

Carcinogenesis in female C57Bl/6J mice chronically exposed to sodium arsenate (As^V) in drinking water for 2 years

M. Krishnamohan¹, A. A. Seawright¹, M. R. Moore^{1,2} & J. C. Ng¹

¹*The University of Queensland, National Research Centre for Environmental Toxicology (Entox), Australia*

²*Water Quality Research Australia, Adelaide, Australia*

Abstract

Arsenic is a ubiquitous element in the environment and has been classified as a human carcinogen primarily based on epidemiological evidence. It has been estimated there are over 100 million people globally being exposed to elevated arsenic from both natural and anthropogenic sources. Surprisingly, positive carcinogenicity animal studies were lacking until recent years. We aim to validate inorganic arsenate carcinogenic effect in C57Bl/6J mice, and establish the dose-response relationship using environmental concentrations of arsenic similar to those found in typical endemic-areas. Mice were given 0, 100, 250 or 500 µg As/L in the form of sodium arsenate in drinking water *ad libitum* over 2 years. Tumours occurred after about 18 months of arsenic exposure otherwise the animals appeared to be normal in their appearance and behaviour. Incidences of all types of tumours and non-tumourous lesions in the treated groups were higher than those observed in the control group. The induction of tumours was in a dose-response manner for some tumour types. Enlargement of the mesenteric lymph node due to hyperplasia or neoplasia of lymphoid elements was commonly observed. Apart from abdominal cavity lymph nodes, tumours were frequently observed in the liver, spleen and intestinal wall, and to a lesser extent in the lung with various other tissues also occasionally affected. Of the non-tumourous lesions, haemorrhagic ovarian cysts occurred more frequently in the treated groups than in the control group. Our results suggest that the C57Bl/6J mouse model can be a useful adjunct for further mechanistic studies of arsenic carcinogenesis. This bioassay data may also be considered for the risk evaluation of chronic exposure to inorganic arsenic.



Keywords: arsenic, arsenate, carcinogenesis, tumours, lymphoma, mice, drinking water, chronic exposure, risk assessment.

1 Introduction

Inorganic arsenic is classified as a human carcinogen [1, 2]. Over 100 million of people globally are at risk of exposure to elevated levels of arsenic in drinking water [3]. Although it is well known that arsenic is toxic to both humans and animals, the mechanism underlying its chronic toxicity remains unclear. Several long-term animal studies in which daily doses of sodium arsenate and sodium arsenite were given in the drinking water (up to 400 µg/L) had been found to be negative in rats, mice, beagles and cynomolgus monkeys, suggesting the apparent non-carcinogenicity of arsenic in animal models [4].

Adenocarcinoma was induced in the stomach of the rats implanted with 8 mg of arsenic trioxide in a capsule, by surgical implantation [5]. Hamsters administered with 3 mg As/kg of arsenic trioxide using charcoal carbon and 2 mM H₂SO₄ (a carrier to increase retention) by intra-tracheal installation once weekly for 15 weeks had low incidences of carcinomas, adenomas, papillomas and adenomatoid lesions of the respiratory tract [6]. Though these earlier animal studies demonstrated the carcinogenicity of inorganic arsenic in animals, they could not be taken as reliable animal models [7].

However, in more recent years, there have been several positive observations showing the carcinogenic effects of inorganic arsenic in mice. The first inorganic arsenic carcinogenicity study with chronic, low-dose exposure was done by Ng et al. [8] in C57BL/6J mice exposed to 500 µg As/L sodium arsenate in drinking water for 2 years. The authors reported increased incidence of tumours in various organs of treated mice but not in the control group. In another study [9], sodium arsenate in the drinking water (0, 1, 10 and 100 mg/L) administered to male A/J mice for 18 months resulted in an increase of lung tumour multiplicity and size in a dose response manner. Sodium arsenite was also proved to a carcinogen via the transplacental pathway [10, 11]. In this current study we aimed to confirm the study by Ng et al. [8] and further evaluate the carcinogenic effect of inorganic arsenic by including a lower and wider range of sodium arsenate concentrations. The water arsenic concentrations are similar to those reported to have caused arsenicosis in As-endemic areas [12].

2 Materials and methods

Sodium arsenate (Na₂HAsO₄) was purchased from Ajax Chemical, Australia. Animal experimental protocols were approved by the Queensland Health Animal Ethics Committee (AEC No. NRC 2/99/19). Female C57Bl/6J mice, aged 4 weeks, were divided into six groups of 70 each, 5 mice per cage, and were given drinking water containing 100, 250 or 500 µg As/L as sodium arsenate *ad libitum* for 24 months. A group of 105 control mice was given demineralised water containing <0.1 µg As/L. Female mice were used in our previous inorganic arsenic carcinogenicity study [8]. The stock solution of sodium arsenate was



prepared and kept in the refrigerator for one month. The working solutions of 100, 250 and 500 $\mu\text{g As/L}$ were prepared every week. Five animals from the control group were sacrificed at time zero, while 5 animals from the control and all the treatment groups were sacrificed after 2, 6 and 12 months exposure for the biomarker studies [13]. The animal care facility was operated at a controlled temperature set at 21–23 °C, with 13 filtered air changes per hour, a 12/12 h light/dark cycle, and year-round relative humidity of approximately 60%. All animals were kept in standard polycarbonate cages with stainless steel wire-mesh tops equipped with polycarbonate plastic drinking bottles and stainless steel sip-tubes, and given a commercial rodent diet *ad libitum* (Norco Pty Ltd, Brisbane, Australia).

Arsenic concentrations of the drinking water and rodent diet were monitored by HPLC–ICP–MS. The volume of the drinking water consumed by the mice in each cage, and the body weight of each mouse, were measured weekly. Five animals were sacrificed at time zero from the control group and five from each treatment group were sacrificed after 2, 6, 12 and 18 months for interim pathological examinations. Mice that became sick or which had developed significant skin lesions were also sacrificed by an overdose of carbon dioxide at various times up until the conclusion of the study at 104 weeks. Gross pathological changes, including tumours, were recorded and photographed. Tissue samples were collected for histopathological examination in a 10% buffered neutral formalin during necropsy. Paraffin sections were prepared from formalin-fixed tissues and stained with haematoxylin and eosin.

3 Results

3.1 General observations

Tumours occurred after about 18 months of arsenic exposure; otherwise no abnormal appearance or behaviour was noticed in any of the animals. A total of 9 animals out of 85 were found dead in the control group; and 5, 8 and 11 animals out of 55 were found dead in the 100, 250 and 500 $\mu\text{g As/L}$ groups respectively. No reason was found for the sudden death of some animals. Other animals died because of fatal haemorrhage from a blood-filled ovarian cyst or from large internal tumours.

3.2 Tumour incidence and gross pathology

Percentage incidences of all types of tumours and non-tumourous lesions in the treated groups were higher than those observed in the control group at the end of 2 years. Enlargement of the mesenteric lymph node due to hyperplasia or neoplasia of lymphoid elements was commonly observed. Apart from abdominal cavity lymph nodes, tumours were frequently observed affecting the liver, spleen and intestinal wall with various other tissues also occasionally affected. Of the non-tumourous lesions, haemorrhagic ovarian cysts were observed more frequently in the test groups than in the control group. Examples of tumours in



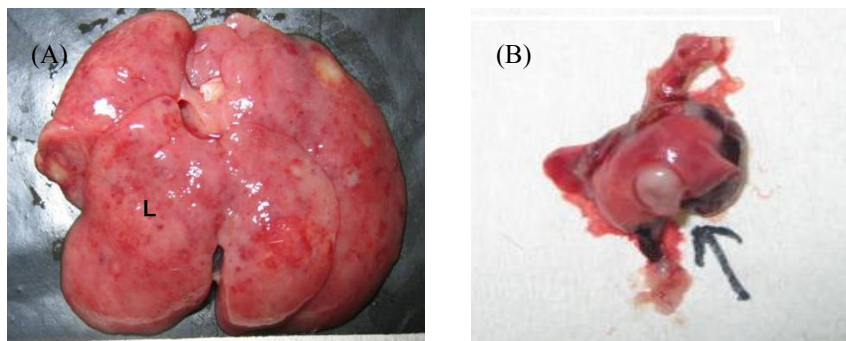


Figure 1: A mouse liver (A) diffusely invaded by tumour tissue. Pale tumour foci were surrounded by hyperaemic zones from the 500 $\mu\text{g As/L}$ treatment group; a mouse lung (B) shows a large nodular tumour (arrow) from the 250 $\mu\text{g As/L}$ treatment group.

Table 1: Percentage of tumour incidences observed in the control and sodium arsenate treated female C57Bl/6J mice at the end of 2 years.

Tumour incidence (%)	Control	100 $\mu\text{g/L}$	250 $\mu\text{g/L}$	500 $\mu\text{g/L}$
No. of mice exposed to As for more than 12 months	85	55	55	55
Mice with all types of tumours	15	25	62	56
Mice with lymphoma	11.7	15	31	29
Lymph nodes only	4.7	2	5.5	7
Organs & lymph nodes	7	13	25.5	22
Mice with other types of tumours	4	9	24	15
Mice with multiple tumours	1	2	7	13

the liver and lung are shown in Figure 1. There were significantly higher incidences of all types of tumours in the treated groups compared to the control group (Table 1) in a dose response relationship. Multiple tumours include lymphoma, plasmacytoma and histiocytic sarcoma.

Histological examination showed that lymphoma was the major type of tumour observed in both treated and control groups. Mesenteric lymph node was the major lymph node affected in the experiment animals. There was a significant difference in the incidence of lymphoma in the treated groups compared to the control group. A significant dose-response relationship was observed in total lymphoma incidence including lymph nodes and organs

($p=0.0021$), and lymphoma invading the organs ($p = 0.0033$). A significant dose-response relationship was observed in the incidences of other types of tumours ($p = 0.0018$) and multiple tumours ($p = 0.0016$).

After the mesenteric lymph node, liver and spleen were the major organs affected by As^V treatment. A higher incidence of liver, spleen and gastrointestinal tract lymphoma was observed in the treated groups compared to the control group. However, there was no evidence of a dose-response relationship (data not shown). Lung lymphoma was seen in all the treated groups but not in the control group. Uterus lymphoma was seen only in the 250 $\mu\text{g As/L}$ group. Submandibular lymph nodes, thoracic lymph nodes, mammary glands, pancreas and subcutaneous lymph node were affected in at least one of the treated groups but not in the control group.

Plasmacytoma was observed in the liver, spleen, kidney, lung, GI tract, pancreas and subcutaneous glands of at least one of the treated groups but not in the control group. A higher incidence of histiocytic sarcoma was observed in the liver and spleen of treated groups compared to the control group. No urinary tract or bladder tumour was observed, except in one mouse in the 500 $\mu\text{g As/L}$ group that developed bladder histiocytic sarcoma. Tubulosomal adenoma of the ovary was observed in the 250 and 500 $\mu\text{g As/L}$ treated groups.

Other types of tumour such as Harderian gland adenoma, pituitary adenoma, subcutaneous fibrosarcoma and hepatocellular adenoma were found only in the treated groups and not in the control group. Hepatocellular carcinoma was higher in the 250 $\mu\text{g As/L}$ group compared to the control group and it was not observed in the 100 and 500 $\mu\text{g As/L}$ treated groups. Non-haematopoietic cancers observed are shown in Table 2.

3.3 Histology

Histological examinations (H&E) of the tumours showed various lymphoma patterns. These are illustrated in Figure 2.

Table 2: Percentage distribution of non-haematopoietic cancers in the control and sodium arsenate-treated mice.

Type	Control	100 $\mu\text{g/L}$	250 $\mu\text{g/L}$	500 $\mu\text{g/L}$
Hepatocellular carcinoma	1	0	4	0
Hepatocellular adenoma	0	2	9	5
Subcutaneous fibrosarcoma	0	0	2	0
Harderian gland adenoma	0	4	2	0
Pituitary adenoma	0	4	0	0



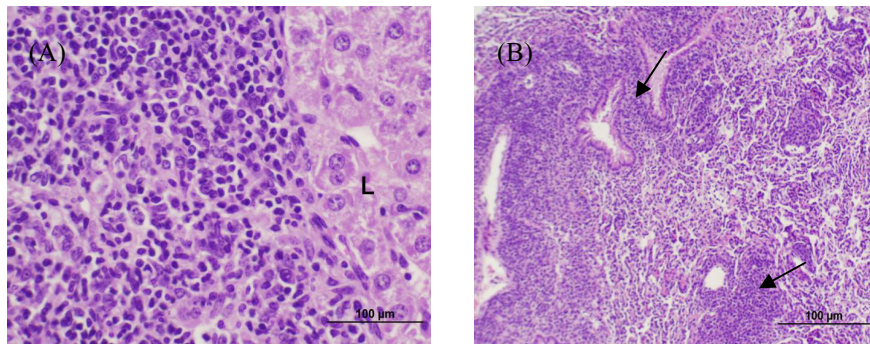


Figure 2: Lymphoid metastasis in the liver (A) from a mouse treated with 500 $\mu\text{g As/L}$. The cells are mostly small, dense and variable in shape. Low power illustration of lung (B) showing dense accumulations of lymphoid cells surrounding blood vessels and bronchi (arrows) from a mouse treated with 250 $\mu\text{g As/L}$ as sodium arsenate in drinking water for two years.

4 Discussion

Although Dimethyl arsenic acid (DMA,) a major metabolite of inorganic arsenic, had been shown to cause bladder cancers in rats [14], inorganic arsenic alone has not been proved to be a carcinogen in any of the known animal models until recently [8]. The carcinogenic effect of arsenic has since been shown to be able to cross the placenta and subsequently induce tumours in the off-spring of pregnant dams exposed to relatively high concentrations of arsenic [10, 11]. Ng et al. [8] reported that when female C57Bl/6J mice were given 500 $\mu\text{g As/L}$ as sodium arsenate (As^{V}) in the drinking water for up to 26 months, the tumour incidence in all organs was 41.1%. No tumours were reported in the control group. These findings were reported in the Environmental Health Criteria on Arsenic and Arsenic Compounds [15], which stated that “this was the first experimental carcinogenicity study in rodents using a relevant route of exposure and relevant exposure level” and that the incidence of tumours was “treatment related”. In the report of this study, the types of tumours were not reported and no dose response effect was determined. The present study was designed to investigate whether the human carcinogen, inorganic arsenic is also carcinogenic in mice at concentrations which are commonly found in As-endemic areas and to confirm the previous study [8]. The overall incidence for all types tumours was 56% which is slightly higher but in general agreement with the tumour incidence (41.1%) reported previously [8]. More significantly our results in mice have also demonstrated a significant dose-response relationship in multiple types of tumours, total lymphoma, lymphoma invading the organs in the mice exposed to sodium arsenate. On the other hand there were *in-vitro* studies which showed that exposure to low concentration of arsenic could be adaptive and protective

[16], causing enhanced cell proliferation and viability [17] rather than cytotoxicity. At higher concentrations toxicity of arsenic can cause an apoptotic response, as was seen in the treatment of promyelocytic leukaemia and multiple myeloma [18, 19]. This, in fact, could be a reason for not encountering tumour production in most earlier animal studies, which employed high doses of arsenicals resulting in cytotoxic effects rather than carcinogenic effects as demonstrated here.

Our results showed that the liver in mice was commonly the site of metastatic spread of haemotopoietic tumours but not hepatocellular tumours in the exposed mice. Pregnant mice exposed to sodium arsenite during 8-18 days of gestation at doses of 42.5 and 85 mg/L in drinking water resulted in dose-dependent increases in tumours of the liver and adrenal glands in male offspring, and of the ovary in female offspring [10]. Male BALB/c mice given 3.2 mg/L arsenic in the drinking water developed fatty liver after 12 months and hepatic fibrosis after 15 months of exposure [20]. Arsenic increased hepatocellular carcinoma (14%), adenoma (23%) and total tumours (31%) compared to the control (0, 2 and 2%) in the male offspring of CD1 mice exposed to 85 mg/L arsenite during the 8th to the 18th day of gestation [21]. Hepatocellular adenoma was observed in all the test groups from the As^V-exposed groups. The percentage incidence in the As^V-exposed group was 2, 9 and 5% compared to the control 0%. In addition, liver degenerative lesions, liver cell vacuolation and fatty infiltration were seen together with preneoplastic proliferative lesions (data not shown) which could lead to cancerous endpoints. These observed lesions were similar to those seen in the liver biopsy samples of hepatomegaly from patients exposed to arsenic in Guizhou, China [22]. Our result also supports other studies where chronic arsenic exposure in the mouse produced liver cell vacuolation and fatty infiltration along with preneoplastic proliferative lesions and chronic inflammation [23, 24].

DMA was also found to be carcinogenic in the rat urinary bladder [25]. In contrast, our gross pathology showed only one bladder tumour (histiocytic sarcoma) in the 500µg As/L group, a relative low incidence of less than 2%. This suggests that sodium arsenate is not a potent carcinogen in C57BL/6J mice.

Our present study is the first long-term, low-dose carcinogenic study of arsenate which is often the major component of arsenic-contaminated drinking water found in endemic areas. It also demonstrates the dose-response relationship in carcinogenic effects of As^V. In the present study lung lymphoma was observed in all the treated groups and none in the control group. Histiocytic sarcoma was observed in 500 µg As/L exposed mice.

In a study by Salim et al. [26], a significant increase in tumour multiplicity (malignant lymphoma) was recorded in both p53^{+/-} knockout and C57BL/6J wild type male mice exposed to 50 or 200 mg/L DMA^V for 18 months. The present results support the findings of these studies, that arsenate also increases the incidence of lymphomas, and with higher potency than dosing with DMA.

In conclusion, our study clearly shows that sodium arsenate is a complete carcinogen to C57BL/6J mice, causing multi-organ tumours without any promoter. In all previous animal studies apart from that by Ng et al. [8] the doses



used were relatively high compared to those associated with human exposures. This study and others [8, 10, 11] dismiss the notion that no animal model exists for arsenic carcinogenesis. These studies have also settled the debate on whether arsenic, a human carcinogen, is carcinogenic to laboratory animals. Our results can contribute to the risk evaluation of chronic arsenic exposure and the development of arsenic exposure standards.

Acknowledgement

Entox is a partnership between Queensland Health and the University of Queensland.

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