

Neural tube defects in rodents caused by a tap water contaminant

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ABSTRACT

In May of 2006, the Hrubec group suddenly began to observe neural tube defects (NTDs) in embryos of untreated control mice. Unintentional exposure to a teratogenic agent in tap water was identified as the cause. We aimed to identify the contaminant, but first we demonstrated that the NTDs were pathological being present on both gestational day 9 and 10. We also found that a second species, rats, developed NTDs when exposed to tap waters. Disinfection by-products (DBPs) arise when natural organic matter in municipal water sources reacts with disinfectants used in the water treatment process. Purge and trap gas chromatography-mass spectrometry (PT GC-MS) and animal exposure studies were used to determine if the teratogenic contaminant was a DBP. Since the distribution pattern of DBPs did not match the distribution pattern of NTDs, we concluded that a DBP was not likely to be responsible for the observed malformations. Pharmaceuticals and personal care products have emerged as ubiquitous contaminants of ground and surface waters, and have been detected in drinking water. In order to analyze for these compounds, we submitted different water samples to a commercial water analysis lab (AXYS Analytical Services, Sidney, BC, Canada). Several pharmaceuticals were identified in a number of samples, including a known teratogenic drug used to treat mood disorders and seizures: carbamazepine. Further analysis for carbamazepine was conducted in-house. Carbamazepine was found in several ground, surface, and tap waters, at various concentrations. To establish whether or not carbamazepine was responsible for NTDs in our mice, we conducted 2 dosing studies. Carbamazepine was provided to mice at concentrations detected in tap water, as well as approximately 2 x and 1000 x that concentration. Both studies found no significant differences in NTD rates among the dose groups. As no dose effect was observed, we concluded that CBZ was not directly responsible for the malformations. The identity of the teratogenic contaminant is not known at this time, but is unlikely to be a DBP or low concentrations of the pharmaceutical carbamazepine.

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I. LITERATURE REVIEW

The need for clean water sources exceeds the supply in many parts of the world. Currently, the World Bank estimates that more than 80 countries experience clean water shortages. Two principal threats to the integrity of fresh water sources are pathogenic and chemical contamination. During 1999 and 2000, the United States Geological Survey measured the concentrations of 95 organic wastewater contaminants from 139 streams across 30 states. Organic wastewater contaminants were found at 80% of the sites sampled, though none of the contaminants exceeded drinking water standards (Wilkinson et al. 2002). Water contamination can occur from point or non-point sources. Point sources include discharges from waste water treatment facilities and factories, whereas non-point sources of contamination involve forestry, urban and agricultural runoff. Agricultural runoff is especially hazardous, given the possibility for pathogenic contamination. In 1993, a waterborne outbreak of the pathogen, *Cryptosporidium parvum*, struck Milwaukee, Wisconsin. As a result of productivity losses and medical fees, the total cost of this outbreak was estimated at 96.2 million US dollars (Corso et al. 2003). Nevertheless, the etiology of these pathogens is typically understood and the public is well informed.

In 1974, the US Congress passed the Safe Drinking Water Act that covers 91 contaminants, which includes only some of the disinfectants, disinfection by-products (DBPs), inorganic chemicals, microorganisms, organic chemicals, and radionuclides used in the United States. There are tens of thousands of chemicals used in the United States, only some of which are monitored by the United States Environmental Protection Agency (EPA), but many whose health effects continue to be unclear. The National Primary Drinking Water Regulations established by the United States EPA have maximum contaminant levels (MCL) in place for a number of contaminants. Disinfection by-products are among a troubling group of emerging contaminants, some of them probable human carcinogens. The MCLs for the following DBPs are as follows: bromate (0.010 mg/L), chlorite (1.0 mg/L), haloacetic acids (0.060

mg/L), and total trihalomethanes (0.080 mg/L). Disinfection by-products arise when natural organic matter present in the source water reacts with disinfectants used in the water treatment plants. For instance, *N*-nitrosodimethylamine (NDMA) emerges when dimethylamine (an organic compound) reacts with chlorine in the presence of ammonia ions (Andrzejewski et al. 2005). In 1954, NDMA caused fatal liver necrosis in rats, rabbits, mice, guinea-pigs, and dogs when given in doses of 20 to 40 mg/kg (Barnes and Magee 1954). The US EPA has set a non-regulatory maximum admissible concentration of NDMA in drinking water at 7 ng/L; however, it has yet to set an official MCL.

Chemical contamination of surface waters by organic compounds such as pharmaceuticals and personal care products has also become a major problem with poorly understood consequences. Therapeutic drugs are created specifically to cure, treat, and/or prevent a disease, and are thus pharmacologically active. Upon ingestion, pharmaceuticals are metabolized by the body and then excreted via the urine and feces. Current methods employed by waste water treatment facilities fail to remove many of these compounds, allowing them to contaminate drinking water sources (Metcalf et al. 2003). Pharmaceuticals also enter the environment by agricultural runoff and sewage leaks, in addition to inappropriate disposal techniques. A combination of these factors has resulted in contamination of drinking water sources (O. A. Jones et al. 2005). At this point, the EPA has begun to investigate these chemicals; however, no protective legislation is in place. Many believe that these therapeutic drug residues present no appreciable risk, given the low concentrations in drinking water. It has been estimated that lifetime (70 years) exposure to several pharmaceuticals via drinking water constitutes less than 20 percent of a single therapeutic dose, suggesting low risk of adverse effect (Webb et al. 2003). Although the concentration of these contaminants remains low ($\mu\text{g/L}$ to ng/L), little is understood about the risk associated with chronic exposure at sub-therapeutic levels to multiple pharmaceutical residues.

Neural tube defects (NTDs) are birth defects that arise early in fetal development. During the embryonic period, the neural folds rise, fold toward midline, and begin to fuse together, forming the brain and spinal cord. Failure of the neural tube to close properly will result in an NTD at the location. Complications from NTDs can range from life-long disability to death. In some, the effects are mild and often inapparent. According to the Centers for Disease Control and Prevention, about 3,000 babies born annually are affected by NTDs; however, this number does not account for miscarriages or abortions as a result of NTDs.

Combinations of genetic and environmental factors are responsible for causing NTDs. Low socioeconomic status has been associated with an increased risk for NTDs (Wasserman et al. 1998), presumably as a result of inadequate nutrition during the periconceptual period. In addition, there is geographical variation in the distribution of NTDs, with the highest incidence in southern Appalachia (Greenberg et al. 1983). Increased risk of NTDs has also been associated with maternal obesity (Rasmussen et al. 2008); however, this may be attributable to the pre-diabetic state hyperinsulinemia (Hendricks et al. 2001). Maternal exposure to cigarette smoke has also been suggested as a risk factor for NTDs (Suarez et al. 2008). Folic acid supplementation during the periconceptual period decreases the occurrences of NTDs (Smithells et al. 1980). The mechanism by which folic acid supplementation prevents NTDs remains unknown. Abnormalities in the folate metabolism pathway have been suggested as the mechanism of genetic transmission for mothers of children with NTDs (Steegers-Theunissen et al. 1994).

The United States Food and Drug Administration (FDA) has developed pregnancy categories for pharmaceuticals in order to protect the developing fetus. Facts about the embryotoxicity of pharmaceuticals inform physicians about which pharmaceuticals should not be prescribed for pregnant women. The pregnancy categories reflect drugs that should be avoided during pregnancy, given the

likelihood for fetal injury. Pharmaceuticals that have demonstrated no risk to the fetus in the first (or later) trimester based on well controlled and adequate studies are Category A. Category B pharmaceuticals fail to demonstrate risk to the fetus and there are no adequate, well-controlled studies in pregnant women. Category C drugs have not been shown to be harmful to the fetus, although, animal studies have indicated adverse results. Potential therapeutic benefits of Category C pharmaceuticals may necessitate use in pregnant women; however, the use of Category B drugs is often preferred. Category D pharmaceuticals show positive evidence of human fetal risk, but again the potential therapeutic benefits may compel use. Finally, Category X pharmaceuticals are known to cause both animal and human abnormalities when taken during pregnancy and the hazard associated with use outweighs the potential benefits.

Drugs that cause NTDs often fall in Categories D and X. Maternal exposure to the anticonvulsant drugs phenytoin, phenobarbital, primidone, valproic acid, and carbamazepine have been associated with NTDs in humans (Hernandez-Diaz et al. 2000; Robert and Guibaud 1982). The aforementioned drugs are folic acid antagonists that block the protective action of folic acid. The mechanism by which folic acid protects against NTDs remains unknown. Several studies have evaluated the teratogenicity of anticonvulsant drugs, and indicate that maternal exposure leads to NTDs (Ceci et al. 1996; Janz 1975; Jentink et al. 2010; K. L. Jones et al. 1989; Matalon et al. 2002; Smithells et al. 1980; Wide et al. 2004).

Carbamazepine (CBZ) is an anticonvulsant drug typically prescribed to treat epilepsy, bipolar disorder, and trigeminal neuralgia. The Food and Drug Administration (FDA) has classified carbamazepine as a pregnancy category D drug, which indicates that it may not be safe for use during pregnancy. Carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide) is an iminostilbene derivative (Figure 1). Carbamazepine works by stabilizing the inactive form of presynaptic sodium channels, which then decreases the firing of sodium dependent action potentials (Macdonald and Kelly 1995). Upon

ingestion, CBZ is oxidized by the hepatic microsomal enzymes to its active metabolite, CBZ 10,11-epoxide (Bertilsson 1978) (Figure 1). Carbamazepine induces the expression of the hepatic microsomal enzymes that metabolize it, and is thus an autoinducer (Eichelbaum et al. 1985). Autoinduction results in a need to increase dosage just to maintain an effective blood level of carbamazepine. Approximately 2-3 % of CBZ remains unchanged and is excreted in urine.

Evidence of the teratogenicity of CBZ in humans is by and large anecdotal, given the inability to validate teratogenicity scientifically in humans. Nonetheless, the association has been strengthened by a number of cohort and case-control studies. Since the mid-seventies, numerous epidemiologic studies have reported increased teratogenic risk associated with CBZ exposure during pregnancy (Ceci et al. 1996; Janz 1975; Jentink et al. 2010; K. L. Jones et al. 1989; Matalon et al. 2002; Wide et al. 2004). One case-control study found that CBZ exposure during the first or second months after the last menstrual period increased NTD risk by six-fold, whereas the absence of CBZ reduced risk (Hernandez-Diaz et al. 2001). When data from five prospective studies totaling 1,379 children was collected and reanalyzed by meta-analysis, researchers found a strong association between congenital abnormalities and CBZ (Samren et al. 1997).

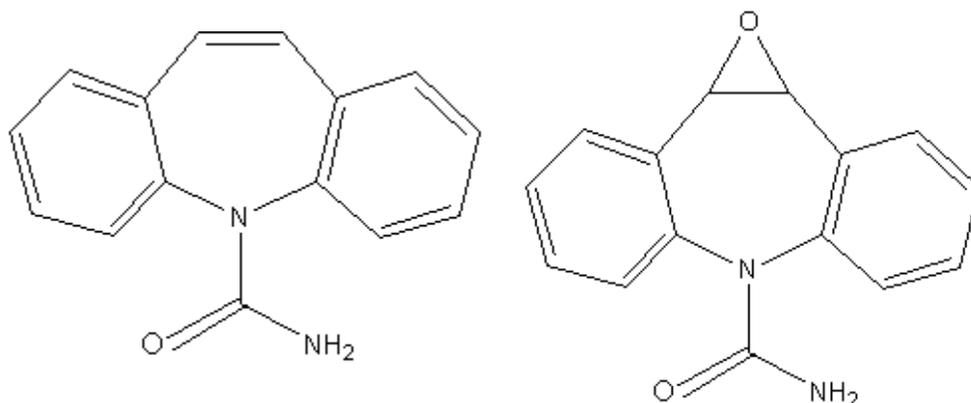


Figure 1. Structures of L: Carbamazepine and R: Carbamazepine-10,11-epoxide

Animal studies are extremely valuable, as they provide insight about the compound's possible effect on humans. The more species in which a compound has an effect, the more likely it is to affect humans. There are few studies evaluating the teratogenicity of CBZ in rodents. These studies indicate that CBZ is teratogenic in both mice and rats. One study demonstrated the teratogenicity of CBZ in mice, and found slight increases in the rate of cleft palate and growth retardation in mouse fetuses treated during the period of organogenesis (Sullivan and McElhatton 1977). An additional study also confirmed the teratogenic effects of CBZ (Eluma et al. 1984). Researchers also found that CBZ was teratogenic in rats at the therapeutic and double the therapeutic dose (El-Sayed et al. 1983). Clearly additional research in this area is necessary, especially to locate a threshold dose.

As highlighted in the popular press, scientific investigations have demonstrated CBZ in ground and surface waters. In 2008, the Associated Press reported pharmaceuticals in the drinking water supplies of 41 million Americans. To date, no study has evaluated the risk posed by CBZ at concentrations found in drinking water contamination. At concentrations present in drinking water, CBZ has been shown to disrupt natural ecosystems (Lienert et al. 2007). Although the ecotoxicological risk of CBZ at these concentrations has been assessed, the teratogenic risk to rodents or humans has not been. Carbamazepine has been detected in groundwater at concentrations up to 610 ng/L (Drewes et al. 2002), and up to 1075 ng/L in surface waters (Heberer et al. 2002).

Carbamazepine is practically insoluble in water, and thus persists in the environment. Carbamazepine has been detected ubiquitously in aquatic environments, which can be attributed to its low biodegradability (Ternes 1998). Andreozzi et al. determined that compared to other pharmaceuticals (sulphamethoxazole, diclofenac, ofloxacin and propranolol) in a salt- and organic-free (bi-distilled) water environment, CBZ had at least a 10-fold greater calculated half-life at the highest latitudes (50 ° N) in winter (Andreozzi et al. 2003). The presence of CBZ in the environment is so

extensive that it has been proposed as a possible anthropogenic marker (Clara et al. 2004).

Carbamazepine is used as a chemical marker to determine the contribution of wastewater to surface water contamination.

In addition to surface and ground waters, CBZ has been found in tap waters around the world. A study conducted in 2008 found that tap water serving about 28 million had a median concentration of 4.1 ng/L of CBZ, and the highest concentration detected was 51 ng/L (Benotti et al. 2009). Conventional drinking water treatments (coagulation, flocculation, filtration, adsorption, and chlorination or chloramination) fail to remove CBZ from sources, with a removal efficiency of less than 10% (Zhang et al. 2008). Biodegradation of CBZ has been achieved using UV irradiation (Kosjek et al. 2009), as well as ozonation (Andreozzi et al. 2002).

The persistence of CBZ in the environment may pose a threat to aquatic wildlife. This is significant since it illustrates the detrimental effects that CBZ has on live organisms. Although studies have investigated the effect CBZ has on aquatic wildlife, the concentration has been at higher (mg/L) than environmentally relevant levels. To date, the lowest concentration that demonstrated a negative effect in an aquatic species was 25 ug/L (Ferrari et al. 2003). Because most studies were conducted at doses higher than those found in the environment, little is understood about the impact that CBZ has on aquatic wildlife.

Current literature suggests that CBZ is not only teratogenic, but incredibly persistent in the environment. Its corruption of drinking water supplies makes it an important emerging contaminant. There is a lack of research on the potential teratogenic effects CBZ may have at the concentrations detected in tap water samples. This study analyzed the concentration of CBZ and other contaminants in drinking water and evaluated the teratogenicity of environmentally relevant contaminant exposures.

II. **Ambient concentrations of disinfection by-products in tap water do not cause neural tube defects in rodents.**

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ABSTRACT

In May of 2006, we suddenly began to observe neural tube defects (NTDs) in embryos of untreated control mice. Unintentional exposure to a teratogenic agent in tap water was identified as the cause. We aimed to identify the contaminant and further characterize the malformations in both mice and rats. We compared NTD rates in gestational day (GD) 9 and GD 10 mice, and found the neural tube still open on GD 10 when it should normally be closed. In addition, rats developed NTDs when exposed to tap waters. Disinfection by-products (DBPs) arise when natural organic matter in source water reacts with disinfectants used in the water treatment process. DBPs have been associated with neural tube defects in both humans and rodents. Purge and trap gas chromatography-mass spectrometry (GC-MS) and animal exposure studies were used to determine if the contaminant was a DBP. The distribution pattern of DBPs analyzed by GC-MS did not fit the distribution pattern of NTDs. We concluded that a DBP was not responsible for the NTDs in our mice.

INTRODUCTION

The water treatment process is complex and typically involves 5 steps: coagulation, sedimentation, filtration, disinfection, and storage. The coagulation process involves aggregation of particles that then settle out of the water column by sedimentation. Smaller particles are then filtered out using layers of sand, gravel, and charcoal. During disinfection, bacteria and other microorganisms are typically destroyed by treating the water with chlorine, chloramines, ozone, or chlorine dioxide. Reservoirs house the water during the storage phase, in order to give the disinfectants enough time to work. Water is then disseminated through distribution systems to businesses and residences.

In 1974, the United States Congress passed the Safe Drinking Water Act. This legislation covers 91 contaminants including some disinfection by-products (DBPs), inorganic chemicals, microorganisms, organic chemicals, and radionuclides used in the United States. There are, however, tens of thousands of chemicals in use in the US today, only some of which are monitored by the United States Environmental Protection Agency (EPA). The health effects from exposure to many of these chemicals are unclear. The National Primary Drinking Water Regulations, developed by the US EPA, establishes maximum contaminant levels (MCL) for a number of contaminants, including DBPs. Disinfection by-products, some of which are suspected human carcinogens, arise when natural organic matter in the source water reacts with disinfectants used during the water treatment process. The Stage 1 Disinfection Byproduct Rule was enacted in 1998 to combat high levels of DBPs in finished waters. This rule requires that DBP's, especially potentially carcinogenic trihalomethanes (THMs), fall below a set MCL level. Most water treatment facilities have chosen to change their disinfection process from chlorination to chloramination, in order to reduce production of chlorine DBPs such as trihalomethanes. Chloramination is similar to chlorination, but includes the subsequent addition of ammonia, which reacts with chlorine to form chloramines. Several byproducts are still formed during

the chloramination process (Chang and McAuley 1998; Lewis et al. 1998); however, they have not been identified in the Disinfection Byproduct Rules and their health effects remain unknown.

N-Nitrosodimethylamine (NDMA) is a DBP that emerges when dimethylamine (an organic compound) reacts with chlorine in the presence of ammonia ions (Andrzejewski et al. 2005). In 1954, NDMA caused fatal liver necrosis in rats, rabbits, mice, guinea-pigs, and dogs when given in doses of 20 to 40 mg/kg (Barnes and Magee 1954). The US EPA has set a non-regulatory maximum admissible concentration of NDMA in drinking water at 7 ng/L; however, it has yet to set an official MCL.

In 2005, the Blacksburg-Christiansburg-VPI Water Authority changed its disinfection process from chlorination to chloramination. Approximately 6 months after this water treatment change, our lab began to observe neural tube defects (NTDs) in untreated control mice. The NTDs were not observed in any control animals ($n > 1700$) prior to this time period, but were detected in approximately 10% of all offspring after this time. We eliminated bedding, food, and strain and source of mice as possible causes and identified tap water as the source of NTDs (Mallela et al. 2010).

Neural tube defects are birth defects that arise early in fetal development. During the embryonic period, the neural folds rise, fold toward midline, and fuse together, forming the brain and spinal cord. Failure of the neural tube to form properly will result as an NTD at that location. Complications from NTDs can range from life-long disability to death. In some, the effects are mild and often inapparent. According to the Centers for Disease Control and Prevention, about 3,000 babies born annually are affected with observable NTDs (Pirmohamed et al. 1992).

We demonstrated that NTDs could be eliminated by providing mice with distilled deionized (DDI) water for 2 generations (F_2 -DDI) (Mallela et al. 2010). The NTDs could be induced again when F_2 -DDI mice were provided tap water from many different geographical locations prior to breeding. A dose effect was demonstrated in mice exposed for longer periods of time (Mallela et al. 2010). The

anomalous NTDs arose 6 months after chloramination replaced chlorination in the water treatment process, and coincided with the appearance of nitrates in the water, which is an indicator of chloramination DBPs (Regan et al. 2003).

This study was conducted to further characterize the cause of the anomalous NTDs and determine if changes in the DBPs were responsible for the NTDs in mice.

MATERIALS AND METHODS

Animals

All animals (CD-1 mice and Sprague Dawley and Wistar rats) were bred in-house for 2 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-hour light/dark cycle, 20 – 25 °C temperature, 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. F₂ females were randomly assigned to a water treatment or control group. Each group was provided test water for 8 weeks (mice) or 12 weeks (rats) prior to breeding and throughout gestation. Females were caged overnight with a male of the same strain. The presence of a vaginal plug the next morning confirmed mating and was designated as gestational day (GD) 0. On GD 9 or 10 (mice) or GD 11 (rats), pregnant females were euthanized by CO₂ inhalation and the uterus opened. Embryos were dissected from the uterus and excised from the yolk sac. Any resorptions were noted and live embryos were staged by GD, somite count, and extent of heart and limb bud development. Embryos were assessed for neural tube defects (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.

Test Water

Test waters (Table 1) were collected and maintained in amber glass bottles (I-Chem 200 series, Rockwood, TN) and stored for no more than 4 weeks at 2 °C.

Location	Water Treatment Process	Source	Key
Blacksburg, VA	Untreated River Water	River 1	UT
Blacksburg, VA	Chlorinated Pre-Distribution	River 1	CPD
Blacksburg, VA	Chloraminated Pre-Distribution	River 1	CAPD
Blacksburg, VA	Municipally Chloraminated	River 1	MCA
Blacksburg, VA	Millipore Direct Q System (Billerica, MA)	River 1	MQ
Blacksburg, VA	Barnstead (Dubuque, IA) Nanopure Ultrapure Water System	River 1	BU
Blacksburg, VA	Vaponics VSS-30TI still	River 1	DDI
Riner, VA	Municipally Chlorinated	Well	MCW
Dublin, VA	Municipally Chlorinated	River 2	MC1
Silver Spring, MD	Municipally Chlorinated	River 3	MC2

Table 1. Waters used for animal exposures and chemical analysis. Waters were selected based on location and treatment regime, in order to determine associated differences. All waters were collected in clean amber glass bottles (I-Chem 200 series, Rockwood, TN) and stored for no more than 4 weeks at 2 °C.

DBP mouse exposure

Water with different sources and treatment processes were compared. Two separate river systems (river 1 and river 2) and a well water source were tested. Water was collected from the disinfection treatment plant at different locations during the treatment and distribution process in order to obtain water samples with a variety of DBPs. Timing of water sampling ensured the same initial water column was followed throughout the treatment process. UT was collected at the plant intake. Water CPD was collected 6 hours later after completion of the chlorination process. Ammonia was then added in the treatment process and water MCA was collected from the distribution system 12 hours later after the ammonia addition at a location with a known 12 hour transit time. Waters UT and CPD were autoclaved to ensure the water was pathogen free prior to treating the mice. A sample of water MCA was also autoclaved prior to giving to the mice to control for any changes to the contaminant from the autoclaving process. After each was municipally chlorinated, waters MCW and MC1 were collected.

GC-MS Analysis of Waters

Waters UT, CAPD, MCA, DDI, and DDI spiked with EPA 521 Nitrosamine mix standard were processed using an Eclipse 4660 Purge-and-Trap Sample Concentrator (College Station, TX) and analyzed by Shimadzu (Columbia, MD) GC-2010 /MS-QP2010S for volatile organic compounds (VOCs). A 30 m Rxi-5MS (Restek, Bellefonte, PA) fused capillary silica column (0.25 mm internal diameter, 1.4 μ m film thickness) was used for GC separation. Fifty ppb of the internal standard 4-Bromofluorobenzene was injected. After injection of the internal standard, each sample was injected into the purge-trap port, where it was purged with helium (8.5 psi) at ambient temperature (24 °C). The purging allowed volatile materials to move to the headspace and travel along a pressure gradient. The total time of the purge and trap cycle was 11 minutes. Adsorbed volatiles were heated for 4 minutes at 210 °C before samples

were introduced into the GC-MS for separation. Mass spectra were then interpreted using a Shimadzu GC-MS-QP 2010 System Package (Columbia, MD).

NDMA Analysis

Samples were prepared following EPA Method 521 (Determination of Nitrosamines in Drinking Water by Solid Phase Extraction and Capillary Column Gas Chromatography with Large Volume Injection and Chemical Ionization Tandem Mass Spectroscopy (MS/MS) http://www.epa.gov/microbes/m_521.pdf). Once prepared, samples were protected from light and kept at -15°C until analysis with a Varian Saturn 6890 equipped with an auto injector and a HP-5MS 5% phenyl methyl siloxane column, with methane/helium for ionization/carrier gases. MS ion source was electron impact. The initial temperature of the oven was set at 40°C , and the maximum temperature was 350°C . The total sample run time was 17.67 minutes. The GC constant flow was set at 1.5 mL/min and the injector temperature at 200°C and pressure at 11.90 psi.

Further analysis of the NDMA SPE samples was conducted using a Thermo Focus GC with a DSQ II MS and a DB-5 (nonpolar, low-bleed) column, then using a DB-1701 column.

Statistics

For the bioassay experiments, the unit of analysis was the dam. NTD rates in offspring were calculated as an average per dam and then averaged for each treatment. Differences in NTD rates in control and treated dams were reported percent mean \pm standard error. Wilcoxon rank sum (Statistix, Tallahassee, FL) was used to assess the differences between GD 9 and GD 10 mouse mean NTD rates. Kruskal Wallis (Statistix, Tallahassee, FL) was used to assess group differences in mean NTD rates of mice and as well as rats in exposure studies. Differences were considered significant at $p \leq 0.05$.

RESULTS

NTD rates by gestational day

To determine whether sufficient time was provided for NT closure prior to assessing embryos, NTD rates were compared on GD 9 and GD 10. NTDs were noted in all groups of mice and were lower on GD 10. Rates of NTDs in the offspring of mice provided water MC1 were significantly higher on GD 9 than GD 10: $10.0 \pm 1.9\%$ and $4.0 \pm 1.5\%$, respectively ($p = 0.01$) (Figure 1). Rates of NTDs in offspring of mice provided water MC2, however, were not significantly different between GD 9 and 10: $25.0 \pm 3.4\%$ and $18.0 \pm 2.5\%$, respectively ($p = 0.08$) (Figure 1). The number of litters containing at least one NTD decreased from GD 9 to GD 10 with water MC1 but not MC2. On GD 9 and 10, the percent of litters with NTDs was 76.9 and 35.7, respectively, for mice provided water MC1. For mice provided water MC2, the percent of litters with NTDs was 91.7 and 100 on GD 9 and 10, respectively. The most common NTDs observed for all water treatments were spina bifida and split face defects. These results indicate that NTDs were present on both days, though slightly less on GD 10.

GD 11 Rat Exposure Study

To assess whether a second species was affected by the water contaminant, 2 strains of rats were exposed to tap water and assessed for NTDs. Sprague-Dawley rats provided laboratory purified water MQ did not develop NTDs. GD 11 Sprague-Dawley rats provided water MC2, MCA, and MCW developed NTDs at rates of $2.2 \pm 1.2\%$, $0.7 \pm 0.7\%$, and $0.9 \pm 0.9\%$, respectively. Wistar rats provided water MC2 developed NTDs at a rate of $2.1 \pm 1.5\%$ (Figure 2). These results indicate that rats can also develop NTDs when exposed to an unknown contaminant in tap water. There was no statistical difference in NTD rates for Sprague-Dawley rats on any water type ($p = 0.34$). There was no statistical

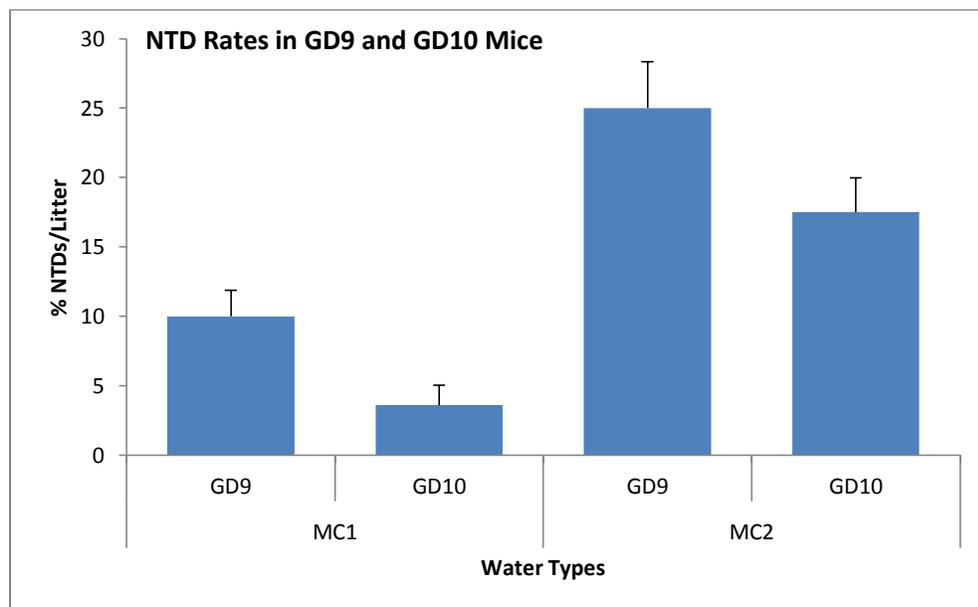


Figure 1. Differences in NTD rates in GD 9 and GD 10 mice exposed to waters MC1 and MC2. Values represent the mean percentages \pm SE of embryos affected with NTDs per litter with 12-16 litters per treatment. Animals were staged by somite count and extent of limb bud development, to assess stage of development. For mice provided municipally chlorinated river 2 water, there was a significant difference between GD 9 and 10, but not for mice provided water municipally chlorinated river 3 water. Statistical significance was set at $p \leq 0.05$.

difference between the Wistar and Sprague-Dawley strains across all 3 waters ($p = 0.59$), thus neither strain was more genetically susceptible than the other. For Sprague-Dawley rats, the percent of litters with at least one NTD per litter was 37.5, 9.1, and 11.1 for waters MC2, MCA, and MCW, respectively. For Wistar rats provided water MC2, the percent of litters with NTDs was 25. The most common NTDs observed among all waters were spina bifida and split face defects.

Analysis of Water Samples for the DBP NDMA

We analyzed waters known to cause NTDs for the probable carcinogen, NDMA, to determine if it was responsible for NTDs in rodents. We were unable to detect NDMA using the Varian Saturn 6890 and a HP-5MS 5% phenyl methyl siloxane column. Further analysis conducted on a more sensitive instrument (Thermo Focus GC with a DSQ II MS) with a DB-5 (nonpolar, low-bleed column) also did not

identify the compound. Subsequent analysis using a DB-1701 column noted that the NDMA peak was very low and not identifiable by the GC-MS software library. These analyses indicated that the NDMA present in the water samples was too low to be detected by our instruments, and was not pursued any further as the cause of the defects.

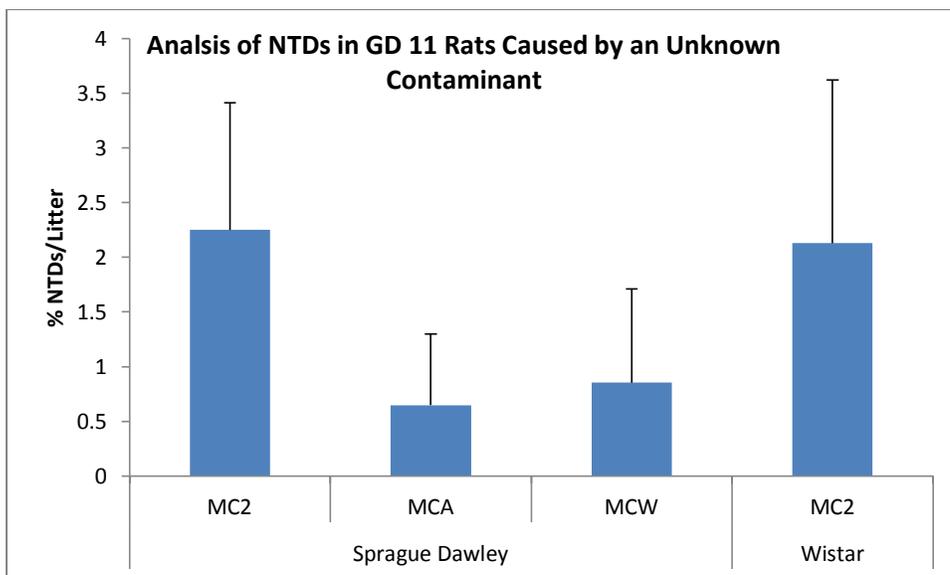


Figure 2. NTD rates in GD 11 rats provided municipally chlorinated river 3, municipally chloraminated river 1, and municipally chlorinated well for 12 weeks. Values represent the mean percentages \pm SE of embryos affected with NTDs per litter with 5-17 litters per treatment. Animals were staged by somite count and extent of limb bud development, in order to confirm gestational day. There were no significant differences in NTD rates among strains and water types. Statistical significance was set at $P \leq 0.05$.

Purge-Trap GC-MS analysis for VOC DBPs

The aim of this experiment was to determine if a VOC DBP was present in waters known to cause NTDs. Three compounds of interest were identified using the Shimadzu GC-MS-QP 2010 System Package (Columbia, MD). The peak areas and retention times are summarized in Table 2a and b. Compound A was identified in waters MCA and CAPD; but was not detected in water MQ or DDI. Conversely, Compound B was detected in waters MCA, CAPD, and MQ. Compound C was detected in waters CAPD and MCA. Compounds A, B, and C were also higher in CAPD than MCA which should

contain higher concentrations of chloramine DBPs. These findings suggest that the contaminant is not a VOC DBP.

Retention Time	CAPD	MCA	MQ	DDI
5.32 Compound A	144339	99138	0	0
5.93 Compound B	29427	27415	12035	0
6.46 Compound C	22110	12043	0	0

Table 2a. Peak area and retention times of 3 compounds of interest detected by PT-GC-MS in water samples subjected to different treatment schematics.

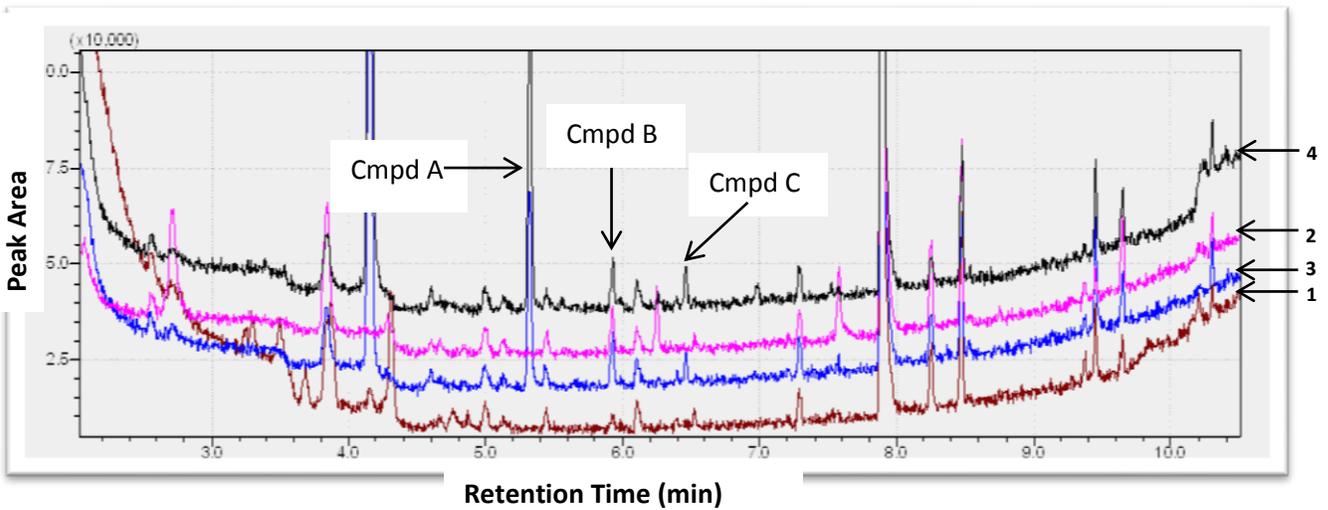


Table 2b. Merged chromatograms of four water samples subjected to purge-trap extraction showing retention times for unknown compounds A, B, and C. Compound identification 1) Water. MQ 2) Water. MCA. 3) Water DDI. and 4) Water CAPD.

Association of NTDs with Water Treatment Regime

Mice were exposed to waters with different treatment regimes in order to assess if there was an association between defect rate and treatment regime. All river water sources produced embryos with NTDs, whereas dams who received water DDI did not have embryos with NTDs. This difference was statistically significant. Water treatment with chlorine or chloramine did not affect the rates of NTDs. Neural tube defect rates were present at a rate of 12 % in embryos of dams receiving water UT.

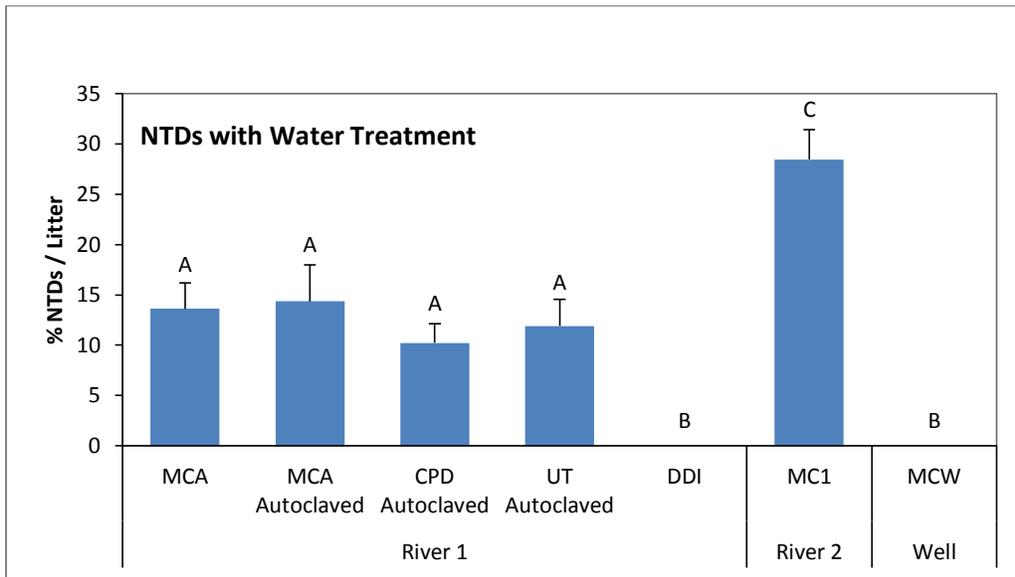


Figure 3. Rates of NTDs in offspring of mice provided water from 3 sources was collected: River 1, River 2, and a municipal well. River 1 water was sampled at different times in the treatment process, in order to obtain a variety of DBPs. NTD rates were calculated per litter with an N of 10 to 12 dams per water treatment. Bars with different letters indicate statistical difference in NTD rates ($p \leq 0.05$).

Embryos of dams receiving waters CPD and MCA had NTD rates of 10% and 14% respectively, and the treatments did not statistically alter the rate of malformations. Autoclaving the water did not alter the malformation rate with 14% NTDs in the offspring of dams provided either water MCA or autoclaved water MCA. Dams that received water MC1 had embryos with significantly higher rates of NTDs than those receiving waters MCA, CPD, UT, and DDI (Figure 3). The similar NTD rates in offspring of mice provided waters MCA, CPD, UT, and DDI regardless of treatment regime indicated that the contaminant

was not likely a DBP. Additionally, water MCW did not produce embryos with NTDs in support of this conclusion.

DISCUSSION

In humans, it is difficult to identify an exact cause for a NTD. Combinations of genetic and environmental factors cause NTDs. Risk factors for NTDs include low socioeconomic status, presumably as a result of inadequate nutrition during the periconceptual period (Wasserman et al. 1998). Geographically, the highest incidence of NTDs is in Southern Appalachia (Greenberg et al. 1983). Maternal obesity and diabetes have been associated with increased risk of NTDs (Hendricks et al. 2001; Rasmussen et al. 2008). Environmental exposures to pesticides, cigarette smoke, mercury, paints, and beauty salon/hair products have also been suggested as risk factors for NTDs (Matte et al. 1993; Rull et al. 2006; Suarez et al. 2008; Thulstrup and Bonde 2006). Abnormalities in the folate metabolism pathway have been suggested for mothers of children with NTDs (Stegers-Theunissen et al. 1994) and folic acid supplementation prevents the occurrences of many NTDs (Smithells et al. 1980). This finding prompted mandatory folic acid fortification of grains in the US and other countries.

Neurulation begins in mice on GD 8.5 and is completed early on GD 9. Thus, by GD 10, the neural tube should be closed. Failure of the neural tube to close properly, results in an NTD at that location. Observation of mice prematurely could lead to false positives and inaccurately inflated NTD rates. It is important to evaluate the embryos on both GDs 9 and 10 to preclude this issue. NTD malformations, although lower on GD 10, were still present, indicating that the NTDs are in fact pathologic and not an artifact caused by premature examination of embryos. Lower NTD rates on GD 10 indicate that in addition to causing the defect, the contaminant also delays neural tube closure. Variability in NTDs between water types suggests a dose response relationship between concentration of contaminant and rates of NTDs. Overall, these data indicate that the NTDs are not simply a result of examining the embryo too early.

Both Wistar (W) and Sprague Dawley (SD) rats were exposed to waters known to cause NTDs in mice in order to determine whether rats could get NTDs from exposure to tap water. Both strains of rats developed NTDs, indicating that the contaminant has the ability to affect a second species. As there was no significant difference between NTD rates in the strains, both strains were equivalently susceptible to the contaminant. Both rats and mice were affected by the contaminant. This increases the likelihood that the contaminant may pose a threat to humans.

The early 20th century marked the first time chlorination was used as a disinfection method (Cutler and Miller 2005); however, it wasn't until 1974 that the first DBPs, chloroform and other trihalomethanes, were identified (Rook 1976). Disinfection by-products are formed when chlorine used in the disinfection process reacts with organic compounds present in the water. Chloramination of drinking water frequently stimulates the growth of nitrifying bacteria in distribution systems (Regan et al. 2003). We first observed NTDs after chloramination replaced chlorination in the water treatment process, and the NTDs coincided with the appearance of nitrates in the water indicating the presence of chloramination DBPs. This finding that led us to believe the contaminant was a DBP.

Epidemiological studies have demonstrated a possible link between DBPs and congenital abnormalities. Women living in areas with high levels of chlorination DBPs exhibited an increased risk of birth defect [odds ratio (OR) = 1.22; 95% confidence interval (CI), 1.01-1.48], compared with women living in areas with low levels of chlorination DBPs (Chisholm et al. 2008). One population-based cross sectional study found an elevated fetal risk of developing ventricular septal defects, cleft palate, and cranial NTDs when exposed to DBPs in utero (Hwang et al. 2008).

Rodent studies have also indicated the teratogenic effects of DBPs. Studies in whole embryo culture of CD-1 mouse embryos exposed from 50 to 2500 μ M concentrations of haloacetic acid DBPs found NTDs and dysmorphogenesis (Hunter et al. 2006). This dose is significantly higher than standard

concentrations in tap water, which are typically 0.15-0.40 μM (Malliarou et al. 2005). A 24 hour exposure of CD-1 mice in whole embryo culture to haloacetic acids affected neural tube closure and produced dysmorphogenesis (Hunter et al. 1996). Oral administration of the DBP trichloroacetic acid produced developmental abnormalities in soft tissue and the skeleton (Smith et al. 1989). At concentrations higher than found in tap water, DBPs have been shown to accumulate in fetal brain tissues and cause degeneration of neurons and cause NTDs (Ahmed et al. 2005). This work indicates that a change in concentration or type of DBP may have initiated the NTDs observed in our control mice.

N-nitrosodimethylamine (NDMA) forms when dimethylamine (an organic compound) reacts with chlorine in the presence of ammonia ions (Andrzejewski et al. 2005); NDMA is an emerging DBP resulting from chlorine or chloramine disinfection during water treatment. *N*-nitrosodimethylamine causes fatal liver necrosis in rats, rabbits, mice, guinea-pigs, and dogs when given in doses of 20 to 40 mg/kg (Barnes and Magee 1954). The US EPA has set a non-regulatory maximum admissible concentration of NDMA in drinking water at 7 ng/L; however, has yet to set an official MCL. *N*-nitrosodimethylamine is typically found in waters in ng/L concentrations, due this directive. Both of our instruments failed to detect NDMA in our water samples. These results suggest such low concentrations of NDMA are unlikely to be the cause of the NTDs.

Several DBPs such as trihalomethanes are VOCs. Purge-trap GC-MS is commonly used to measure VOC DBPs, and is frequently used to detect trihalomethanes in mixtures (Budziak et al. 2007; Golfinopoulos and Nikolaou 2001; McNeal et al. 1995; Xu et al. 2011). The water types CAPD, MCA, and DDI were waters known to cause NTDs. After December 2009, DDI water started causing NTDs in our mice. We did not detect compounds A, B, or C in DDI water, but detected all compounds in the water types CAPD and MCA. Since distribution of the DBPs identified by PT GC-MS did not fit the distribution pattern of NTDs, we don't believe these VOC DBPs are a cause of the defects.

There is a contaminant in surface water from a number of river systems and a wide geographical area that is causing NTDs in rodents. This contaminant is not likely a DBP formed during the water treatment process, as untreated river water did not differ in NTD rates compared to treated waters. This would mean that a teratogen is present in surface waters and is capable of surviving the water treatment process intact and is retained in the tap water. The observation that the teratogen is not a DBP is strengthened by the fact that NTD rates were not significantly different in mice provided with two different treatment methods: chlorinated vs. chloraminated. Chlorination and chloramination tend to produce different concentrations and different kinds of DBPs. If the teratogen was a DBP, the concentration would likely vary with the different treatment processes and result in differing rates of malformations. We did see a difference in NTD rates between water CPD and water MC1. The higher NTD rates induced by water MC1 could indicate differences resulting from the length of chlorine treatment, but it could also indicate a higher concentration of the contaminant present in the two river basin systems. These results, coupled with the fact that water MCW did not produce NTDs, indicate that the rates of NTDs vary more with the water source than with the treatment method. Even though the appearance of NTDs coincided with the presence of chloramination DBPs, we do not believe the water contaminant is a DBP.

In summary, NTDs were present on both GD 9 and 10 in mice, indicating that the defects were not simply a result of examining embryos too early. Rats also developed NTDs, demonstrating that the contaminant in tap water also affected a second species. Concentrations of the DBP NDMA were too low to detect in waters known to cause NTDs, thus it was eliminated as a possible cause of the NTDs. Our PT GC-MS data suggested that while VOC DBPs were present in all categories of water samples, a single teratogenic contaminant was not identifiable in waters known to cause NTDs. For this reason, VOC DBPs were excluded. Untreated river water caused similar NTD rates in mice than treated water. This indicates the contaminant in tap water is not likely a DBP as it is present in the untreated river

water. Taken together our data indicates that the contaminant present in tap water that causes NTDs in rodents is not likely a DBP.

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III. Presence of carbamazepine in drinking water does not appear to cause neural tube defects in mice

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ABSTRACT

In May of 2006, we suddenly began to observe neural tube defects (NTDs) in embryos of untreated control mice. Unintentional exposure to a teratogenic agent in tap water was identified as the cause. Pharmaceuticals and personal care products have emerged as ubiquitous contaminants of ground and surface waters and have recently been found in drinking water. In order to analyze for these compounds and narrow our search, we submitted water samples to a commercial water analysis lab (AXYS Analytical Services Sidney, BC, Canada). Several pharmaceuticals were identified in a number of samples, including carbamazepine (CBZ), a drug used to treat seizures and mood disorders and also a known teratogen. Further analysis of CBZ was conducted in-house. CBZ was found in several ground and surface water samples at concentrations ranging from 0.11 ng/L to 16 ng/L. To establish whether or not CBZ was responsible for NTDs in our mice, we conducted 2 dosing studies. CBZ was provided to mice at concentrations detected in tap water, and at approximately 2 x and 1000 x this dose. Both studies found no significant differences in NTD rates among dose groups. Since no dose effect was observed, we concluded that CBZ was not directly responsible for the malformations.

INTRODUCTION

Carbamazepine (CBZ) is an anticonvulsant drug typically prescribed to treat epilepsy, bipolar disorder, and trigeminal neuralgia. Carbamazepine (5H-dibenz[b,f]azepine-5-carboxamide) is neutral at environmentally relevant concentrations (Figure 1). Carbamazepine works by stabilizing the inactive form of presynaptic sodium channels, decreasing the firing of sodium dependent action potentials (Macdonald and Kelly 1995). Upon ingestion, CBZ is oxidized by hepatic microsomal enzymes to its active metabolite, CBZ 10,11-epoxide (Bertilsson 1978) (Figure 1). Carbamazepine induces the expression of these hepatic microsomal enzymes and thus is an autoinducer. Autoinduction results in a need to increase dosage over time to maintain a therapeutic blood level of drug. Approximately 2-3 % of CBZ remains unchanged and is excreted in urine.

The Food and Drug Administration (FDA) has classified CBZ as a pregnancy category D drug, which indicates that it may not be safe for use during pregnancy. Evidence of CBZ teratogenicity in humans is largely anecdotal, because it is unethical to validate the fetal toxicity in humans. Nonetheless, the association has been strengthened by a number of epidemiological studies. Since the mid-seventies, a number of case control and cohort studies have reported increased teratogenic risk associated with CBZ exposure during pregnancy (Ceci et al. 1996; Janz 1975; Jentink et al. 2010; K. L. Jones et al. 1989; Matalon et al. 2002; Wide et al. 2004). One case-control study found that CBZ exposure during the first or second months after the last menstrual period increased NTD risk by six-fold, whereas the absence of CBZ reduced risk (Hernandez-Diaz et al. 2001). When data from five prospective studies totaling 1,379 children was collected and reanalyzed by meta-analysis, a strong association between congenital abnormalities such as neural tube defects and CBZ was found (Samren et al. 1997).

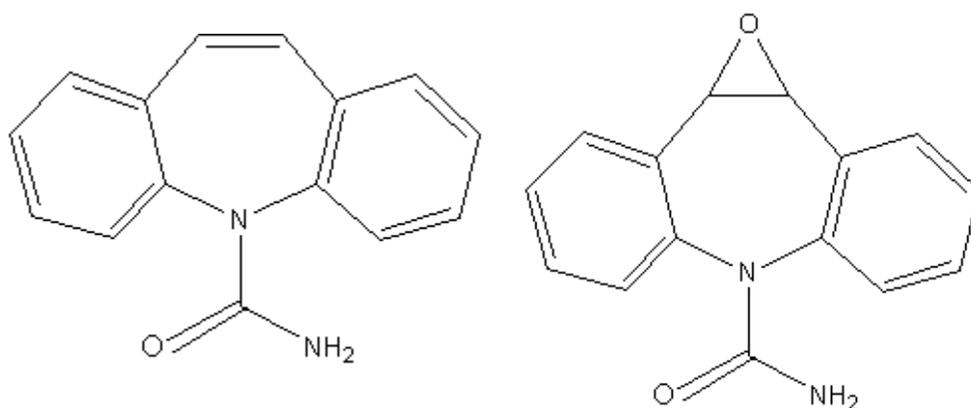


Figure 1. Structures of L: Carbamazepine and R: Carbamazepine-10,11-epoxide

There are few studies evaluating the teratogenicity of CBZ in rodents. Slight increases in the rate of cleft palate and growth retardation were seen in mouse fetuses exposed during the period of organogenesis (Sullivan and McElhatton 1977). Increased palatal defects were also observed when CBZ was administered to pregnant mice on days 8-13 (Paulson et al. 1979). Furthermore, intraperitoneal injection of clinically relevant doses of CBZ during organogenesis induced eye malformations in mice (Afshar et al. 2010). Carbamazepine dose levels of 225, 338, 563 mg/kg all produced embryotoxic and teratogenic effects, and decreased fetal weights (Eluma et al. 1984). The minimal rodent study, coupled with the epidemiological data indicating teratogenicity to humans, demonstrates a need for additional research to evaluate CBZ teratogenicity.

Carbamazepine has low solubility in water, and thus persists in the environment. It has been detected ubiquitously in aquatic environments, because of its low biodegradability (Ternes 1998). One study comparing a variety of pharmaceuticals in a salt- and organic-free (bi-distilled) water environment found that CBZ had at least a 10-fold greater calculated half-life than other pharmaceuticals (Andreozzi et al. 2003). The presence of CBZ in the environment is so extensive that it has been proposed as a possible anthropogenic marker (Clara et al. 2004). Carbamazepine has been detected in groundwater at concentrations up to 610 ng/L (Drewes et al. 2002), and up to 1075 ng/L in surface waters (Heberer et al.

2002). Carbamazepine has also been detected in tap waters around the world. A study conducted in 2008 found that tap water serving a population of 28 million had a median concentration of 4.1 ng/L of CBZ with the highest concentration being 51 ng/L (Benotti et al. 2009).

To date, no study has evaluated the risk posed to humans by CBZ at concentrations found in drinking water as a contaminant. Its common presence in drinking water supplies makes it an important emerging contaminant. This lack of scientific research investigating possible health effects of CBZ at the concentrations detected in tap water samples is troubling, considering the ubiquitous nature of this contaminant. To begin to address this issue, we analyzed the concentration of CBZ and other contaminants in drinking water, and evaluated the teratogenicity in mice of environmentally relevant CBZ exposures.

MATERIALS AND METHODS

Test Water

Water samples were collected and maintained in amber glass bottles (I-Chem 200 series, Rockwood, TN) and stored for no more than 4 weeks at 2°C. Water samples tested are listed in Table 1.

Animals

CD-1 mice were bred in-house for 2 generations (F₂) 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-hour light/dark cycle, 20-25 °C temperature, and 30-60% relative humidity. National Toxicology Program (NTP) diet and water were provided *ad libitum*. Females were randomly assigned to a water treatment or control group. Each group was provided test water for 8 weeks prior to breeding and throughout gestation. Females were caged overnight with an unrelated male. The presence of a vaginal plug the next morning confirmed mating and was designated as gestational day (GD) 0. On GD

10, pregnant females were euthanized by CO₂ inhalation, and the uterus was opened. Embryos were dissected from the uterus and excised from the yolk sac. Any resorptions were noted and live embryos were staged by GD, somite count, and extent of heart and limb bud development. Embryos were assessed for neural tube defects (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.

AXYS Analysis

Water was collected into supplied containers, held at 2°C and shipped within 24 hours to AXYS for analysis of pharmaceuticals and personal care products. See Table 1 for list of waters analyzed. See Appendix 1 for a list of analytes screened.

CBZ Analysis of Ground and Surface Waters

See Table 1 for the list of waters analyzed. All glassware was prewashed with test water prior to use. Sample water (500 or 1000 mL) was measured with a volumetric flask and was spiked with 1.00 mL of 5×10^{-9} M CBZ-d₁₀ (CBZ- d₁₀) as an internal standard (IS). Carbamazepine was extracted from water samples by Oasis® HLB cartridges (WAT106202, Waters, Milford, MA) preconditioned with LC-MS grade methanol. The water sample was loaded and passed through the cartridge at an approximate flow rate of 10 mL/minute using a PrepSep 12 port vacuum manifold (Fisher Scientific Pittsburg, PA) and an Air Cadet pressure station (Cole Parmer Instrument Chicago, IL). After the entire sample had been applied, the cartridge was dried, and the target compound was eluted from the column with 6 mL of LC-MS grade methanol. The eluent was then evaporated under a stream of nitrogen and the residue was reconstituted with 100 µL of LC-MS grade methanol-water (70:30) containing 0.1% formic acid. Samples

were stored at –30 °C until LC-MS analysis. Samples were analyzed by reverse-phase high-pressure liquid chromatography (RP-HPLC) using an Agilent 1100 Series (Agilent Technologies, Paulo

Water Source/Treatment
River 1 Untreated (Surface water) * ^o
River 1 Chloraminated Pre-Distributed (Surface water) * ^o
River 1 Chloraminated Tap (Surface water) * ^o
River 2 Chlorinated Tap (Surface water) * ^o
River 3 Chlorinated Tap (Surface water) * ^o
Municipally Chlorinated Well (Ground water) * ^o
Private Well 1 (Ground water) * ^o
Private Well 2 (Ground water) * ^o
Omnisolv (Gibbstown, NJ) Laboratory Purified HPLC Water* ^o
JT Baker (Deventer, The Netherlands) Laboratory Purified LC-MS Water* ^o
River 1 Elix Milli Q Academic (Milford, MA) Purification System* ^o
River 1 Millipore Direct Q (Milford, MA) Purification System* ^o
River 1 DDI Vaponics VSS-30TI still* ^o
River 1 DDI passed through charcoal filter Barnstead (GAC) ^o
River 1 DDI passed through Oasis HLB Solid-Phase Extraction Cartridge ^o
River 1 Barnstead (Dubuque, IA) Nanopure Purification System* ^o

Table 1. List of all waters analyzed. Waters were analyzed by LC-MS for pharmaceuticals and personal care products by AXYS Analytical Services (Sidney, BC, Canada) and in-house by LC-MS. Waters marked with an * were analyzed in-house, and waters analyzed by AXYS are marked with ^o.

Alto, CA) coupled to an ion-trap mass-spectrometer (Qtrap 3200, Applied Biosystems, Foster City, CA) equipped with a Luna (Phenomenex Inc, Torrance, CA) 2.5 μ C-18 HST (100 x 3.0 mm) analytical column. The instrument was equipped with an electrospray ionization (ESI) source operating in the positive ion mode. The mobile phase used was LC-MS grade methanol-water (70:30) containing 0.1% formic acid.

Carbamazepine ions were identified by selected reaction monitoring (SRM), with Q1 set at 237 m/z for the parent ion (MH⁺) and Q3 at 194 m/z (MH⁺-CONH₂).

Efficacy of CBZ Extraction

Efficacy of CBZ extraction by Waters Oasis[®]HLB was assessed by comparing the amount of CBZ in test water to the amount leached. Two volumes of test water (500 mL and 1000 mL) were tested separately. Water was passed through a cartridge (first pass) and the passed through water was then collected and passed through a second cartridge (second pass). CBZ was then eluted from both cartridges and compared. This determines the amount of CBZ not retained in the cartridge via first pass.

Standards were comprised of CBZ at 0.1, 0.2, 1.2, 2.4, 5.9, 12, 24, 59, and 120 µg/L and 12 µg/L of the deuterated CBZ internal standard (IS). In Figure 2, “Average STD CBZ- d₁₀” represents the average peak area of the constant 12 µg/L IS contained in each standard measured by LC-MS. “Average Sample CBZ- d₁₀” represents the amount of IS detected after the sample was subjected to concentration via the Oasis[®]HLB cartridge.

Mouse CBZ Dosing Study

CBZ Dosing Study in Mice with Municipally Chlorinated Well Base Water

The mice were assigned to one of the following four groups: 1) Municipally chloraminated Blacksburg tap water collected from the College of Veterinary Medicine at Virginia Tech; 2) Municipally chlorinated well water spiked with 15 ng/L CBZ in methanol; 3) Municipally chlorinated well water spiked with 1500 ng/L CBZ in methanol; 4) Municipally chlorinated well water spiked with methanol served as the vehicle control (0.00015% v/v). All water was collected and spiked at one time point to ensure precision. Mice were maintained on test waters for 8 weeks prior to breeding and throughout gestation. Mice were sacrificed on GD 10, and the embryos were examined for malformations.

CBZ Dosing Study in Mice with DDDI Base Water

DDI water (Vaponics VSS-30TI still) collected from the College of Veterinary Medicine glasswares lab was distilled a second time to remove CBZ (DDDI) before it was spiked with methanol or CBZ. The mice were assigned to one of the following four groups: 1) Chloraminated tap water; 2) DDDI spiked with 6 ng/L CBZ in methanol; 3) DDDI spiked with 1500 ng/L in methanol; 4) DDDI spiked with methanol, which served as the vehicle control (0.00015% v/v). Mice were maintained on test waters for 8 weeks prior to breeding and throughout gestation. Mice were sacrificed on GD 10 and the embryos examined for malformations.

Statistics

For the bioassay experiments, the unit of analysis was the dam as is required for rodent teratogenicity studies. NTD rates in embryos were calculated as an average per dam and then averaged for each treatment. Differences between control and treated dams were reported as the mean \pm standard error. A Kruskal Wallis test was used to assess the differences between CBZ dosed treatment groups. Differences were considered significant at $p \leq 0.05$.

RESULTS

AXYS Analysis

Samples	Compound	Conc. Found (ng/L)
River 1 Untreated (Surface water)	Caffeine	33.7
	Carbamazepine	1.80
	Clarithromycin	1.97
	Diphenhydramine	0.913
	Erythromycin	0.423
River 1 Chloraminated Pre-Distributed (Surface water)	ND	
River 1 Chloraminated Tap (Surface water)	Caffeine	19.2
Municipally Chlorinated Well (Ground water)	ND	
River 2 Chlorinated Tap (Surface water)	ND	
River 1 DDI Vaponics VSS-30TI still	ND	
River 1 DDI passed through Oasis HLB Solid-Phase Extraction Cartridge	Caffeine	21.2
River 1 DDI passed through charcoal filter Barnstead (GAC)	ND	
River 1 Barnstead (Dubuque, IA) Nanopure Purification System	ND	

Table 2. Pharmaceuticals and personal care products in water samples detected by AXYS (Sidney, BC, Canada) analysis. Water was collected into supplied containers, held at 2 °C and shipped within 24 hours to AXYS. Concentration are listed as ng/L. ND=none detected.

Nine test water samples were analyzed using LC-MS/MS for a panel of pharmaceuticals and personal care compounds by AXYS (Sidney, BC, Canada) to determine the extent of contamination. Analysis by AXYS found contaminants in the following 3 samples: 1) River 1 untreated; 2) Municipally chloraminated tap water; and 3) DDI passed through Oasis HLB solid-phase extraction cartridge (Table 2). Of the detected compounds, the following are known teratogens: Carbamazepine, Clarithromycin, Caffeine, and Erythromycin.

In-house CBZ Analysis

Purified and Municipal Waters Analyzed In-House for CBZ by LC-MS	Collection Date	[CBZ] ng/L
Omnisolv (Gibbstown, NJ) Laboratory Purified HPLC Water	Feb 2011	ND
JT Baker (Deventer, The Netherlands) Laboratory Purified LC-MS Water	Aug 2009	1.7 ± 0.69
River 1 Vaponics VSS-30TI still DDI	Aug 2009	ND
	May 2010	1.4 ± 0.022
	Dec 2010	ND
River 1 Barnstead Nanopure System (Dubuque, IA)	Dec 2010	ND
River 1 Elix Milli Q Academic (Milford, MA)	Oct 2010	ND
River 1 Millipore Direct Q System (Billerica, MA)	Oct 2010	ND
River 1 Untreated (Surface water)	Aug 2009	2.5 ± 0.27
	Dec 2010	0.28 ± 0.15
River 1 Chloraminated Pre-Distributed (Surface water)	Dec 2010	0.11 ± 0.0008
River 1 Chloraminated Tap (Surface water)	Aug 2009	1.4 ± 0.15
	Dec 2010	0.11 ± 0.0060
	May 2011	5.4 ± 0.98
	June 2011	7.0 ± 0.18
River 2 Chlorinated Tap (Surface Water)	Aug 2009	ND
	May 2010	1.6 ± 0.18
River 3 Chlorinated Tap (Surface water)	Aug 2009	0.90 ± 0.01
Private Well 1 (Ground water)	May 2011	16 ± 1.3
Private Well 2 (Ground water)	May 2011	9.5 ± 0.90
Municipally Chlorinated Well 3 (Ground water)	Aug 2009	ND
	Jan 2011	ND
	June 2011	ND

Table 3. Carbamazepine concentration in purified and municipal waters concentrated by Oasis HLB cartridge and analyzed using LC-MS. All samples were spiked with 5 nM of CBZ d₋₁₀ as an internal standard and concentrated 5000-fold.

Carbamazepine was detected in LC-MS grade commercial water; DDI, River 1 untreated; River 1 chloraminated tap; River 2 chlorinated tap; River 3 chlorinated tap; and two private wells. The lowest concentration of CBZ was detected in Laboratory Purified HPLC water, whereas the highest

concentration was found in private well 1. Out of all municipally provided waters, municipally chlorinated well water was the only one found to be negative for CBZ. Interestingly, the 2 private wells sampled had the highest concentrations of CBZ. Carbamazepine was not detected in laboratory purified waters from the Barnstead Nanopure System (Dubuque, IA), Millipore Direct Q System (Billerica, MA), and Elix Milli Q Academic (Milford, MA) System; however, CBZ was detected in Vaponics VSS-30TI distilled deionized water. The concentration of CBZ detected in river 1 untreated was 0.28 ng/L, and decreased after treatment to 0.11 ng/L in the river 1 chloraminated pre-distribution sample. Furthermore, similar concentrations of CBZ were detected in river 1 chloraminated pre-distribution and tap (post-distribution) waters when measured at the same time point.

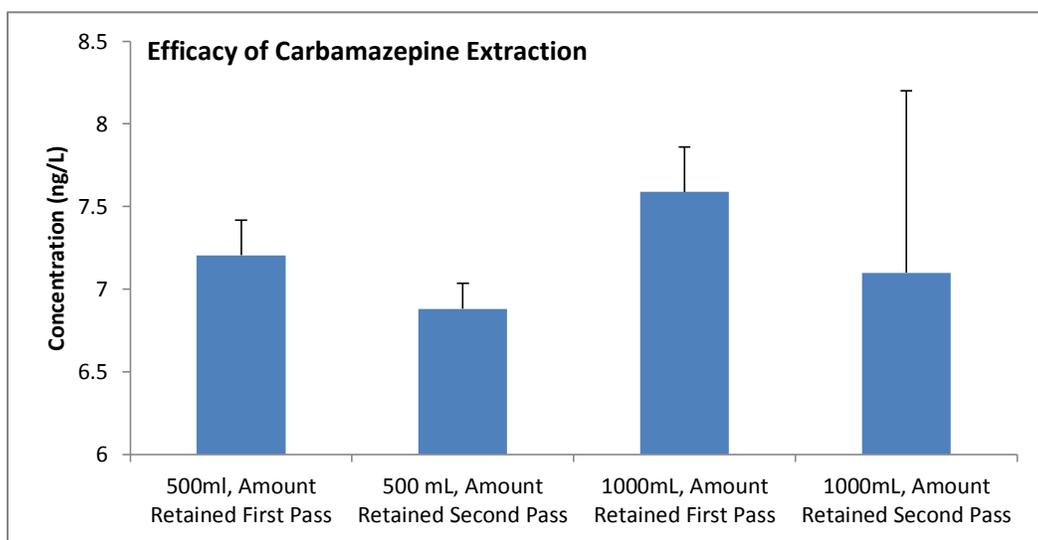


Figure 2a. Amount of CBZ retained from Blacksburg tap water (June 2011) in first cartridge and second cartridge for 500 and 1000 mL volume samples. The concentration in the second pass represents CBZ that passed through the cartridge on the initial first pass.

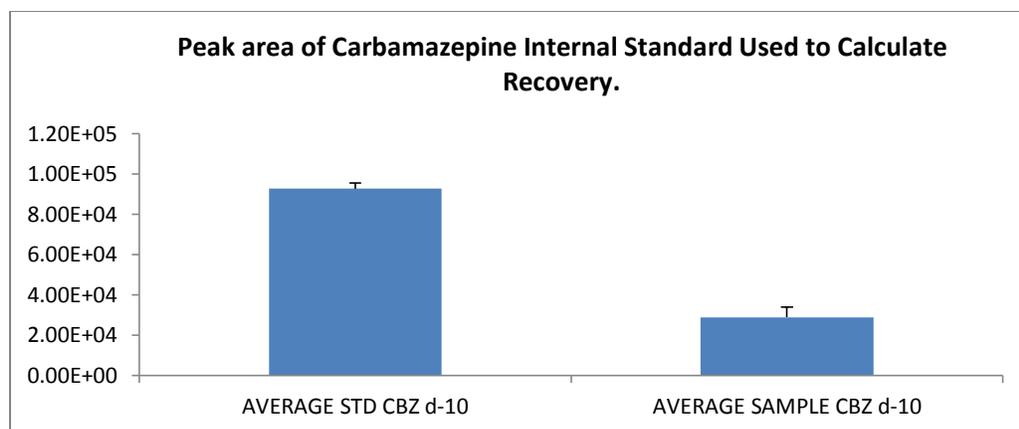


Figure 2b. “Average STD CBZ- d₁₀” represents the average peak area of the constant 50 nM IS contained in each standard measured by LC-MS. “Average Sample CBZ- d₁₀” represents the amount of IS detected after the sample was subjected to concentration via the Oasis®HLB cartridge. CBZ- d₁₀ recovered from Oasis®HLB cartridge was estimated at 31.2%.

In order to assess the efficacy of CBZ extraction, we measured the amount of CBZ not retained by the cartridge on the first pass and compared internal standard peak areas between standards and samples. Similar concentrations of CBZ were retained on the first pass for both 500 mL and 1000 mL samples. Additionally, the concentration of CBZ retained on the second pass was only slightly less than that retained on the first pass, for both 500 mL and 1000 mL sample (Figure 2). Average CBZ- d₁₀ recovered from Oasis®HLB cartridges was estimated at 31.2%, indicating a relatively low recovery.

CBZ Dosing Study in Mice with Municipally Chlorinated Well Base Water

The dosing study was conducted to determine whether CBZ could cause NTDs at concentrations of 5 x and 500 x of that found in tap water. Neural tube defects were noted in all groups of mice. The NTD rate for embryos of dams exposed to river 1 chloraminated tap water was $3.8 \pm 1.2\%$. Embryos exposed to vehicle had NTD rates of 4.7 ± 1.5 . The NTD rate for the 15 ng/L CBZ exposed mice was $8.6 \pm 1.9\%$ and 5.4 ± 2.2 for the 1500 ng/L exposed CBZ mice (Figure 3). NTD rates between groups were not significantly different ($p = 0.18$ Kruskal wallis). Neural tube defects were not expected in the vehicle control water. To account for possible CBZ contamination of the municipally chlorinated well base water at the time of collection, the experiment was repeated with different base water.

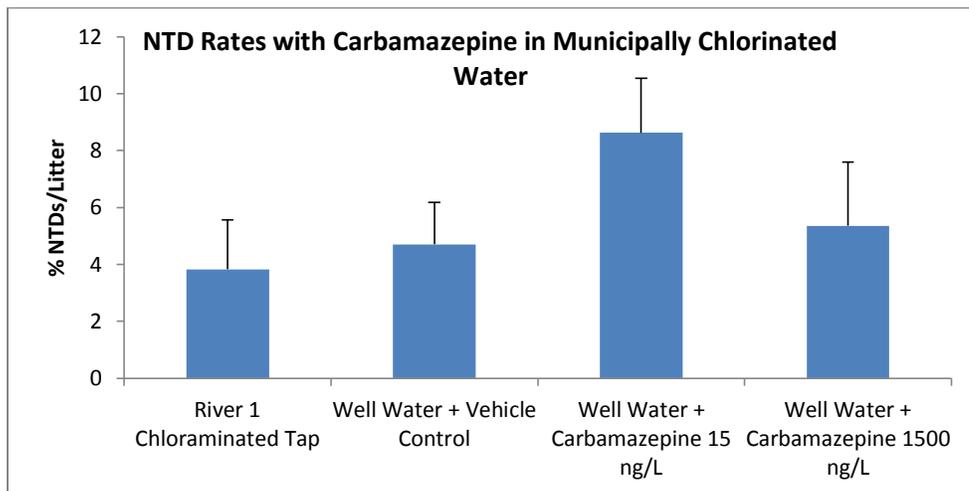


Figure 3. Differences in NTD rates of mice exposed to carbamazepine in municipally chlorinated well base water. Groups tested were: Blacksburg, VA tap water, vehicle (methanol), 15 ng/L CBZ and 1500 ng/L CBZ. Mice were maintained on test waters for 8 weeks and embryos examined on gestational day 10. NTD rates, represented as mean \pm SE, were calculated per litter with an N of 8 to 13 dams per water treatment. NTD rates between groups were not statistically different ($p = 0.18$ Kruskal wallis).

CBZ Dosing Study in Mice with DDDI Base Water

The dosing study was repeated again using DDDI water as a base to test whether or not CBZ caused NTDs. Carbamazepine analysis of the distillate revealed that it was negative for CBZ. As in the first dosing study, NTDs were noted in all groups of mice at similar rates to the previous experiment. The NTD rate for embryos of dams exposed to river 1 chloraminated tap water was $5.3 \pm 1.9\%$. Embryos exposed to vehicle had NTD rates of 7.6 ± 2.1 . The NTD rate for the 6 ng/L CBZ mice was $3.6 \pm 1.3\%$ and 7.4 ± 1.4 for the 1500 ng/L CBZ mice (Figure 4). NTD rates between groups were not significantly different ($p = 0.21$ Kruskal Wallis). No dose response was observed, with the increased concentration of CBZ. Additionally, DDDI known to be negative for CBZ caused malformations, reducing the likelihood that CBZ was responsible for the malformations.

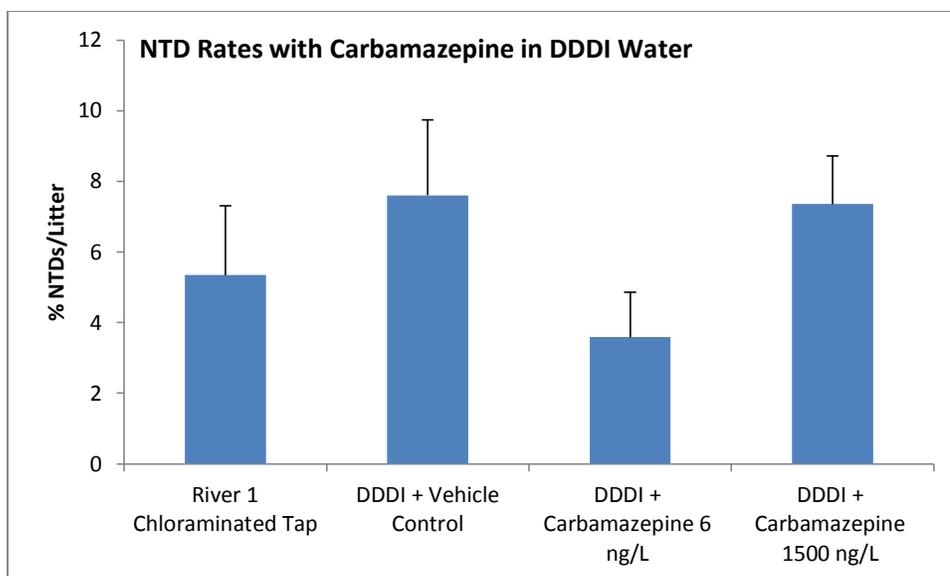


Figure 4. Differences in NTD rates of mice exposed to carbamazepine in DDDI base water. Groups tested were: River 1 chloraminated tap, vehicle (methanol), double distilled deionized water (DDDI) spiked with 6 ng/L CBZ, and 1500 ng/L CBZ. Mice were maintained on test waters for 8 weeks and embryos examined on gestational day 10. NTD rates, represented as mean±SE, were calculated per litter with an N of 11 to 15 dams per water treatment. NTD rates between groups were not statistically different ($p=0.21$ Kruskal wallis).

DISCUSSION

In May 2005 our lab began to see NTDs in untreated control mice (Mallela et al. 2010). Prior to that time, NTDs were not observed in control animals, but were detected in approximately 10% of all embryos after this time. We eliminated bedding, food, and strain and source of mice as possible causes and identified exposure to tap water as the source of NTDs (Mallela et al. 2010).

Drinking water contamination has become a global issue. Some common water contaminants include DBPs, phthalates, synthetic estrogens and other pharmaceuticals, atrazine, DDT, benzene, toluene, xylene, tributyl tin, arsenic, fluoride, selenium, and heavy metals (Schwarzenbach et al. 2006). Many studies have indicated widespread contamination of river and surface waters from pharmaceuticals (Hummel et al. 2006; Metcalfe et al. 2003; Zuccato et al. 2000; Zuccato et al. 2005). The pharmaceutical CBZ has been found ubiquitously in drinking water (Segura et al. 2011) and is correlated with teratogenicity in rodents and humans. The Food and Drug Administration approved CBZ

for the treatment of bipolar disorder in 2005, which may account for its increased concentrations in the environment. Environmental concentrations of CBZ are typically in the ng/L range; however, have been detected up to 0.258 µg/L in finished waters (Stackelberg et al. 2004). These concentrations are in contrast to the much higher therapeutic dose of 400–1200 mg/day. At therapeutic levels, maternal CBZ exposure has the potential to increase the risk of birth defects two-fold (Diav-Citrin et al. 2001), whereas embryotoxic effects have been observed in rat cultures of whole embryos at doses as low as 60 µg/mL (Hansen et al. 1996). The health effects of environmentally relevant concentrations of CBZ are not known.

Several studies have indicated the inability of traditional water treatment processes to remove CBZ from wastewater and raw water (Stackelberg et al. 2004; Ternes et al. 2002; Vieno et al. 2006). As a result, CBZ is frequently detected in finished drinking water supplies (Kleywegt et al. 2011). A United States Geological Survey study demonstrated that many pharmaceuticals, including CBZ, are capable of surviving the water treatment process (Stackelberg et al. 2004). Degradation of CBZ has been achieved using UV irradiation (Kosjek et al. 2009) and ozonation (Andreozzi et al. 2002). Most treatment facilities, however, still use chlorination or chloramination to preclude higher equipment and operational costs associated with ozonation (Canizares et al. 2009). Conventional drinking water treatments (coagulation, flocculation, filtration, adsorption, and chlorination or chloramination) fail to remove CBZ from sources, with a removal efficiency of less than 10% (Zhang et al. 2008). For this reason, our lab pursued CBZ as a suspect contaminant in tap water responsible for causing NTDs.

Commercially available LC-MS grade water was positive for CBZ. Laboratory purification systems also failed to remove CBZ from source waters. Carbamazepine is generally rejected from nanofiltration membranes as result of size exclusion interactions with the membrane (Nghiem et al. 2005). Carbamazepine may not be rejected by certain nanofiltration membranes of commercial water

purification systems and thus CBZ may permeate into the filtered water (Schafer et al. 2006). We found this to be the case, since CBZ was present in some purified waters.

The analysis of pharmaceuticals and personal care products conducted by AXYS Analytical Services detected CBZ and other pharmaceuticals. The concentration of CBZ differed compared to our in-house analysis. AXYS identified CBZ in raw river water at a concentration of 1.8 ng/L, whereas we detected it from the same site at 2.5 ng/L. Carbamazepine enters this source in a variety of ways. After CBZ is taken therapeutically, it is excreted in feces and urine and enters sewage treatment plants contaminating the effluent (Kummerer 2009). AXYS detected CBZ in only 20% of raw river or municipal waters analyzed, whereas we detected the compound in 80% of the test waters. The AXYS method detection limit for CBZ was 1.8 ng/L, whereas we were able to detect CBZ at 0.11 ng/L. It is likely that the assay methodology utilized by AXYS was different from our own assay used to identify CBZ. For this reason, we may have detected CBZ in water samples where AXYS was unable. Analysis by AXYS detected no CBZ in municipal tap water even when it was present in the water coming into the treatment plant. This indicates that the water treatment process decreased CBZ concentration below that detectable by the AXYS methodology.

We were still able to detect CBZ in municipal drinking waters. Concentrations of CBZ changed over collection dates, suggesting that the concentration of CBZ is not stable throughout the year. The identification of CBZ in different geographical locations suggests a widespread contamination problem. Carbamazepine was identified in private wells but not in municipally treated well water. This indicates that the aquifer may be contaminated with CBZ, but that municipal water treatment methods may reduce the concentration below detectable limits. Fram and Belitz (2011) found 1.5% of 1231 groundwater samples contaminated by CBZ. Carbamazepine has even been used to assess the extent of sewage leakage into aquifers by concentration with solid phase extraction and analysis via silica gel

column chromatography (Nakada et al. 2008). One study indicated a low leaching possibility of CBZ, because it is non-mobile and remains in upper soil layers; however, this contradicts with the finding that CBZ is often identified in groundwater (Oppel et al. 2004). Overall, CBZ is frequently detected in groundwater samples and remains a contaminant of significance.

Since there was no significant difference in NTD rates between mice exposed to different concentrations of CBZ, including the vehicle control, it is possible that at the time of collection, the municipally chlorinated well and DDDI waters were positive for CBZ. Takao et al. (2008) recorded daily and seasonal fluctuations of CBZ in sewage water and found that concentrations were typically higher in the spring and winter. Residential sewage water samples analyzed over a span of 14 hours found significant variations, such as a recorded concentration of 91.4 ng/L at 10:00 am and 11.7 ng/L at 12:00 pm (Takao et al. 2008). These observations suggest that while water collected at one time point may have been negative for CBZ, water collected even a couple hours later may have been positive. It is therefore possible that CBZ was present on occasion in the municipally chlorinated well water, even though none was detected on the dates measured. Another possible source of contamination was the vehicle, as all water spiked with methanol caused NTDs. Inhaled methanol has been shown to cause NTDs in CD-1 mice (Rogers et al. 1993).

When testing the efficacy of Waters Oasis® HLB cartridge, we found that not all CBZ in water samples was retained by the cartridge on the first pass. Furthermore, we estimated the recovery to be only 31.2%. A considerable amount of CBZ passed through and was leached from the cartridge during the first pass. Additionally, equivalent amounts of CBZ (approximately 7 ng/L) were extracted from both 500 mL and 1000 mL samples, indicating that the cartridge may have reached saturation point, after which no more CBZ was retained. Several studies have used Oasis® HLB cartridges with good, but variable, recovery of spiked samples. One study achieved good recovery of CBZ in plasma using Oasis®

HLB; however, less than 1 mL total volume was applied to the cartridge (Mandrioli et al. 2001). A second study found that using MeOH as an elution solvent, as we did in our experiment, showed absolute recoveries much less than 100% (Ollers et al. 2001). Analysis of CBZ using Waters Oasis®HLB cartridges may not be the most quantitative method of measuring CBZ in water samples; however, our data demonstrated good precision. Samples were run in duplicate with relatively low standard error, indicating reproducibility of the results. Alternative techniques may be required to maximize accuracy, in order to determine the true concentration of CBZ in the samples.

The possibility remains that a mixture of compounds is responsible for the NTDs. Mixtures of pharmaceuticals or other contaminants may produce additive or synergistic effects leading to increased toxicity over the individual contaminants. Increased concentrations of plasma vitellogenin were observed when juvenile rainbow trout were provided with mixtures of environmentally relevant concentrations of various estrogenic compounds compared to the individual compounds (Thorpe et al. 2001). So although we did not observe a direct correlation of NTDs with CBZ exposure, it is possible the NTDs are caused by a mixture of teratogenic agents, and CBZ could still be a component of this mixture.

The highest concentration of CBZ detected by our in-house analysis was in a ground water sample, at a 16 ng/L. Rabiet et al. (2006) detected CBZ in ground waters at concentrations of 43.2 and 13.9 ng/L (Rabiet et al. 2006). Carbamazepine levels detected in our study are typically lower than those detected by other studies for surface and ground waters; however, we still detected CBZ in approximately 70% of samples. We were unable to correlate NTD rates with different concentration of CBZ. The DDDI base water known to be negative for CBZ still caused NTDs. Neural tube defect rates have typically shown a dose effect with exposure length (Mallela et al. 2010), but all NTD rates in this study were approximately the same indicating a similar exposure dose. It is possible that another

contaminant is present in these waters and is responsible for NTDs. Although CBZ is a persistent contaminant, it does not appear responsible for NTDs in our rodents.

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IV. CONCLUSIONS FOR THESIS

In May of 2006, we suddenly began to observe neural tube defects (NTDs) in embryos of untreated control mice. Unintentional exposure to a teratogenic agent in tap water was identified as the cause. We aimed to identify the contaminant and further characterize the malformations in both mice and rats. Neural tube defects were present on both GD 9 and 10 in mice, indicating that the defects were not simply a result of examining embryos too early. Rats also developed NTDs, demonstrating that the contaminant in tap water also affected a second species. Concentrations of the DBP NDMA were too low to detect in waters known to cause NTDs, thus were eliminated as a possible cause of the NTDs. Our PT GC-MS data suggested that while VOC DBPs were present in all categories of water samples, a single teratogenic contaminant was not identifiable in waters known to cause NTDs. For this reason, VOC DBPs were excluded. Untreated river water caused significantly higher NTD rates in mice than treated waters. This indicates the contaminant in tap water is not likely a DBP as it is present in the untreated river water. Taken together, our data indicates that the contaminant present in tap water that causes NTDs in rodents is not likely a DBP.

The highest concentration of CBZ detected by our in-house analysis was in a ground water sample at a 16 ng/L. Rabiet et al. (2006) detected CBZ in ground waters at concentrations of 43.2 and 13.9 ng/L (Rabiet et al. 2006). Carbamazepine levels detected in our study are typically lower than those detected by other studies for surface and ground waters; however, we still detected CBZ in approximately 70% of samples. We were unable to correlate NTD rates with different concentration of CBZ. The DDDI base water known to be negative for CBZ still caused NTDs. Neural tube defect rates have typically shown a dose effect with exposure length (Mallela et al. 2010), but all NTD rates in this study were approximately the same indicating a similar exposure dose. It is possible that another

contaminant is present in these waters and is responsible for NTDs. Although CBZ is a persistent contaminant, it does not appear responsible for NTDs in our rodents.

These studies indicate the need for better quantification of CBZ. We were unable to accurately measure the concentration of CBZ in test waters, thus the actual concentrations remain unknown. Further studies are necessary to determine what other contaminant(s) are responsible for the NTDs in our rodents. Additionally, future studies must address the concept that CBZ may be acting as a component of a mixture. In summary, the identity of the contaminant remains unknown, and additional research needs to be conducted in order to evaluate what it may be.

APPENDIX I

Barnstead Nanopure Ultrapure Water System River 1: BU

Carbamazepine: CBZ

Chloraminated Pre-Distribution River 1: CAPD

Chlorinated Pre-Distribution River 1: CPD

Disinfection By-Product: DBP

Environmental Protection Agency: EPA

Food and Drug Administration: FDA

Gestational Day: GD

Internal Standard: IS

Liquid Chromatography Mass Spectrometry: LC-MS

Maximum Contaminant Level: MCL

Millipore Direct Q System River 1: MQ

Municipally Chloraminated River 1: MCA

Municipally Chlorinated River 2: MC1

Municipally Chlorinated River 3: MC2

Municipally Chlorinated Well: MCW

Neural Tube Defect: NTD

None Detected: ND

Purge and Tap Gas Chromatography-Mass Spectrometry: PT GC-MS

Untreated River 1 Water: UT

Vaponics VSS-30TI Still River 1: DDI

