidation in erythrocytes and cell membrane. *Cell Biol. Int.*, 1995, **19**, 987–993.
25. Williamson, J. R. *et al.*, Hyperglycemic pseudohypoxia and diabetic complications. *Diabetes*, 1993, **42**, 801–813.
26. Nourooz-Zadeh, J., Rahimi, A., Tajaddini-Sarmadi, J., Tritschler, H., Rosen, P., Halliwell, B. and Betteridge, D. J., Relationship between plasma measures of oxidative stress and metabolic control in non-insulin dependent diabetes mellitus. *Diabetologia*, 1997, **40**, 647–654.
27. Inada, C., Yamada, K., Takane, N. and Nonaka, K., Poly(ADP-ribose) synthesis induced by nitric oxide in a mouse beta-cell line. *Life Sci.*, 1995, **56**, 1467–1474.
28. Lyons, T. J., Oxidised low density lipoproteins – a role in the pathogenesis of atherosclerosis in diabetes. *Diabetic Med.*, 1991, **8**, 411–419.
29. Sinclair, J. A., Free radical mechanisms and vascular complications of diabetes mellitus. *Diabet. Rev.*, 1993, **2**, 7–10.
30. Lyons, T. J., Lipoprotein glycation and its metabolic consequences. *Diabetes*, 1992, **41**, 67–73.

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