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Drinking water treatment is not associated with an observed increase in neural tube defects in mice

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Abstract

Disinfection by-products (DBPs) arise when natural organic matter in source water reacts with disinfectants used in the water treatment process. Studies have suggested an association between DBPs and birth defects. Neural tube defects (NTDs) in embryos of untreated control mice were first observed in-house in May 2006 and have continued to date. The source of the NTD-inducing agent was previously determined to be a component of drinking water. Tap water samples from a variety of sources were analyzed for trihalomethanes (THMs) to determine if they were causing the malformations. NTDs were observed in CD-1 mice provided with treated and untreated surface water. Occurrence of NTDs varied by water source and treatment regimens. THMs were detected in tap water derived from surface water but not detected in tap water derived from a groundwater source. THMs were absent in untreated river water and laboratory purified waters, yet the percentage of NTDs in untreated river water were similar to the treated water counterpart. These findings indicate that THMs were not the primary cause of NTDs in the mice since the occurrence of NTDs was unrelated to drinking water disinfection.

Keywords

Neural tube defects (NTDs); Birth defects; Teratogens; Purge-trap gas chromatography-mass spectroscopy (PT GC-MS); Disinfection by-products (DBPs); Trihalomethanes (THMs)

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Conflict of interest None of the authors have conflicts of interest.

Introduction

Two principal threats to the integrity of fresh water sources are pathogens and chemical contamination. In 1974, the US Congress passed the Safe Drinking Water Act that regulates 91 contaminants including disinfectants, disinfection by-products (DBPs), inorganic chemicals, microorganisms, organic chemicals, and radionuclides used in the USA. There are numerous chemicals used in the USA whose health effects continue to be unclear, and only some of which are monitored by the US Environmental Protection Agency (EPA).

The National Primary Drinking Water Regulations established by the EPA have maximum contaminant levels (MCL) in place for a number of contaminants (EPA 2012). The Stage 1 Disinfection Byproduct Rule (US EPA 816-B-05-004) was enacted in 1998 to decrease high levels of DBPs in finished waters. DBPs form when natural organic matter in source water reacts with disinfectants used during the water treatment process. Among these by-products, the EPA regulates concentrations of total trihalomethanes (THMs) at 80 µg/L annual average, as these are considered to be probable human carcinogens (Graves et al. 2001; Dunnick and Melnick 1993). The principal monitored THMs are chloroform, bromoform, bromodichloromethane (BDCM), and chlorodibromomethane (CDBM). Many municipal water treatment facilities have elected to change their disinfection process from chlorination to chloramination in order to reduce production of trihalomethanes. Chloramination is similar to chlorination, but includes the subsequent addition of ammonia, which reacts with chlorine to form chloramines. By-products are still formed during the chloramination process (Le Roux et al. 2011; Krasner et al. 2006); however, many of these have not been identified.

Neural tube defects (NTDs) are birth defects that arise early in fetal development during initial formation of the nervous system. During the embryonic period, the neural folds raise and fuse together, creating the brain and spinal cord. Failure of the neural tube to close properly will result in an NTD. According to the Centers for Disease Control and Prevention, about 3,000 babies born annually are affected with major NTDs (CDC 2004). These numbers do not include spina bifida occulta which is a mild form or “hidden” NTD. Because symptoms are mild or absent, this malformation is rarely reported, underestimating the actual number of NTDs. Complications from major NTDs can range from lifelong disability to death. Combinations of genetic and environmental factors such as maternal obesity, low socioeconomic status, and cigarette smoke have been associated with an increased risk of NTDs (Suarez et al. 2008; Wasserman et al. 1998). Folic acid supplementation during the periconceptional period has been shown to decrease the occurrence of NTDs (Smithells et al. 1980).

In 2005, the local water treatment plant changed its disinfection process from chlorination to chloramination. Approximately 6 months after this water treatment change, NTDs were observed for the first time in litters of untreated control mice. Litters exposed to tap water since this change have averaged 3–30 % NTDs. NTDs could be eliminated by providing the mice with deionized distilled water for two generations (F₂), indicating that the contaminant was removed by the purification process. NTDs could once again be induced when F₂ mice were provided tap water from different geographic locations prior to breeding and gestation.

This process isolated exposure to tap water as the variable responsible for the NTDs (Mallela et al. 2010).

Animal and epidemiological studies provided evidence to suggest that THMs may be responsible for the NTDs. Human epidemiological studies have demonstrated a positive correlation between THMs from tap waters and abnormal reproductive outcomes, including NTDs (Dodds and King 2001; Klotz and Pyrch 1999; Bove et al. 1995). Exposure to THMs has also been shown to negatively alter rodent embryonic development (Ruddick et al. 1983; Murray et al. 1979; Schwetz et al. 1974; Narotsky et al. 2011). Taken together, these results suggest that THMs could be associated with the observed developmental defects.

Concentrations of THMs should decrease after switching to chloramination; however, adverse reproductive outcomes associated with THM exposure indicated that these DBPs could not be disregarded as potential causes of the NTDs. The objective of this experiment was to investigate if THMs could in fact be eliminated as a cause of the observed malformations. Mice were exposed to waters from different sources and treatment regimens. THM concentrations and NTD formation in exposed mice were assessed.

Materials and methods

Animals

CD-1 mice (Charles River Labs, Raleigh, NC) obtained at 5–6 weeks of age were maintained on distilled deionized water (DDI) and bred in-house for two generations to ensure no exposure to tap water prior to the experiment, as described in Mallela et al. (2010). Animals were maintained in a climate-controlled room under a 12-h light/dark cycle, 20–25 °C, and 30–60 % relative humidity. National Toxicology Program (NTP) diet and water were provided ad libitum. Animals were provided water in glass bottles that were changed weekly. At 8–10 weeks of age, F₂ females were randomly assigned to a water treatment or DDI control group. Each group was provided test water or DDI for 8 weeks prior to breeding and throughout gestation. Animals were bred, and the presence of a vaginal plug the next morning confirmed mating and was designated as gestational day zero. Pregnant females were euthanized by CO₂ inhalation on gestational day 9, and the uteri were removed and transferred to Dulbecco's modified Eagle medium (DMEM). All embryos were carefully staged by gestational day, somite count, number of branchial arches, and extent of heart and limb bud formation to ensure sufficient development for normal neural tube closure. The cranial neural tube is usually closed by the 13 somite stage. Embryos with <15 somites or only two branchial arches were not included for evaluation. Due to our photoperiod and breeding schedule, most embryos had >17 somites at evaluation. Embryos were dissected from the uterus and yolk sac and assessed for NTDs under a stereozoom microscope (Olympus SZX7, Melville, NY). All animal experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the College of Veterinary Medicine at Virginia Tech, an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited facility.

Water collection

Waters were collected in certified I-Chem amber bottles (New Castle, DE) from three municipal areas in Virginia, supplied respectively by two separate river systems (river 1 and river 2) and a municipal well (Table 1). RAW R1 was collected at the treatment plant intake, and chloraminated TAP R1 was collected after distribution from a tap. TAP R1 was further purified by a Vaponics (Rockland, MA) VSS-30TI deionization/distillation still and collected as PURE R1. TAP R2 and TAP W3 were both from treatment plants utilizing chlorination; water was collected from respective distribution systems. RAW R1 was autoclaved prior to being provided to the mice to eliminate pathogens. A sample of TAP R1 was also autoclaved to control for possible changes to the contaminant from the autoclaving process.

Statistical analysis of mouse data

Evaluation of test embryos for NTDs was performed with the examiner blind to the treatment group. The dam served as the unit of analysis with 11–12 dams per treatment group. Percentage of NTDs per litter was calculated for each dam and then averaged per treatment to give average percentage per dam per litter. Differences in NTDs between control and treated dams were reported as a mean percent with standard error. Differences in the mean percentage of NTDs were analyzed using a Kruskal-Wallis test and Tukey's means comparison test (Statistix, Tallahassee, FL). Differences were considered significant at p 0.05.

Purge-trap GC-MS water analysis

All water samples were analyzed for volatile organic compounds (VOCs) by EPA Method 524.2 using an Eclipse 4660 Purge-and-Trap Sample Concentrator (College Station, TX) coupled with a Shimadzu (Columbia, MD) GC-2010 / MS-QP2010S. An Rtx-VMS (Restek, Bellefonte, PA) fused capillary silica column (30 m×0.25 mm i.d., 1.4- μ m film thickness) was used for GC separation. Analytical quality control was assessed using THM standard solutions (2,000 μ g/mL 501 THM mix in P&T grade methanol; Restek, Bellefonte, PA) fortified with 50 μ g/L of internal standard (4-bromofluorobenzene). Samples were heated to 40 °C, held for 2 min, increased 20 °C/min until 250 °C, held for 2 min, purged for 11 min, baked for 5.5 min, and desorbed on the purge-and-trap for 2 min at 24 °C. Mass spectra were interpreted using a Shimadzu GC-MS-QP 2010 System Package (Columbia, MD). EPA Method 524.2 with an Rtx-VMS column (Restek, Bellefonte, PA) has the ability to detect THMs and the compounds listed in Table 2.

Results and discussion

Occurrence of NTDs differed between the water exposures (Fig. 1). NTDs were significantly higher in mice exposed to water from river 2 (TAP R2) compared to water from river 1 (TAP R1). Mice receiving RAW R1, TAP R1, and TAP R1 autoclaved exhibited 12–14 % NTDs in offspring and were not statistically different. Animals exposed to groundwater from a municipally chlorinated well (TAP W3) did not exhibit NTDs. These results suggest that the contaminant responsible for NTDs was present in raw water, and that municipal water processing did not alter its concentration. The contaminant(s) also appears to be heat stable,

since similar numbers of NTDs in TAP R1 and TAP R1 autoclaved were observed. Deionized and distilled PURE R1 water did not cause NTDs, suggesting the contaminant can be removed by laboratory purification.

In addition to THMs, the compounds listed in Table 2 are detectable using EPA method 524.2; however, only two THMs were identified. Chloroform was detected in TAP R1 and TAP R2 at average concentrations of 39 and 49 $\mu\text{g/L}$, respectively (Table 3). BDCM was present in TAP R1 at an average concentration of 5 $\mu\text{g/L}$ but was not detected ($<5 \mu\text{g/L}$) in TAP R2. Chloroform and BDCM were not detected in RAW R1 and the chlorinated groundwater sample TAP W3. Both chloroform and BDCM were removed by laboratory purification and were undetectable ($<5 \mu\text{g/L}$) in PURE R1. The other two regulated THMs, bromoform and CDBM, were not detected in any samples.

The purpose of this study was to evaluate if the observed NTDs were associated with THM exposure. Different water sources produce varied concentrations and types of DBPs after treatment (Hua and Reckhow 2007). Compared to chlorination, chloramination decreases the amount of THMs formed (Goslan et al. 2009; Krasner 2009; Lu et al. 2009); however, THMs are still needed to be considered due to the research linking THMs with detrimental developmental outcomes.

Through human epidemiological studies, Klotz and Pyrch (1999) reported an increased incidence of NTDs in one population of first-trimester pregnant mothers exposed to tap water with THM concentrations ranging from 40–80 $\mu\text{g/L}$. Bove et al. (1995) observed abnormalities in gestational growth and development including central nervous system defects associated with exposure to THMs. A retrospective study conducted by Dodds and King (2001) found an increased risk of NTDs when cohorts were exposed to BDCM at concentrations of 20 $\mu\text{g/L}$ or greater.

Results from animal studies are conflicting. Rats exposed to 0, 50, 150, 450, or 900 mg/kg and rabbits exposed to 0, 15, 150, 450, or 900 mg/kg of BDCM did not significantly exhibit any embryotoxic effects (Christian et al. 2001). Rats treated with BDCM on gestational days 6–10 at 75 $\text{mg kg}^{-1}\text{day}^{-1}$ exhibited full litter resorption; however, exposure on gestational days 11–15 at 75 or 100 $\text{mg kg}^{-1}\text{day}^{-1}$ had no effect (Bielmeier et al. 2001). Fetotoxicity was observed in rats exposed during gestational days 6–15 to chloroform at 100, 200, and 400 mg/kg. Rat embryos exposed to inhaled chloroform had an increase in resorption, delayed fetal development, and other embryonic malformations, and oral administration of chloroform to rats during gestation caused significant decreases in fetal weights (Ruddick et al. 1983; Murray et al. 1979; Schwetz et al. 1974). Fetotoxicity was also observed from CDBM and bromoform at 50, 100, or 200 $\text{mg kg}^{-1}\text{day}^{-1}$ (Ruddick et al. 1983). These inconsistent results have made it difficult to establish causality between THMs and developmental outcomes observed in epidemiological studies. While epidemiological studies can associate THM exposure with increased risk of adverse reproductive outcomes, animal studies are often needed to establish a causal relationship between these factors.

Complicating the picture is the fact that mixtures of trace contaminants, including DBPs, can produce additive or synergistic effects, leading to increased toxicity (Jordan et al. 2012;

Hernandez et al. 2012; Andrews et al. 2004; Narotsky et al. 2011). Studies evaluating the cytotoxicity of DBP mixtures have observed 1.5–4-fold increases in toxicity compared to the individual compounds (Zhang et al. 2011). Rat whole-embryo culture studies have indicated that the developmental toxicity of DBPs is additive; greater number of embryonic defects, like NTDs, have been observed when DBPs are applied as a mixture (Andrews et al. 2004). Trace contaminants with different chemical structures and modes of action can additively and/or synergistically produce significant toxic effects or below no observable adverse effect levels when combined into a mixture (Hermens et al. 1985; Faust et al. 2003; Narotsky et al. 2011). It is plausible that a complex range of trace contaminants that may still include DBPs is responsible for the mouse's NTDs caused by surface water exposure.

THM concentrations were unrelated to changes in NTD occurrences. Increased NTDs were observed in mice receiving TAP R1 and TAP R2, which had detectable concentrations of THMs, but were also observed in mice that received RAW R1, which yielded non-detectable concentrations of THMs. This suggests that the contaminant is present in the surface water before the water treatment process. Average concentrations of chloroform and BDCM in TAP R1 were approximately 40–50 % less after the water treatment plant switched to chloramination and never exceeded EPA standards (Table 4). In humans, elevated risk of adverse reproductive outcomes has typically been associated with THM concentrations exceeding current regulatory standards (Levallois et al. 2012; Wright et al. 2003). In the current study, THM concentrations never exceeded regulatory standards and an association between THMs and NTDs was not observed. This is in contrast to several human epidemiological studies. It is unclear whether this difference is due to the lower THM concentrations, lower sensitivity of mice to THMs, or other experimental factors. Taken together, these findings indicate that THMs were not the primary cause of NTDs in the mice, but rather the contaminant is a component of surface water. Surface waters are often contaminated by substances present in wastewater and urban or rural rainwater runoff including pharmaceuticals, personal care products, petroleum products, and herbicides and pesticides.

Conclusions

The NTD-inducing contaminant is a component of surface waters and does not appear to be a by-product of water disinfection. THMs detected in drinking water samples were within EPA standards and not the primary cause of NTDs in the mice. Identification of other water contaminants as causative agents for the NTDs will require further study. Future research should focus on contaminants that find their way into surface waters prior to the water treatment plant.

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Fig. 1.

Occurrence of NTDs in offspring of mice provided water from various water sources and treatments. Mean NTD $\pm$ SEM was calculated per litter with $N=10-12$ dams per water treatment. Significant differences in NTD rates among groups were determined by a Kruskal-Wallis test ($p < 0.05$) and Tukey's mean comparison test; Bars with similar letters are not significantly different

Table 1

Source water, location, and treatment schemes used for animal exposures and chemical analysis

Location	Water treatment process	Source	Key
City 1	Untreated river water	River 1	RAW R1
City 1	Municipally chloraminated	River 1	TAP R1
City 1	Deionized distilled (Vaponics (Rockland, MA) VSS-30TI still)	River 1	PURE R1
City 2	Municipally chlorinated	River 2	TAP R2
City 3	Municipally chlorinated	Well	TAP W3

Table 2

Additional compounds detectable using EPA method 524.2 for volatile organics and an Rtx-VMS (Restek, Bellefonte, PA) fused capillary silica column (30 m×0.25 mm i.d., 1.4-μm film thickness)

Compound	Compound
1,1,1,2-Tetrachloroethane	Bromomethane
1,1,1,-Trichloroethane	Carbon tetrachloride
1,1,2,2-Tetrachloroethane	Chlorobenzene
1,1,2-Trichloroethane	Chloroethane
1,1,-Dichloroethane	cis-1,2-Dichloroethane
1,1,-Dichloropropene	cis-1,3-Dichloropropene
1,2,3-Trichlorobenzene	Dibromomethane
1,2,4-Trichlorobenzene	Methylene chloride
1,2,4-Trimethylbenzene	Napthalene
1,2-Dichlorobenzene	<i>n</i> -Butylbenzene
1,2-Dichloroethane	<i>n</i> -Propylbenzene
1,3,5-Trimethylbenzene	o-Xylene
1,3-Dichlorobenzene	sec-Butylbenzene
1,3-Dichloropropane	Styrene
1,4-Dichlorobenzene	tert-Butylbenzene
2,2-Dichloropropane	Tetrachloroethene
2-Chlorotoluene	Toluene
4-Chlorotoluene	Trans-1,2-dichloroethene
Benzene	Trans-1,3-dichloropropene
Bromobenzene	Trichlorofluoromethane
Bromochloromethane	

Table 3
Sample size, mean, median, and range of chloroform and BDCM concentrations in samples analyzed

Sample ^a	Percentage of NTDs ^b	N ^c	Chloroform (µg/L)			BDCM (µg/L)		
			Mean	Median	Range	Mean	Median	Range
RAW R1	12	2	ND	ND	ND	ND	ND	ND
TAP R1	14	23	39	38	29–57	6	5	5–8
PURE R1	0	18	ND	ND	ND	ND	ND	ND
TAP W3	0	1	ND	ND	ND	ND	ND	ND
TAP R2	28	2	49	49	40–57	ND	ND	ND

^aBromoform and CDBM were not detected (ND) in any samples

^bNTD rates from the animal exposure study are included for reference

^cNumber of water samples analyzed

Historical THM data obtained from the local water utility before and after the change to chloramination as a disinfection procedure in 2005

Table 4

Sample ^a	Chloroform (µg/L)			BDCM (µg/L)		
	N	Mean	Median	Range	Mean	Median
1999	12	71	71	38–152	6	6
2000	14	54	49	25–93	6	5
2001	16	65	56	18–119	9	8
2002	16	58	49	27–120	6	6
2003	16	69	72	ND–145	8	8
2004	16	58	69	21–105	6	6
2005	16	38	30	22–72	5	5
2006	16	36	24	13–103	4	4
2007	16	31	29	15–62	4	4
2008	16	29	27	15–47	4	4
2009	16	29	22	17–59	4	3
2010	16	28	27	11–52	4	4
2011	16	30	27	15–49	4	4
2012	12	32	25	21–52	4	4

^a Average concentrations of bromoform and CDBM were below detection limits (ND) in all samples