Results of a Long-Term Carcinogenicity Bioassay on Sprague-Dawley Rats Exposed to Sodium Arsenite Administered in Drinking Water

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ABSTRACT: **Arsenic (As) is a metal found in nature whose acute and chronic toxic effects have been known for decades. Hundreds of millions of people are at risk of exposure to As and its various chemical forms which can occur in the occupational and general environment in air, water, soil, food, and medicines. Several epidemiological studies have shown that prolonged exposure to As can induce various types of malignant tumors in humans, namely, skin, lung, liver, kidney, and bladder cancers. These effects have been observed particularly in geographic areas where people are exposed to well water with high concentrations of As. While the risks of As at high concentrations are well documented, there is still a great deal of uncertainty regarding the risk of exposure to As at very low levels. This uncertainty is due to the absence of adequate epidemiological data and the insufficiency of experimental data currently available. Given the limited evidence demonstrating the carcinogenic potential of As in animals, a long-term carcinogenicity bioassay on sodium arsenite ($NaAsO_2$) was performed at the Cesare Maltoni Cancer Research Center (CMCRC) of the European Ramazzini Foundation (ERF). $NaAsO_2$ was administrated with drinking water at concentrations of 200, 100, 50, or 0 mg/L, for 104 weeks to Sprague-Dawley rats (50/sex/group), 8 weeks old at the start of the study. The animals were monitored until spontaneous death at which time each animal underwent complete necropsy. Histopathological evaluation of all pathological lesions and of all organs and tissues collected was routinely performed on each animal. The results demonstrate that in our experimental conditions $NaAsO_2$ induces sparse benign and malignant tumors among treated rats. The types of tumors observed are infrequent in the strain of Sprague-Dawley rats of the colony used in our laboratory, namely, lung adenomas and carcinomas, kidney adenomas/papillomas and carcinomas, and bladder carcinomas.**

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Notably, an elevated incidence of these types of oncological lesions is also observed among people living in geographical areas where As is present at higher concentrations in drinking water.

KEYWORDS: **sodium arsenite; carcinogenicity; long-term bioassays; rat**

INTRODUCTION

Arsenic (As) is a metalloid widely distributed in the earth's crust, found particularly in igneous and sedimentary rocks. It can exist in four valency states: −3, 0, +3, and +5. Under reducing conditions, the +3 valency state as arsenite may be the dominant form; the +5 valency state as arsenate is generally the more stable form in oxygenized environments.[1] Arsenic compounds usually occur in trace quantities in rock, soil, water, and air. However, concentrations may be higher in certain areas as a result of weathering and anthropogenic activities including metal mining and smelting, fossil fuel combustion, and pesticide use.[2]

In soils, a global average concentration level of 5 mg/kg was estimated by Koljonen,[3] but concentrations may vary considerably among geographic regions. In some areas of South-West England, near old smelters or mining areas, concentrations of As range from 24 to 161,000 mg/kg.[4]

In natural waters, As is mostly found in inorganic forms as oxyanions of trivalent arsenite or pentavalent arsenate. Background concentrations of As in groundwater range from less than 0.5 to 5000 μg/L.[5] Most high levels of As occur naturally, but cases of As pollution by mining are numerous, albeit localized. In surface waters, levels of dissolved As range from 0.1 to 1.7 μg/L in uncontaminated stream waters,[6] however, in some areas characterized by volcanic activity, As levels may reach 3000 μg/L.[7]

Concentrations in air in remote locations range from less than 1 to 4 ng/m^3; however, in cities, concentrations may reach up to 200 ng/m^3 and may be greater than 1000 ng/m^3 in the vicinity of industrial sources, particularly near nonferrous smelters.[2] It has been estimated that the atmospheric flux of As is about 75,540 tons/year of which 60% is of natural origin and the remaining 40% is derived from anthropogenic sources.[8]

The estimated world production of As (expressed as As trioxide equivalent) was 35,000 tons in 2002, representing a slight decrease compared to the world production at the end of 1990s (about 40,000 tons in 1998 and 1999).[9] As is mostly used for the production of wood preservatives, but also for the production of some agricultural chemicals, pesticides (mainly herbicides), glass, nonferrous alloys, and semiconductors.[9] In the United States, As consumption is predicted to decline drastically because of regulations aimed toward ceasing use of chromated copper arsenate as a wood preservative.[9]

In both humans and rodents, ingested As compounds are quickly absorbed and enter the bloodstream. Inhaled As is not absorbed as readily as ingested

As, even though a study on smelter workers showed an 80% absorption rate of inhaled As.[10] Once absorbed, arsenate is reduced to arsenite in the blood through reactions with glutathione and then transported to the liver. Arsenite is detoxified by methylation in the liver both enzymatically by methyltransferases [to monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA)] and nonenzymatically (to methanearsonic acid).[11] An unusual feature of the metabolism of As is that there are deep interspecies differences in methylation: humans are more sensitive to As toxicity than are several other species because As methylation in humans is believed to be less efficient.[11,12]

In humans, ingestion of high levels of As (>1 g) induces acute effects characterized by severe gastrointestinal damage which may result in shock, multi-organ failure, and death.[2] Subacute exposure affects primarily the respiratory, gastrointestinal, cardiovascular, nervous, and hematopoietic systems.[1] Chronic As toxicity is mainly manifested in the skin in forms, such as hyperpigmentation and hyperkeratosis, with pigmentation affecting trunks and limbs and keratosis affecting hands and feet. Chronic lung disease, peripheral neuropathy, and peripheral vascular disease have been frequently associated with chronic exposure to As.[1]

Few studies have been conducted to evaluate the immunotoxicity of As. From studies on humans, it appears that inorganic As is immunotoxic[13,14] and As-induced apoptosis could be a major mechanism of immunosuppression.[15]

Reproductive and developmental effects have been studied in humans and in experimental animals. Human data, albeit limited, suggest that exposure to high concentrations of As in drinking water during pregnancy may increase the risk of fetal and neonatal mortality.[16,17] The results in rodents show that MMA and DMA have developmental toxicity effects.[1]

In humans, As is a chromosomal mutagen (an agent that induces mutations involving more than one gene). Micronuclei, chromosomal aberration, and aneuploidy have been detected in both peripheral lymphocytes, and urothelial cells of people exposed to elevated levels of As.[1]

In prokaryotic *in vitro* systems, As has been reported to be nonmutagenic.[18–20] In mammalian and human cell *in vitro* systems, As has been shown to be genotoxic. Moore *et al*.[21] reported sodium arsenite and, to a lesser extent sodium arsenate, to induce chromosomal aberrations, micronuclei polyploidy, and endoreduplication in the L5178Y/TK ± mouse lymphoma assay. Chromosome alteration in Chinese hamster ovary cells are also reported to be induced by arsenite.[22] In human lymphocytes and fibroblasts, inorganic As was reported to induce dose-dependent chromosomal aberrations and DNA-protein cross-links; the effects were observed to be more potent with sodium arsenite than with sodium arsenate.[23,24]

Epidemiological studies on cancer risks in relation to As exposure in drinking water include mostly ecological studies that can provide important information on causal inference due to large exposure contrasts and limited population migration. Several epidemiological studies have shown that prolonged exposure

to As in drinking water can induce various types of malignant tumors in humans, namely, tumors of the skin, lung, liver, kidney, and urinary bladder. Many reports several decades ago described skin cancers following ingestion of arsenical medicine, exposure to arsenical pesticides, and As-contaminated water.[25–27] Typical As-associated skin tumors include squamous-cell carcinoma and multiple basal-cell carcinoma.[27–29] Arsenic in drinking water was reported to increase the risk for lung cancer in epidemiological studies which included large population groups and different levels of exposure.[30–32] Exposure to As was also reported to induce liver angiosarcoma and to increase the risk for liver cancer, mainly hepatocarcinoma.[30,31,33] Moreover, a positive correlation between exposure to As in drinking water and cancers of the kidney and urinary bladder was found in many epidemiological studies.[30–32,34]

Various As compounds have been tested for carcinogenicity by oral administration in rats and mice, by intratracheal administration in hamsters, and by transplacental exposure in mice.[1]

In a Fischer rat study, groups of 36 males, 10 weeks of age at the start of the experiment, were administered DMA at concentrations of 0, 12.5, 50, and 200 ppm in drinking water for 104 weeks. A statistically significant increase of the incidence of transitional cell carcinomas of the urinary bladder was observed in the two highest dose groups.[35]

Studies were also conducted on mice to evaluate the potential carcinogenicity of different As compounds. DMA was administered in drinking water at various concentrations to different mice strains, namely: (*a*) groups of 10–14 male A/J mice were given 0, 50, 200, or 400 ppm beginning at 6 weeks of age and lasting for 25 or 50 weeks, respectively. After 50 weeks, an increase (although nonsignificant) in the incidence of lung tumors was observed in treated mice[36]; (*b*) groups of 20–30 K6/ODC transgenic mice were administered 0, 10, or 100 ppm of DMA beginning at 7 weeks of age and lasting for 5 months. One additional group was administered sodium arsenite at 10 ppm. The incidence of squamous skin tumors increased in all treated groups compared to the control[37]; (*c*) groups of 29–30 male $p53^{+/-}$ heterozygous or $p53^{+/+}$ mice were exposed to 0, 50, 200 ppm DMA in drinking water for 80 weeks. In the $p53^{+/-}$ mice, a nonsignificant increase in the incidence of total tumors was observed, while in $p53^{+/+}$ mice a significant increase in the incidence of total tumors was observed, but with no dose-dependence.[38]

In an other study, groups of 25 male and female C3H mice were exposed exclusively during fetal life to 0, 42.5, and 85 ppm of inorganic As. Males and females were then observed for 72 and for 90 weeks, respectively. A dose-related increase in the incidence of hepatocarcinomas and adrenal cortical adenomas was observed in males; in females, a dose-related increase in the incidence of total ovarian tumors (benign and malignant) and lung carcinomas was observed.[39]

The carcinogenicity of As trioxide, calcium arsenate, and As trisulphide was evaluated in groups of 30 male Syrian golden hamsters, treated by intratracheal

instillation beginning at 8 weeks of age, once a week for 15 weeks and followed until death (113–121 weeks after the initial instillation). A group of 20 males served as a control. Each compound, containing 0.25 mg As, was suspended in 0.1 mL of saline buffer; the controls received buffer solution alone. A statistically significant increase of the incidence of lung tumors (benign and malignant combined) was observed in animals treated with calcium arsenate.[40]

IARC reviewed the aforementioned studies and concluded that there is sufficient evidence in experimental animals to determine the carcinogenicity of DMA, but there is limited evidence to confirm the carcinogenicity of sodium arsenite, calcium arsenate, and As trioxide.[1]

Given the limited evidence demonstrating the carcinogenic potential of As and the importance of obtaining additional experimental data to better assess the carcinogenic risks among people exposed to As in drinking water, a long-term carcinogenicity bioassay on sodium arsenite ($NaAsO_2$) was performed at the Cesare Maltoni Cancer Research Center (CMCRC) of the European Ramazzini Foundation (ERF). The results of the study are presented in this article.

MATERIALS AND METHODS

The sodium arsenite ($NaAsO_2$) used was produced by Sigma of St. Louis, MO and supplied by Prodotti Gianni of Milan, Italy. Its purity was 98% by titration. $NaAsO_2$ was administered in drinking water at concentrations of 200, 100, 50, or 0 mg/L *ad libitum* for 104 weeks to male (M) and female (F) Sprague-Dawley rats (50/sex/group), 8 weeks old at the start of the experiment. Rats were bred from the colony used at the CMCRC/ERF laboratories for nearly 30 years. Extensive historical data are available on the tumor incidence among untreated rats. Each morning, leftover solution from the previous day was removed and glass drinking bottles were washed and refilled with fresh solution. Control animals received tap water. Animals were fed with the standard Corticella diet (Corticella S.p.A., Bologna, Italy), used for more than 30 years in our laboratories. The experiment was performed according to Good Laboratory Practices using the Standard Operating Procedure (SOP) of the CMCRC/ERF.

After weaning at 4–5 weeks of age, the experimental animals were randomized in order to have no more than one male and one female from each litter in the same group. Animals were then housed in groups of five in makrolon cages (41 cm $\times$ 25 cm $\times$ 15 cm) with stainless-steel wire tops and a shallow layer of white wood-shavings as bedding. Cages were kept in rooms used exclusively for this experiment at a temperature of 21 $\pm$ 2°C and relative humidity of 50–60%. A light/dark cycle of 12 hours was maintained using both natural and artificial light sources.

Mean daily drinking water and feed consumption were measured once weekly per cage for the first 13 weeks, and then every 2 weeks until 111

weeks of age. Body weight was measured individually once weekly for the first 13 weeks and then every 2 weeks until 111 weeks of age. Measurement of body weight continued every 8 weeks until the end of the experiment. The animals were clinically examined for gross changes every 2 weeks for the duration of the study.

The biophase ended at 159 weeks, with the death of the last animal at 167 weeks of age. Upon death, all animals underwent complete necropsy. Histopathology was routinely performed on the following organs and tissues of each animal from each group: skin and subcutaneous tissue, the brain (three sagittal sections), pituitary gland, Zymbal glands, salivary glands, Harderian glands, cranium (five sections, with oral and nasal cavities and external and internal ear ducts), tongue, thyroid, parathyroid, pharynx, larynx, thymus and mediastinal lymph nodes, trachea, lung and mainstem bronchi, heart, diaphragm, liver, spleen, pancreas, kidneys, adrenal glands, esophagus, stomach (fore and glandular), intestine (four levels), urinary bladder, prostate, gonads, interscapular brown fat pad, subcutaneous and mesenteric lymph nodes, and other organs or tissues with pathological lesions. All organs and tissues were preserved in 70% ethyl alcohol, except for bones, which were fixed in 10% formalin and then decalcified with 10% formaldehyde and 20% formic acid in water solution. The normal specimens were trimmed, following SOP. Trimmed specimens were processed as paraffin blocks and 3–5 μm sections of every specimen were obtained. Sections were routinely stained with Hematoxylin-Eosin.

All slides were examined microscopically by the same group of pathologists, following the same criteria of histopathologic evaluation and classification. A senior pathologist reviewed all tumors and all other lesions of oncologic interest. Multiple tumors of different types and sites, of different types in the same site, of the same types in bilateral organs, of the same types in the skin, subcutaneous tissue or mammary glands, or at distant sites of diffuse tissue (i.e., bones and skeletal muscle) were plotted as single/independent tumors. Multiple tumors of the same type in the same tissue and organ, apart from those mentioned above, were plotted only once.

Three statistical tests were used to analyze neoplastic and non-neoplastic lesion incidence data. The χ^2 test and the Fisher's exact test[41] were used to evaluate differences in tumor incidence between treated and control groups. The Cochran-Armitage trend test[42,43] was used to test for linear trends in tumor incidence.

RESULTS

A dose-related lower intake of water containing various levels of $NaAsO_2$ was observed in both male and female rats (FIG. 1). In females, water consumption became similar between the group treated at 50 mg/L and the control after 88 weeks of age. A dose-related lower intake of feed was also observed in both male and female rats (FIG. 2). This difference was less marked between the

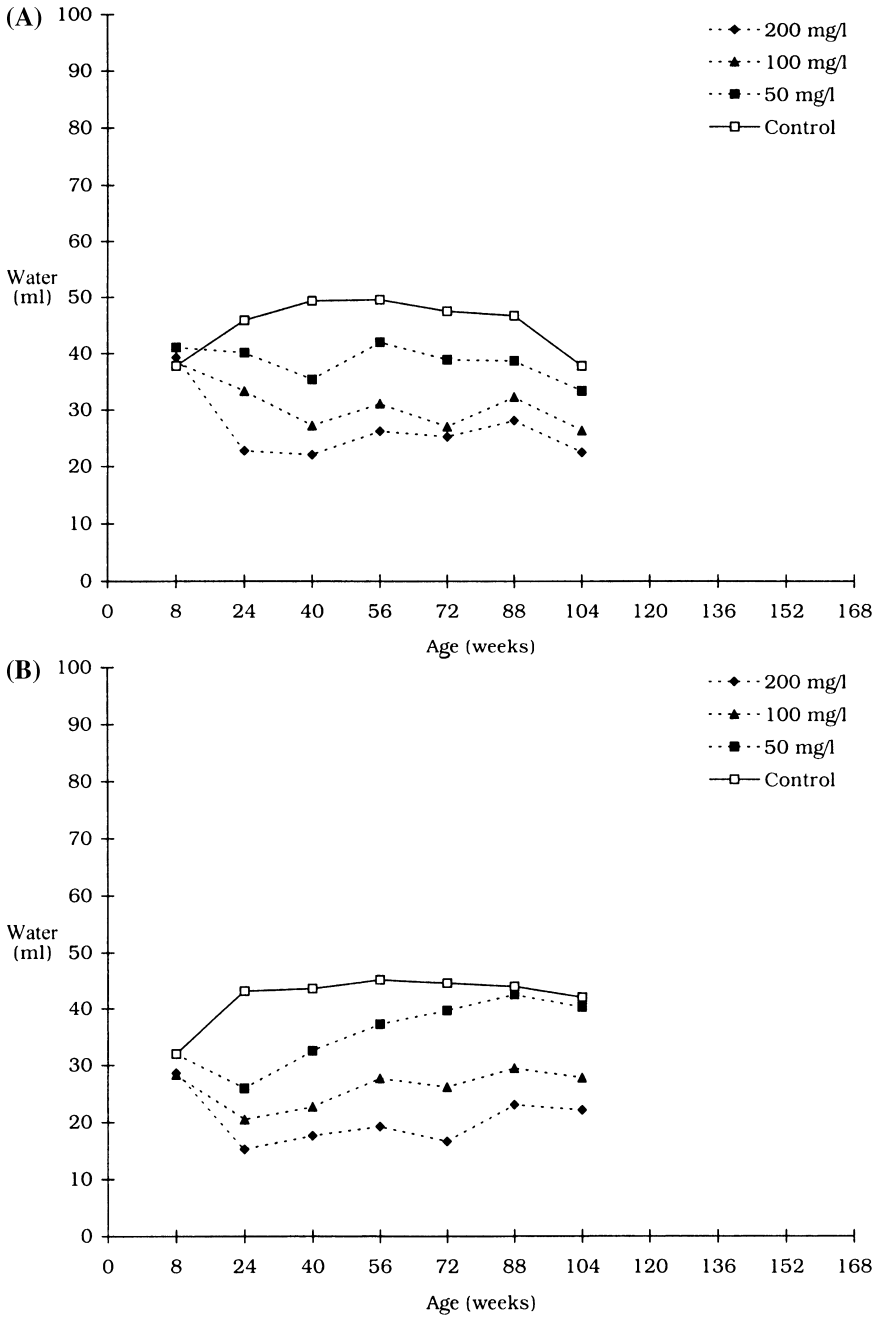

FIGURE 1. Mean daily water consumption in male (**A**) and female (**B**) Sprague-Dawley rats (- - ♦ - -200 mg/L; - - ▲ - - 100 mg/L; - - ■ - - 50 mg/L; □ Control).

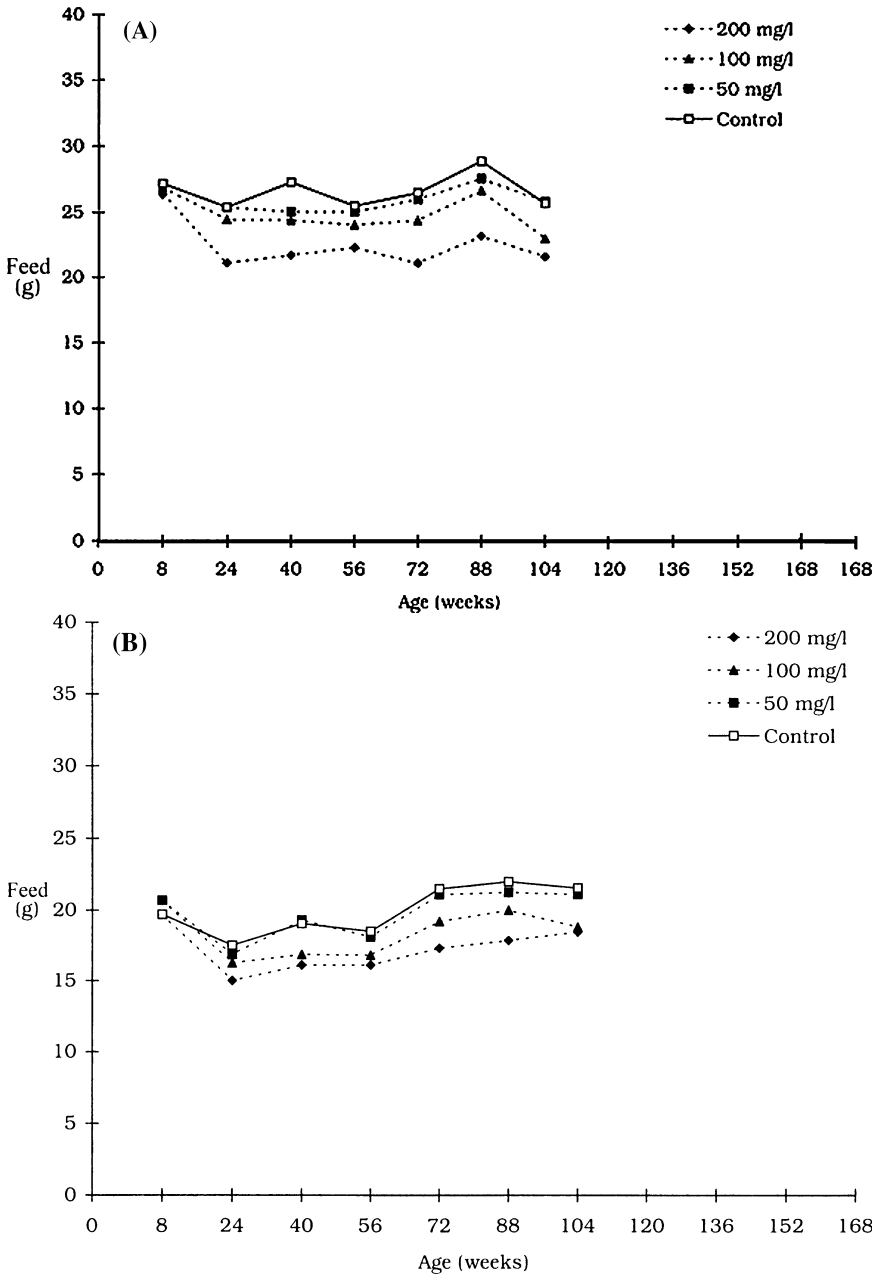

FIGURE 2. Mean daily feed consumption in male (**A**) and female (**B**) Sprague-Dawley rats (- - ♦ - - 200 mg/L; - - ▲ - - 100 mg/L; - - ■ - - 50 mg/L; □ Control).

group treated at 50 mg/L and the control in both males and females. A dose-related difference in mean body weight was observed in males. The difference was more evident in the males treated at 200 mg/L (circa 15% when compared with controls). Differences in mean body weight were also observed in females of the groups treated at 200 and 100 mg/L. Mean body weight was about 20% less in females treated at 200 mg/L compared with control and about 10% less in females treated at 100 mg/L. No treatment-related differences in body weight were observed in females treated at 50 mg/L. Differences in survival rates were observed in both males and females; a slight decrease in the survival rate was observed in males treated at 200 and 100 mg/L, particularly from 40 weeks of age until 88 weeks of age, whereas in females, a decrease in survival rate was observed from 104 weeks of age until the end of the experiment.

Long-term exposure of sodium arsenite administered in drinking water to Sprague-Dawley rats has been shown to induce toxic effects on the kidneys at concentrations as high as 200 mg/L and, to a lesser extent 100 mg/L and 50 mg/L. Nephropathies were characterized by diffuse acute/chronic inflammation, tubular enlargement with deposits of ialin casts and marked fibrosis around glomeruli with distension of Bowman's space.

The main oncologic results of the experiment are reported in TABLE 1 for males and TABLE 2 for females. Among males treated at 100 mg/L, a slightly increased incidence of animals bearing malignant tumors and a statistically significant increased number of total malignant tumors ($P < 0.05$) were observed when compared to controls. Sparse very infrequent benign and malignant tumors were observed in the treated groups, namely, one adenocarcinoma of the lung in a male treated at 200 mg/L; one carcinoma of the kidney and one papilloma of the pelvis in a male treated at 100 mg/L and two papillomas of the renal pelvis in another rat treated at the same dose. Renal pelvis papillomas were also observed in two males treated at 50 mg/L.

Among females treated at 100 mg/L, a slightly increased incidence of animals bearing malignant tumors and an increased number of total malignant tumors were observed when compare to controls. Among the females treated at 200 mg/L, one adenocarcinoma of the lung was observed. The same group also included two animals bearing kidney adenomas, two bearing kidney carcinomas, and one bearing a renal pelvis carcinoma. In the group treated at 100 mg/L, three animals were observed bearing kidney adenomas, one bearing a kidney carcinoma and one bearing a renal pelvis papilloma. One animal bearing a bladder carcinoma was also observed among the females treated at 100 mg/L.

It must be noted that among the untreated Sprague-Dawley rats used in our laboratories over the last 20 years (2265 males and 2274 females), the overall incidence of lung adenomas was 0.2% in males (range: 0–2.0%) and 0.1% in females (range: 0–1.0%), while the overall incidence of lung carcinomas was 0.1% in both males (range: 0–1.0%) and females (range: 0–1.3%). The overall incidence of the kidney adenomas was 0.1% in males (range: 0–1.3%)

TABLE 1. Incidence of the preneoplastic and neoplastic lesions in male Sprague-Dawley rats treated with sodium arsenite in drinking water from 8 weeks of age for 104 weeks and then observed until spontaneous death

		Malignant tumors				Animals bearing neoplastic lesions of the lung[a]				Animals bearing neoplastic lesions of the kidney[a,b]										Animals bearing neoplastic lesions of the bladder			
		Tumor-bearing animals		Total tumors		Adenomas		Adeno-carcinomas		Adenomas		Carcinomas		Pelvis papillomas		Pelvis carcinomas		Total		Papillomas		Carcinomas	
Dose ppm (mg/L)	Animals at start	No.	%	No.	Per 100 animals	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
200	50	15	30.0	16	32.0	0	–	1	2.0	0	–	0	–	0	–	0	–	0	–	0	–	0	–
100	50	23	46.0	28	56.0*	0	–	0	–	0	–	1	2.0	2 (3)	4.0	0	–	2[c] (4)	4.0	0	–	0	–
50	50	17	34.0	19	38.0	1	2.1	0	–	0	–	0	–	2	4.0	0	–	2	4.0	0	–	0	–
0	50	17	34.0	19	38.0	0	–	0	–	0	–	0	–	0	–	0	–	0	–	0	–	0	–

*Statistically significant ($P < 0.05$) using χ^2 test.

[a]The tumor rates are based on the number of animals examined.

[b]Between parentheses the number of tumors (one animal can bear more than one tumor).

[c]One animal bears both kidney carcinoma and papilloma of the renal pelvis.

TABLE 2. Incidence of the preneoplastic and neoplastic lesions in female Sprague-Dawley rats treated with sodium arsenite in drinking water from 8 weeks of age for 104 weeks and then observed until spontaneous death.

		Malignant tumors				Animals bearing neoplastic lesions of the lung[a]				Animals bearing neoplastic lesions of the kidney[a,b]										Animals bearing neoplastic lesions of the bladder			
		Tumor-bearing animals		Total tumors		Adenomas		Adeno-carcinomas		Adenomas		Carcinomas		Pelvis papillomas		Pelvis carcinomas		Total		Papillomas		Carcinomas	
Dose ppm (mg/L)	Animals at start	No.	%	No.	Per 100 animals	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%	No.	%
200	50	19	38.0	27	54.0	0	–	1	2.1	2	4.3	2	4.3	0	–	1	2.2	5	10.0	0	–	0	–
100	50	25	50.0	31	62.0	0	–	0	–	3 (4)	6.0	1	2.0	1	2.0	0	–	5 (6)	10.0	0	–	1	2.0
50	50	17	34.0	21	42.0	0	–	0	–	0	—	0	–	1	2.0	0	–	1	2.0	0	–	0	–
0	50	18	36.0	23	46.0	0	–	0	–	0	–	0	–	1	2.0	0	–	1	2.0	0	–	0	–

[a] The tumor rates are based on the number of animals examined.
[b] Between parentheses the number of tumors (one animal can bear more than one tumor).

and 0.2% in females (range: 0–2.0%), while the overall incidence of kidney carcinomas was 0.2% in males (range: 0–0.3%) and 0.3% in females (range: 0–1.8%). With regard to historical data on the transitional cell epithelium of the renal pelvis and ureter, no papillomas were observed in either males or females, while only one carcinoma was observed in a female (overall incidence: 0.04% and range: 0–1.0%). No carcinomas in the transitional cell epithelium of the bladder were observed in either males or females.

CONCLUSIONS

In our experimental conditions, it has been shown that sodium arsenite administered for 104 weeks in drinking water to male and female Sprague-Dawley rats, 8 weeks old at the start of the experiment and kept under observation until spontaneous death, induces an increased incidence (albeit not statistically significant) of benign and malignant tumors of the lung, kidney, and bladder.

Because these benign and malignant tumors are extremely rare in our extensive historical controls, the observation of adenomas and carcinomas of the lung, of adenomas and carcinomas of the kidney, of papillomas and one carcinoma of the renal pelvis transitional cell epithelium, and of one carcinoma of the bladder transitional cell epithelium among treated rats should not be considered casual.

The biological significance of these results are reinforced if we consider that these tumors are among the same types observed in humans living in geographical areas with an elevated concentration of As in drinking water.

The aforementioned results have shown that Sprague-Dawley rats represent a good animal model to express the carcinogenic potential of As in drinking water. In light of the utility of this model and the results observed, in our opinion, a life-span mega-experiment exposing large groups of male and female Sprague-Dawley rats to As in drinking water at doses much lower than 50 mg/L, starting from embryonic life and lasting until spontaneous death, is urgently required to provide a more adequate scientific basis to the current exposure standards: 50 μg/L in developing countries and 10 μg/L in industrialized countries.[44,45]

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