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In-vitro Prenatal Toxicity of Trimethylarsine, Trimethylarsine Oxide and Trimethylarsine Sulfide

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The embryolethality and the embryotoxicity of trimethylarsine, trimethylarsine oxide and trimethylarsine sulfide were investigated employing Sprague–Dawley rat embryos with intact yolk sacs. The embryos were removed on day 11 of gestation and grown in a culture medium (Waymouth's 725/1) spiked with the arsenic compounds to concentrations in the range 4–100 mM in the presence or absence of rat liver (S-9) homogenate. After 24 h the yolk-sac circulation and heart beat were monitored (indicator of embryolethality), the crown-to-rump lengths were measured, the neural structures (somites) counted, and the development of the limb buds evaluated (indicators of embryotoxicity). At a trimethylarsine concentration of 18.7 mM 78% of the embryos were dead when no S-9 was present. In the presence of S-9 all embryos survived but were necrotic and malformed. Signs of embryotoxicity were observed at concentrations of 18.7 and 9.3 mM. At the 4.7 mM concentration the embryos grew as well as the control embryos. Trimethylarsine oxide ٧ as lethal at 100 mM and severely embryotoxic at 50 and 25 mM. At all but the lowest concentration (4.5 mM) the embryos looked sick, and were frequently necrotic, deformed and underdeveloped. Trimethylarsine sulfide exhibited severe embryotoxicity at 50 mM concentration in the absence and in the presence of S-9. Signs of acute toxicity were observable at 9 mM concentrations of trimethylarsine and trimethylarsine oxide. Compared with other environmental toxicants that show effects at concentrations orders of magnitude smaller, these arsenic compounds cannot be classified as very toxic.

Keywords: trimethylarsine; trimethylarsine oxide; trimethylarsine sulfide; embryotoxicity; rat embryos

INTRODUCTION

Trimethylarsine [$(CH_3)_3As$], an organic derivative of trivalent arsenic, is an oxygen-sensitive liquid with a distinct, unpleasant odor and a boiling point of 53–55 °C. The liquid has a vapor pressure of 238 Torr at 20 °C. Trimethylarsine is a commercially available compound and may become an important starting material for the production of gallium arsenide.[1] It is known to be the product of the action of molds[2] and other microorganisms[3] on inorganic arsenic compounds. Inhalation of trimethylarsine produced by molds from arsenic-containing green paint pigments caused several deaths during the 19th century. Recently, trace amounts of arsenic were found in natural gas;[4,5] a large fraction of the arsenic in natural gas is trimethylarsine.[6] Little information exists about the biological and toxicological effects of trimethylarsine. This compound was found to be not very toxic when given to mice or hamsters orally.[7]

Arsenite, an inorganic arsenic species containing trivalent arsenic, possesses the highest toxicity among common arsenic compounds. Arsenate, an inorganic pentavalent arsenic species, is less toxic than arsenite in spite of the fact that arsenate is reduced to arsenite by many organisms, including man.[8] The biological methylation of arsenite produces methylarsonic acid, dimethylarsinic acid, and finally trimethylarsine oxide. Methylarsonic acid and dimethylarsinic acid are less toxic than arsenate or arsenite[8] in terms of LD_{50} values. Under anaerobic conditions the pentavalent methylarsenic compounds can be reduced to methylarsines, $(CH_3)_nAsH_{3-n}$ ($n = 1$, 2, 3).[9] Arsenite, arsenate, methylarsonic acid and dimethylarsinic acid have been shown to possess prenatal toxicity and to be teratogenic.[10]

However, no information exists on the prenatal toxicity of methylarsines, trimethylarsine oxide,

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or trimethylarsine sulfide. Because trimethylarsine oxide may be formed from trimethylarsine in an aerobic environment and trimethylarsine sulfide from trimethylarsine and a sulfur source, the toxicities of these compounds are of interest in comparison with the toxicity of trimethylarsine. Trimethylarsine sulfide was identified as a solid deposit in pipelines carrying natural gas that contains trimethylarsine. This paper reports the results of investigations of prenatal toxic effects of trimethylarsine, its oxide and its sulfide on rat embryos.

EXPERIMENTAL

Trimethylarsine was prepared from arsenic trichloride and methylmagnesium iodide in dibutyl ether. The compound was purified by distillation under nitrogen.[11] Trimethylarsine oxide was obtained by oxidation of trimethylarsine with hydrogen peroxide in diethyl ether. Trimethylarsine sulfide was prepared from trimethylarsine and sulfur powder in refluxing ethanol. The compond (m.p. 183 °C) was purified by recrystallization from ethanol.[12]

Waymouth's 752/1 medium, Hanks Balanced Salt Solution (HBSS), penicillin, and streptomycin were obtained from Gibco Inc., Grand Island, NY, USA. Microsomal hepatic supernatant (S-9), prepared from male rats treated with Aroclor, was purchased from Microbiological Associates, Bethesda, MD, USA. Glucose 6-phosphate and NADP were provided by Sigma Chemical Co., St Louis, MO, USA.

Embryo cultures

Nulliparous female Sprague–Dawley rats (225–250 g) were supplied by Harlan–Sprague–Dawley Laboratories, Houston, TX, USA. The rats were bred after having been kept on a 12 h light, 12 h dark cycle for 10 days. Conception was monitored by aspirating vaginal fluids and checking for the presence of sperm. The day on which sperm was found was considered day 1 of gestation. On day 11 of gestation the pregnant rats were etherized and the embryos in their intact yolk sacs removed. The embryos were washed with HBSS and then cultured as described earlier.[13,14] Randomly selected groups of nine embryos were transferred into culture bottles each containing 7.5 ml Waymouth's 725/1 medium appropriately spiked with trimethylarsine, trimethylarsine oxide or trimethylarsine sulfide, 7.5 ml rat serum, 1500 units penicillin, and 1.5 mg streptomycin. To test the arsenic compounds in the presence of bioactivating enzymes, hepatic S-9 (0.225 ml), NADP (5.7 mg) and glucose 6-phosphate (21.2 mg) were mixed into the 15 ml culture medium. The cultures were kept in a 5%-CO_2-in-air incubator maintained at 37 °C. After 24 h the embryos were checked with respect to yolk-sac circulation and heart beat as indicators of embryo viability, and with respect to crown-to-rump length, somite count, and malformations as indicators of embryotoxicity.

Preparation of culture media containing trimethylarsine

Culture media (15 ml each) prepared as described in the preceding section were mixed with 30 µl, 15 µl or 7.5 µl of pure trimethylarsine. The mixtures were shaken in air to achieve homogeneity. The embryos were then placed in these arsenic-containing media.

Preparation of culture media containing trimethylarsine oxide

Trimethylarsine (10.0 g; 0.084 mol) was added to diethyl ether (220 ml) kept in a 500 ml, three-necked, round-bottomed flask equipped with a reflux condenser, a pressure-equalizing dropping funnel and a magnetic stirring bar. Hydrogen peroxide (30%; 9.4 ml; 0.14 mol) was slowly dropped into the vigorously stirred solution. The mixture was stirred for 24 h. The diethyl ether was distilled from the reaction mixture, the residue was dissolved in water, and the aqueous solution was evaporated to dryness at 3.4 Torr in a rotary evaporator heated by a water bath at 72 °C. The white residue was quickly scraped from the bottom of the flask and transferred into a vacuum sublimator. The air-cooled sublimator was heated in an oil bath. Trimethylarsine oxide sublimed at 2.8 Torr at a bath temperature of 140 °C. When all the trimethylarsine oxide had sublimed, the stopcock on the sublimator leading to the vacuum system was closed, the oil bath removed, and the sublimator allowed to cool to room temperature. The sublimator was then transferred into a nitrogen-filled dry box. The stopcock on the sublimator was opened to release the vacuum and fill the sublimator with nitrogen. The sublimator was disassembled and the

Table 1 Embryolethality of trimethylarsine in the presence or absence of microsomal hepatic supernatant (S-9)

Trimethylarsine				Without S-9		With S-9	
[μg (15 ml)$^{-1}$]	(mg l^{-1})	(mol l^{-1})	(mg As l^{-1})	No. of embryos live[a]/total	Embryo survival (%)	No. of embryos (live[a]/total)	Embryo survival (%)
30.0	2.25	0.0187	1.40	4/18	22	18/18	100
15.0	1.12	0.0093	0.70	17/18	94	18/18	100
7.5	0.56	0.0047	0.35	18/18	100	18/18	100

[a] Embryos were judged to be alive when yolk-sac circulation and heartbeat were observed under the microscope.

trimethylarsine oxide scraped from the cold finger onto a watch glass. The sublimator was wiped clean with a paper towel, the trimethylarsine oxide placed in the sublimator, the sublimator reassembled, the stopcock closed, and the sublimator taken from the dry box for resublimation. The resublimed trimethylarsine oxide (65% yield) melted at 189–191 °C. The very hygroscopic compound was stored in a screw-capped bottle under nitrogen in a dry box.

Trimethylarsine oxide was transferred in the nitrogen-filled dry box from the storage bottle into a pre-weighed small vial. The vial was closed, taken from the dry box, and weighed. The trimethylarsine oxide (816 mg; 6.0 mmol) was added to 30.0 ml of the Waymouth's 725/1 medium. The mixture was shaken until the trimethylarsine oxide had dissolved. The resulting solution contained 27.2 mg ml^{-1} trimethylarsine oxide (15 mg ml^{-1} arsenic). Aliquots of this solution (7.50, 3.80, 1.90, 1.40, 0.70, 0.35 ml) were mixed with the volume of arsenic-free Waymouth's medium required to reach a total volume of 7.50 ml. To each of these arsenic-containing 7.50 ml portions were added the other ingredients needed to complete the culture media.

Preparation of the culture medium containing trimethylarsine sulfide

Trimethylarsine sulfide (277.2 mg) was dissolved in 12.0 ml Waymouth's medium. Arsenic-free Waymouth's medium (10.0 ml) was mixed with the arsenic-containing medium (5.00 ml). The resulting solution (7.70 mg ml^{-1} trimethylarsine sulfide; 3.80 mg ml^{-1} arsenic; 0.05 mol l^{-1}), after addition of the other needed ingredients, was used to culture the embryos.

RESULTS AND DISCUSSION

The effects of trimethylarsine on rat embryos were investigated by keeping groups of nine 10-day-old embryos for 24 h in media spiked with trimethylarsine. Usually, the toxicant is introduced into the media as a solution in dimethyl sulfoxide. Trimethylarsine, however, is not sufficiently soluble in this solvent to achieve concentrations of the arsenic compound in the media required for the appearance of toxic effects. For this reason pure trimethylarsine was mixed with the media.

Embryolethality of trimethylarsine

Severe embryolethality with 78% mortality was observed in the groups of embryos cultured in media with a trimethylarsine concentration of 0.0187 mol l^{-1} in the absence of hepatic S-9 (Table 1). When S-9 was present in these media, all embryos survived. Extensive necrosis was noticed on the dead embryos with malformation of the embryonic ultrastructure. In the presence of S-9, the ultrastructure was maintained; however, the growth and the development of the embryos were severely retarded. In media with lower concentrations of trimethylarsine (0.0093, 0.0047 mol l^{-1}), almost all of the embryos survived (Table 1).

Embryotoxicity of trimethylarsine

The embryotoxicity of trimethylarsine was assessed by measuring the crown-to-rump length of the embryos, counting somites, and identifying malformed embryos. The embryotoxicity of trimethylarsine at the concentration of 0.0187 mol l^{-1} in the absence of hepatic S-9 was

not evaluated, because most of the embryos did not survive under these conditions. The embryos cultured in the medium with 0.0187 mol l^{-1} trimethylarsine and hepatic S-9 had significantly smaller crown-to-rump length (2.4 mm) than the controls (3.0 mm) (Table 2). Sixteen of the 18 embryos were malformed. At a trimethylarsine concentration of 0.0093 mol l^{-1} in the absence of S-9, embryonic growth was impaired and most of the embryos showed malformations. In media with 0.0093 mol l^{-1} trimethylarsine but in presence of S-9 and in media with 0.0047 mol l^{-1} trimethylarsine in the absence or presence of S-9, the embryos grew as well as the embryos in the control groups. Malformations were observed only in isolated cases under these conditions. The somite count was approximately the same for the control embryos and the embryos exposed to trimethylarsine (Table 2).

Embryolethality of trimethylarsine oxide

The embryolethality of trimethylarsine oxide was tested at concentrations of 0.0045, 0.009, 0.018, 0.025, 0.050, and 0.10 mol l^{-1}. None of the embryos survived in the media with 0.10 mol l^{-1} of trimethylarsine oxide. At a concentration of 0.05 mol l^{-1} all the embryos survived in the absence of hepatic S-9; in the presence of S-9 significant mortality was observed (Table 3). In the media with trimethylarsine oxide concentrations of 0.025, 0.018, 0.009 and 0.0045 mol l^{-1} the embryos survived.

Embryotoxicity of trimethylarsine oxide

Trimethylarsine oxide possesses pronounced embryotoxicity at non-lethal concentrations. At concentrations of 0.05 and 0.025 mol l^{-1} many embryos were severely deformed. At lower concentrations the percentage of deformed embryos is approximately equal to that for the control groups (Table 3). The embryotoxicity was quantified in terms of embryonic growth (crown-to-rump length) and the somite count. Embryonic growth was drastically reduced in media with high but non-lethal concentrations of trimethylarsine oxide. The average crown-to-rump length increased toward the values characteristic for the control groups with decreasing concentration of the arsenic compound. The embryos in media without hepatic S-9 were generally smaller than the embryos in media with S-9 at all concentrations investigated. At the highest non-lethal concentration (0.05 mol l^{-1}) without S-9 the average crown-to-rump length was only 66% of the lengths of the controls. The corresponding values from the experiments in the presence of S-9 was 70%. The somite counts show similar trends (Table 4). The values for embryonic growth and somite count at 0.018 mol l^{-1} trimethylarsine oxide without S-9 are lower than the values for the next higher and next lower concentrations; the values for media with S-9 follow the general trend (increasing values with decreasing concentration of arsenic compound). These observations were made in two independent sets of experiments. On microscopic examination of the embryos, many were found to lack an observable yolk-sac circulation. This observation was made even in media with low concentrations of trimethylarsine oxide. Although this effect was not as reproducible as one would like, the impairment of the yolk-sac circulation under these conditions seems to be real (Table 4). At all but the lowest concentration of trimethylarsine oxide the embryos looked sick, were frequently necrotic,

Table 2 Embryotoxicity of trimethylarsine in the presence or absence of microsomal hepatic supernatant (S-9)

Trimethylarsine concn (mol l^{-1})	No. of embryos (live/total)	Hepatic S-9	Crown-to-rump length[a] (mm)	Somite count[a]	No. of embryos (malformed/total)
0.0	18/18	Without	3.3 ± 0.2	19.9 ± 0.7	1/18
0.0	18/18	With	3.0 ± 0.3	19.8 ± 0.9	0/18
0.0187	18/18	Without	2.4 ± 0.2	19.3 ± 0.9	16/18
0.0093	17/18	Without	2.4 ± 0.2	17.1 ± 0.8	16/18
0.0093	18/18	With	3.0 ± 0.3	20.5 ± 0.9	2/18
0.0047	18/18	Without	3.1 ± 0.3	21.4 ± 0.5	0/18
0.0047	18/18	With	3.3 ± 0.1	20.2 ± 0.8	0/18

[a] Average ± SEM.

Table 3 Embryolethality of trimethylarsine oxide in the presence or absence of microsomal hepatic supernatant (S-9)

Trimethylarsine oxide		Arsenic	Hepatic S-9	No. of embryos	Embryo survival	No. of embryos	Embryos malformed
(mg ml^{-1})	(mol l^{-1})	(mg ml^{-1})		(live/total)	(%)	(malformed/live)	(%)
0.0	0.0	0.0	Without	18/18	100	02/18	11
			With	18/18	100	02/18	11
13.6	0.10	7.5	Without	00/18	0		
			With	00/18	0		
6.89	0.05	3.8	Without	18/18	100	14/18	78
			With	11/18	61	08/13	62
3.45	0.025	1.9	Without	18/18	100	13/18	72
			With	18/18	100	12/18	67
2.45	0.018	1.4	Without	17/17	100	08/17	47
			With	16/16	100	01/16	6
1.27	0.009	0.7	Without	16/17	94	02/16	12
0.63	0.0045	0.35	Without	09/09	100	02/09	22
			With	08/09	89	02/09	22

deformed and underdeveloped, with cysts in the head region, and appeared opaque.

Embryotoxicity of trimethylarsine sulfide

The effects of trimethylarsine sulfide on rat embryos were investigated only at a concentration of 0.05 mol l^{-1}. In the absence of hepatic S-9 all nine embryos survived, but were sick, deformed and necrotic. All of the embryos lacked yolk-sac circulation. The average crown-to-rump length (2.66 ± 0.06 mm) was only 67% of the length of the controls (4.0 ± 0.1 mm). The somite count (20.1 ± 0.7) was close to the count for the controls (23.1 ± 0.5). The embryos in the same medium but with hepatic S-9 were affected more severely than the embryos in the S-9-free medium. Four out of nine embryos were dead; all the others were deformed, necrotic and opaque. The average crown-to-rump length was low (2.5 ± 0.3 mm) as was the somite count (16.4 ± 1.5).

Table 4 Embryotoxicity of trimethylarsine oxide in the presence or absence of microsomal hepatic supernatant (S-9)

Trimethylarsine oxide (mol l^{-1})	Hepatic S-9	Crown-to-rump length[a] (mm)	Somite count[a]	Embryos without/with heartbeat	Embryos without/with yolk-sac circulation	Embryos with limb buds impaired/not impaired
0.100	Without	All dead	—	18/0	18/0	—
	With	All dead	—	18/0	18/0	—
0.05	Without	2.1 ± 0.1	14.2 ± 1.0	3/6	3/6	7/2
	With	2.4 ± 0.1	16.7 ± 1.4	6/3[b]	9/0[b]	5/4[b]
0.025	Without	2.5 ± 0.1	19.1 ± 0.8	9/9	9/9	2/16
	With	2.8 ± 0.2	19.5 ± 0.7	9/9	9/9	0/18
0.018	Without	2.2 ± 0.1	12.3 ± 0.6	0/17	8/9	6/11
	With	3.3 ± 0.1	20.0 ± 0.8	0/16	5/11	0/16
0.009	Without	2.6 ± 0.1	18.7 ± 1.0	0/16	10/6	5/11
0.0045	Without	2.8 ± 0.1	19.2 ± 0.7	0/9	5/4	0/9
	With	3.1 ± 0.1	20.1 ± 0.3	0/8	0/8	0/8
0.00	Without	3.2 ± 0.1	22.5 ± 0.6	0/27	0/27	0/27
0.00	With	3.4 ± 0.2	22.6 ± 0.6	0/27	0/27	0/27

[a] Average ± SEM.

[b] Five of these nine embryos were judged to be dead.

Comparison of the embryotoxicities

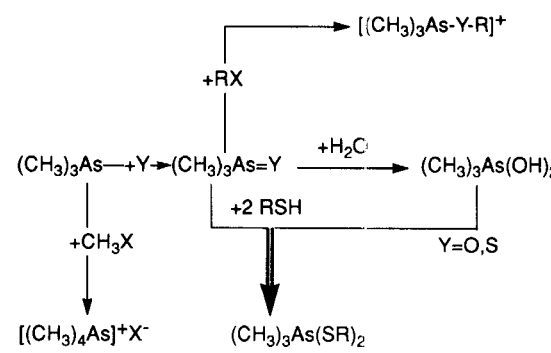
Trimethylarsine appears to be more toxic than trimethylarsine oxide. At a concentration of the arsenic compounds of 0.0187 mol l^{-1} in the absence of hepatic S-9 all the embryos survived when exposed to trimethylarsine oxide, whereas only four out of 18 were alive in the experiments with trimethylarsine (Tables 1, 3). In the presence of S-9 all the embryos survived, irrespective of the nature of the arsenic compound. The collected data give weak indications that S-9 ameliorates the toxicity of trimethylarsine and trimethylarsine oxide (Tables 2, 4). Trimethylarsine sulfide and trimethylarsine oxide at 0.05 mol l^{-1} appear to act very similarly. More embryos survived at this concentration in the absence than in the presence of hepatic S-9 (Table 3).

Chemical aspects of the toxicity of trimethylarsines and its derivatives

The arsenic compounds employed in this study were carefully purified to make sure that only the intended compounds were placed in the culture media. Precautions are particularly appropriate for trimethylarsine oxide that is prepared by oxidation of trimethylarsine. Trialkylarsines can be easily dealkylated oxidatively to dialkylarsinic acids. These acids are considered to have rather low toxicity. To ascertain that pure trimethylarsine oxide was used in the experiments, the oxide was purified by vacuum sublimation. Trimethylarsine sulfide is a stable crystalline compound that can be easily purified by recrystallization. Although the nature of the arsenic compounds added to the media is known, the fate of these compounds in the media under the influence of water, air and the components of the medium is uncertain.

Trimethylarsine is easily oxidized to trimethylarsine oxide. The culture media with the embryos must be kept in an aerobic environment. Under these conditions the trimethylarsine could have been oxidized to trimethylarsine oxide. However, the toxicity data suggest that a rapid and complete oxidation of trimethylarsine to the oxide is unlikely. If this process would proceed rapidly, then the observed effects should have been the same for the two compounds at comparable concentrations. However, the effects are different (Tables 1–4).

Which reactions could change trimethylarsine oxide? Trimethylarsine oxide, present in an aqueous medium as the dihydroxide, can condense with thiol groups and become reduced to trimethylarsine, which in turn can be methylated to an arsonium salt. Whether any of these reactions occurred in the media or in the cells of the embryos is not known. Trimethylarsine given orally to mice or hamsters was partially converted to trimethylarsine oxide. This compound was excreted in the urine. Demethylation of trimethylarsine was not observed.[7] Similar experiments were carried out with trimethylarsine oxide given orally to hamsters[15] and mice.[16] The compound was quickly eliminated in the urine and partly reduced to trimethylarsine, which was detected in the expired air.[15,16] In addition, trimethylarsine can be methylated to tetramethylarsonium salts. Tetramethylarsonium iodide given orally to mice has an LD_{50} value of 890 mg kg^{-1}. Nothing is known about the environmental chemistry of trimethylarsine sulfide. In principle, this sulfide can be methylated to a trimethyl(methylthio)arsonium salt, can be reduced to trimethylarsine and could perhaps react with thiol groups. The hydrolysis of trimethylarsine sulfide to trimethylarsine dihydroxide in the aqueous media employed in the toxicological experiments is unlikely; however, this possibility must be considered. The possible reactions are summarized in Scheme 1.



Scheme 1

In spite of the uncertainty about the nature of the arsenic compounds actually interacting with the biochemical systems of the embryos, the acute prenatal toxicity of trimethylarsine, trimethylarsine oxide and trimethylarsine sulfide is established. Acute toxicity is certainly noticeable at 0.009 mol l^{-1} of the arsenic compounds. Because of the millimolar concentrations required to observe toxic effects, the prenatal toxicity of these arsenic compounds is not very high when compared with other environmental toxicants.

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