DYSREGULATED APOPTOSIS IN TERATOGEN-INDUCED NEURAL TUBE DEFECTS IN MICE

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ABSTRACT

Dysregulation of apoptosis during development is a possible mechanism for teratogen-induced birth defects. Neural tube defects (NTDs) are the second most common fetal malformations. Non-specific stimulation of maternal immune system prevents birth defects. This study investigated the role of dysregulated apoptosis in formation of NTDs from two teratogens: valproic acid (VA) and an unknown teratogen found in tap water. Interferon- γ (IFN γ) was used to stimulate maternal immunity to evaluate the role of altered apoptosis in this protective mechanism.

Apoptosis was evaluated using flow cytometry, Terminal Transferase dUTP Nick End Labeling (TUNEL) assay and gene expression changes by RT^2 Profiler PCR arrays. Additionally, changes in the expression of key signal transduction pathway genes that play a role in development were determined.

Increased apoptosis, suggesting involvement in VA teratogenicity, was observed along the neural tube in both normal and abnormal embryos from VA-exposed dams. Increased apoptosis in normal VA-exposed embryos suggests that VA may alter other cellular processes such as cell proliferation and differentiation in addition to apoptosis. Apoptotic percentages in embryos with NTDs from IFNγ+VA dams were similar to controls, which

indicated resistance to teratogen-induced apoptosis. In IFNγ+VA-exposed embryos with NTDs, immune stimulation failed to prevent apoptosis.

VA initiated both death and survival signaling in the embryos; however, upregulation of the apoptotic genes and down regulation of anti-apoptotic genes of p53 and Bcl2 family tended to shift the balance towards death signaling. This change in gene expression patterns could result in increased apoptosis and NTDs in VA-exposed embryos. Immune stimulation normalized changes in the expression of pro-apoptotic signaling molecules. These results suggest immune stimulation protects embryos from teratogenicity of VA by preventing VA-induced apoptosis.

VA altered the hedgehog, Wnt, retinoic acid and fibronectin signaling pathways in embryos with NTDs. These results suggest that VA also disrupted signaling pathways required for various morphogenic events during organogenesis. Immune stimulation normalized the expression of *Fn1* and *Hspb1* and thus may mediate protection through these signaling pathways.

In tap water exposed embryos, no change in apoptotic pattern was observed by flow cytometry, TUNEL assay and RT-PCR. Also, none of the signal transduction pathway genes tested were significantly altered in tap water-exposed embryos. This suggests that apoptosis is not a mechanism for teratogenicity resulting from exposure to the contaminant in tap water.

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

Neural tube defects (NTDs) are serious birth defects where the malformed brain or spinal cord is not covered by bone or skin. NTDs are the second most common malformation after congenital heart defects affecting 324,000 births every year (March of Dimes global report, 2006). In the United States, the incidence rate is 1 in every 1000 births (CDC). Failure of the neural tube to close during development, results in exencephaly or anencephaly, and spina bifida. Many, if not most, birth defects have a multifactorial etiology where embryonic development of a genetically susceptible fetus is altered by environmental factors. Several environmental, genetic and nutritional factors have been shown to cause NTDs in mice including drugs, physical agents (hyperthermia), vitamin excess or deficiency, and maternal infectious and metabolic diseases. The incidence of NTDs was greatly reduced following folic acid fortification of the diet in United States and other countries. Post folate fortification studies have found no difference in serum folic acid levels between pregnancies affected with NTDs and pregnancies without any birth defects that suggested folic acid levels may have reached threshold levels in preventing NTDs (Mosley et al., 2007). Even though folate intake has several health benefits, increased consumption may have cancer promoting effects (Osterhues et al., 2009). Also, using folate as the sole preventive measure of NTDs is debatable due to the multifactorial etiology of NTDs.

The central nervous system and spinal cord are formed by a process called neurulation that begins with the formation of the neural plate from the neuroepithelium. Changes in shape of neural plate cells cause intrinsic movements at the edges of neural plate to

elevate on either side of the midline and bend towards each other. The edges then fuse to form the neural tube. In mice, differential neural fold elevation causes neural tube closure at multiple sites (Harris and Juriloff, 1999). The primary neurulation process starts at the hindbrain and cervical boundary and then extends into the hind brain cranially and caudally into the spinal cord region. Two other fusion points form between mid - forebrain boundary and at rostral extremity of the forebrain. The spinal cord forms from secondary neurulation by a canalization process (Copp & Green, 2010, review). Failure of neural fold closure at these three sites leads to variety of malformations such as excencephaly, spina bifidia, or split face depending on location of the open neural tube (Harris and Juriloff, 1999). Failure to the close at hindbrain and cervical boundary leads to craniorachischisis. Failure to close between forebrain and midbrain leads to anencephaly, and incomplete closure at rostral extremity of the forebrain leads to split face with anencephaly (Copp & Green, 2010, review).

Neural development is a complicated process involving multiple cells and processes. Teratogens may alter cellular functions or processes such as: cell viability, apoptosis, cell cycle, cell proliferation and interactions between cell surface and extra cellular matrix. Alteration in these processes may lead to neural tube defects (Copp and Greene, 2010). Recent studies identified that alteration in the expression of genes related to these processes may cause neural tube defects; however, specific mechanisms by which these genes cause neural tube defects are not well understood. Identifying specific molecular, biochemical or signaling pathways that are responsible for teratogen-induced NTDs will help in developing intervention methods to prevent NTDs.

This present study investigated the role of altered programmed cell death or apoptosis in formation of teratogen-induced neural tube defects from two teratogens, valproic acid (VA), and an unknown teratogen in tap water. At the beginning of the study, neural tube defects were observed in control mice not exposed to an experimental teratogen. Historically, the CD-1 strain has a low background rate of 1/500 to 1/1000 NTDs (Dr. D Juriloff, U British Columbia; Dr. C Clifford, Charles River Labs; personal communication). Consistent with this low rate of spontaneous NTDs, our lab has examined over 1700 fetuses (130 litters) prior to May, 2006 without encountering a single NTD. Our investigation eventually identified tap water as the source of an unknown teratogen responsible for NTDs in control mice. Studies were initially conducted to characterize the unknown teratogen present in tap water; then the teratogen was used to investigate the role of apoptosis in formation of NTDs. Apoptosis was evaluated to identify the location and amount of apoptosis in head regions of embryos from the two teratogens. Relative expression of genes related to apoptosis and cellular signaling pathways that are important in development were analyzed to identify the molecular mechanisms responsible for NTD formation. Nonspecific maternal immune stimulation was employed to evaluate the role of apoptosis in NTDs.

II. LITERATURE REVIEW

1. Apoptosis in normal development and neural tube formation

Apoptosis or programmed cell death is an essential component of normal fetal development. Apoptosis is a physiological process that plays a major role in tissue remodeling and organogenesis (Shi et al., 1998). Apoptosis also establishes and maintains tissue architecture during development (Meier et al., 2000). Surplus cells and structures formed during development are removed by apoptosis (e.g., oviduct in males) or remodeled (e.g., digit separation). This process enables fetal structures to execute different functions at various stages of development (Meier et al., 2000).

Neural tube closure involves cell migration, proliferation, differentiation and apoptosis (Sabapathy et al., 1999). Disruption of these processes can cause NTDs. Some amount of normal cell death occurs during the formation of neural tube in mice embryos (Schlüter, 1973). Neural folds elevate dorsally and medially and appose each other in order to fuse. During the fusion, the continuity between surface ectoderm and neuroepithelium has to be restructured by apoptosis so that the opposing neural folds can converge to form the neural tube (Copp, 2005). This tissue remodeling by apoptosis is a tightly controlled process. Alteration in this apoptosis process in several knockout mouse strains causes neural tube closure defects (Barrier et al., 2009; Hsu et al., 2008; Shirane et al., 2008; Wong et al., 2008).

Pani et al., (2002) showed that failure to close the neural tube was related to alteration in apoptosis in splotch mouse embryos. This strain carries a loss-of-function of *pax3,* whose

expression may influence several downstream genes that control apoptosis. These authors demonstrated that cells with loss- of- function in *pax3* fail to regulate p53 dependent apoptosis in these embryos. They found an increased apoptotic index using Terminal Transferase dUTP Nick End Labeling **(**TUNEL) assay in embryos with NTDs compared to control embryos. Apoptosis was also reduced by inhibition of p53 in *pax3*-/- embryos that suggested role of altered apoptosis in formation of NTDs in splotch mutant embryos.

Cartilage homeoprotein gene (*Cart-1*) is required for cell viability and proliferation; its loss-of-function caused NTDs in *Cart-1* knockout mice (Greene and Copp 2005). Zhao et al., (1996) demonstrated increased cell death in forebrain as detected by acridine orange staining in *Cart-1* mutant mouse embryos that exhibited NTDs.

Barbera et al., (2002) showed increased apoptosis at the forebrain and midbrain junction in Cited2 (CBP/p300-Interacting Transactivators with glutamic acid (E)/aspartic acid (D)-rich C-terminal domain 2) knockout mice that exhibit NTDs. Forebrain and midbrain regions with increased apoptosis were demonstrated in *Cited2* null mice that indicated increased apoptosis may be associated with formation of NTDs in these embryos. Similar findings were also reported by Bamforth et al., (2001) where increased apoptosis was observed in the midbrain region of GD 9.5 embryos of *Cited2* knockout mice.

Association of NTDs with increased or decreased cell death was shown in several additional knockout mouse models. Decreased apoptosis in *Apaf1*, *Caspase 9* and *p53* knockout mice was associated with NTDs (Reviewed by Copp, 2005). Apaf1 initiates

apoptosis by regulating the activation of apoptotic caspases (Yoshida et al., 1998). Increased apoptosis was associated with NTDs in tubby-like protein 3 (*Tulp3*), *Bcl10* and *Map3k4* knockout mice, (reviewed by Harris and Juriloff, 1999; Ikeda et al., 2001). Specific role of *Tulp3* and *Map3k4* in regulation of apoptosis is not known. Bcl10 induces apoptosis through activation of NF- κB (Ruland et al., 2001). Apoptosis is a normal phenomenon in a developing embryo and it helps in tissue sculpting and removal of unwanted cells (Toder et al., 2002) and any alterations in this tightly controlled biological process may also affect the neuronal development thus resulting in NTDs.

2. Disruption of apoptosis by teratogens to induce NTDs

Rapid proliferation of the neuroepithelial cells during neurulation causes the neural folds to come together and close (Sadler, 2005). These rapidly dividing cells are vulnerable to teratogens. Exposure to teratogens can modulate programmed cell death (Sulik et al., 1988). Studies have suggested that teratogenic deregulation of apoptosis during development is a potential mechanism for birth defects including NTDs.

Several methods can be used to evaluate apoptosis. These techniques detect alterations in the cell membrane integrity, condensation of chromatin, and DNA fragmentation, which are hallmarks of apoptosis. Localization of apoptotic changes can be done using techniques such as electron microscopy, confocal microscopy and TUNEL assay. Quantitative estimation of apoptosis can be achieved using flow cytometry, confocal microscopy and caspase activity. Numerous studies have evaluated teratogen-induced

apoptosis using different techniques; however, most of these studies used localization techniques such as TUNEL assay rather than quantitative estimation.

Thompson et al., (2005) demonstrated increased apoptosis in neural tubes of chicken embryos exposed to cadmium that suggested role of altered apoptosis in cadmium-induced NTDs. Cadmium-induced NTDs in C57BL/6 mouse strain are related to up regulation of p53-dependent mediators *Ccng1* and *Pmaip1* that can cause increased apoptosis. However, this mechanism was not observed in the SWV mouse strain (Robinson et al., 2009). These results indicate that not only do teratogens act differently, but also the same teratogen can have different mechanisms to impair NTD formation in different strains of mice.

Altered apoptosis in cyclophosphamide-induced NTDs in rat embryos was evaluated by Xiao et al., (2007). Increased apoptosis was found in embryonic neuronal cells in response to cyclophosphamide. This suggests increased apoptosis as a possible mechanism for induction of NTDs by cyclophosphamide. Li et al., (1999) demonstrated homocysteine teratogenicity caused NTDs by increased apoptosis in chicken embryos.

Ambroso et al., (1998) found increased apoptosis in medial regions of the anterior neural tube and neural crest in gestational day (GD) 8 embryos from CD-1 mice exposed to 2-methoxyethanol. They demonstrated a dose dependent increase in apoptosis by confocal laser scanning microscopy and also by *in situ* immunohistochemical staining for apoptosis. They proposed increased apoptosis in embryos as a mechanism for the NTDs

in 2-methoxyethanol exposed CD-1 mice. A dose dependent increase in neuroepithelial apoptosis and neural tube defects was also observed from methylmercury chloride teratogenicity (Li and Zhu, 1998). These authors showed increased apoptosis by TUNEL assay and also by electron microscopy in Sprague -Dawley (SD) rat embryos that were exposed to methyl mercury.

Zhao and Reece (2005) evaluated effect of nicotine on mouse embryos during early embryogenesis. They demonstrated increased apoptosis by LysoTracker Red staining of whole embryos and TUNEL assay of tissue sections in ventral neural epithelium in nicotine exposed embryos. These authors also showed that nicotine increases intracellular Ca^{2+} and reactive oxygen species (ROS) leading to oxidative stress, which in turn increased apoptosis and produced NTDs.

Hyperglycemia produces a variety of birth defects including NTDs and acts by altering the apoptotic mechanism. Oyama et al., (2009) showed increased apoptosis in the hindbrain region of GD 9.5 embryos from diabetic dams compared to controls. These authors also observed decreased apoptosis when the diabetic dams were treated with folic acid. Jiang et al., (2008) showed increased apoptotic cells by TUNEL staining in the cranial neural tube of embryos from diabetic mice. Increased apoptosis in diabetic mice could be decreased by treatment of diabetic embryos with iNOS (inductible nitric oxide synthase) (Sugimura et al., 2009). The authors proposed that iNOS was reducing NTDs by decreasing apoptosis in diabetic embryos.

Neural tube formation is a complicated process involving multiple cellular processes. Apoptosis is one such process that plays a major role in neural tube closure. Several studies show that teratogens may disrupt apoptosis in developing embryos by inducing apoptosis in the neuroepithelium. These teratogens include cadmium, cyclophosphamide, nicotine, 2-methoxyethanol, hyperglycemia and possibly others. Intervention strategies such as folic acid and ascorbic acid reduce teratogen-induced apoptosis in embryos and may be protective.

2 a. Valproic acid

Valproic acid (2-propylvaleric acid) is widely used for treatment of epileptic seizures and bipolar disorder (Kostrouchova et al., 2007). Its therapeutic actions are through augmentation of gamma-aminobutyric acid-mediated (GABA) neurotransmission by increased synthesis and decreased degradation of GABA (Johannessen and Johannessen, 2003). Valrproic acid (VA) is also emerging as an anticancer drug to treat a variety of tumour types and is currently in clinical trial testing (Catalano et al., 2004). The drug's antitumor properties are by histone deacetylase (HDAC) inhibition, causing apoptosis and cell cycle arrest (Catalano et al., 2005). Microarray analysis of primary tumors in VA- treated patients demonstrated that VA affects multiple processes such as cell cycle, antigen processing and presentation, apoptosis, and the TGF-β and Wnt signaling pathways (reviewed by Duenas-Gonzalez et al., 2008). Efforts are being made to develop a second generation of VA drugs, such as VA analogues containing a urea moiety, that have less terarogenicity and similar or greater anti-epileptic properties (Okada et al., 2009; Shimshoni et al., 2007).

Administration of VA during early organogenesis can induce fetal malformations including craniofacial, urogenital and skeletal anomalies in animals and humans (Dieterich et al., 1980; Clayton-Smith and Donnai, 1995; Nau et al., 1991). A recent case-control study found an association between use of VA as an antiepiletpic drug during early pregnancy and various birth defects including spina bifida, atrial septal defect, cleft palate, hypospadias, polydactyly, and craniosynostosis in humans (Jentink et al., 2010). VA causes neural tube defects in exposed fetuses at a rate 20 times higher than in the general population possibly through inhibition of cell proliferation or increased apoptosis during neurulation (Eikel et al., 2006; Gurvich et al., 2005; Wiltse, 2005). Recent studies have shown that VA affects cell growth, differentiation, and apoptosis in cancer cells (Aouali et al., 2009; Chen et al., 2009; Bokelmann et al., 2008; Iacomino et al., 2008). VA also induces apoptosis in the developing brain in rats (Bittigau et al., 2003; Koz et al., 2010) and thus likely induces developmental malformation by altering apoptosis.

The mechanism of teratogenicity of VA and its metabolites has not been fully elucidated. Recent studies (Eikel et al., 2006; Menegola et al., 2005) suggest that VA teratogenicity may be related to the ability to inhibit histone deacetylases (HDACs). This action was separate from its therapeutic actions to treat epilepsy and therefore provides a means to differentiate therapeutic and teratogenic actions of this drug. Eikel et al., (2006) demonstrated that teratogenicity of 20 different VA derivatives was through inhibition of HDACs in teratocarcinoma mouse cells that are equivalent to early embryonic cells. In a recent study, Zhang et al., (2010) showed VA altered glutathione homeostasis in CD-1 mouse embryos, thereby causing oxidative stress and NTDs. Oxidative stress-induced

apoptosis in the developing embryo as a mechanism for NTD formation was also observed in studies on other teratogens such as nicotine (Zhao and Reece, 2005) and hyperglycemia (Loeken et al., 2005).

2 b. General contaminants in water

Approximately 292 million people in the United States rely on community water systems for their drinking water needs (U.S EPA, 2008). Most municipal water comes from surface water that is increasingly contaminated with agricultural or man-made pollutants. Contamination of surface water with pathogens and toxicants significantly impacts human and animal health. The US Geological Survey found in 2002 that 80% of the nation's rivers and streams were contaminated with wastewater contaminants. The most frequently detected compounds in surface water were cholesterol, coprostanol (fecal steroid), N,N-diethyltoluamide (DEET – insect repellant), caffeine, triclosan (antimicrobial disinfectant), tri(2-chloroethyl)phosphate (fire retardant), and 4-nonylphenol (sewage treatment surfactant, and endocrine disruptor) (Kolpin et al., 2002). Other ubiquitous contaminants of drinking water include trihalomethanes, haloacetic acid, MTBE, phthalates, synthetic estrogens and other pharmaceuticals, atrazine, DDT, benzene, toluene, xylene, tributyl tin, arsenic, fluoride, selenium, and heavy metals (Schwarzenbach et al., 2006). The Safe Drinking Water Act sets the limits for the maximum allowable concentrations of water contaminants. Safe concentrations in municipal drinking water have not been established for some of these contaminants, nor is their presence monitored (Schwarzenbach et al., 2006). The most important classes of drinking water contaminants associated with adverse developmental or reproductive

effects are pharmaceuticals, endocrine disruptors, water disinfection byproducts and petroleum products.

Recent studies have found trace amounts of pharmaceuticals and endocrine disruptors in both surface and drinking water, although, adverse health effects from this contamination have not been positively identified (Reviewed by Snyder and Benotti, 2010). Pharmaceuticals and their metabolites can enter the environment or waste water from excretion of those compounds, or from disposal of expired and unused pharmaceuticals. Common pharmaceutical compounds such as antibiotics, anti-inflammatory agents, anti-epileptics, antacids, diuretics and β blockers have been found in a variety of water sources including surface and ground water (Fent et al., 2006; Glassmeyer et al., 2009 reviews). In another study, conducted from 19 U.S. water utilities, the most frequently detected contaminants were atrazine, carbamazepine, estrone, gemfibrozil, meprobamate, naproxen, phenytoin, sulfamethoxazole, tris (2-carboxyethyl) phosphine (TCEP) and trimethoprim (Benotti et al., 2009).

Contamination of drinking water sources with agricultural herbicides can also adversely affect reproductive health including intrauterine growth retardation, low birth weight and prematurity (Ochoa-Acuña et al., 2009, Villanueva et al., 2005, Munger et al., 1997). Specifically, atrazine concentrations found in the drinking water were associated with small for gestational age (SGA) births (Ochoa-Acuña et al., 2009). Increases in intrauterine growth retardation (IUGR) were shown to be associated with the presence of triazine herbicides in Iowa (Munger et al., 1997). Specific contaminants may not be

present in toxic concentrations in drinking water, but mixtures can act synergistically such that the resulting combination is toxic. Little is known about the potential toxicity or long term human health effects from chronic exposure to mixtures of these common water contaminants. The stable chemical structure of some organic contaminants, including various pharmaceuticals and endocrine disruptors, may prevent their removal by water treatment systems (Stackelberg et al., 2004, 2007; Falconer, 2006). Occurrence of pharmaceutical or chemical contaminats in finished drinking water may also depend on the type of water treatment (Benotti et al., 2009).

Petroleum products such as diesel, gasoline and kerosene are obtained by refining crude oil, natural gas and coal. Various storage, handling and transport conditions required for the extensive use of petroleum products provide opportunities for spills. Petroleum products may be released into the environment because of leaks from storage tanks and pipes, overfills, spills or intentional disposal. These products can travel through soil and contaminate drinking water sources. Petroleum products contain potentially toxic compounds such as xylene and toluene as well as additives such as lead and methyl tertiary-butyl ether (MTBE). These compounds have various adverse health effects including teratogenesis. Xylene causes low birth weight, cleft palate and other skeletal deformities (Marks et al., 1982; Saillenfait et al., 2003). Toluene is teratogenic and reacts synergistically with acetylsalicylic acid and other compounds that may be present in the water (reviewed by Wilkins-Haug, 1997).

Both epidemiologic and laboratory animal studies suggest an association between water disinfection byproducts and adverse pregnancy outcomes including NTDs (Bove et al., 2002 review; Ahmed et al., 2005, Andrews et al., 2004; Hunter et al., 1996; Hunter et al., 2006). Disinfection of water is necessary to prevent water-borne diseases such as cholera, typhoid and cryptosporidiosis. Common disinfection methods use chemical oxidation by chlorine, chloramines, ozone or chlorine dioxide depending on the water quality and supply system. The disinfectants can react with other compounds present in the water producing toxic by-products referred to as DBPs (disinfection by-products) (Rice et al., 2008). Many of these DBPs have not been identified, raising concerns about safety and health effects of water treatment methods (Richardson et al., 2008 & 2007). The nature and concentrations of DBPs depend on the type of disinfectant used and also on the distribution time of the treated water (reviewed by Bull et al., 2009).

Chlorination, the most common disinfection method, forms by-products including trihalomethanes (THMs) such as chloroform, bromodichloromethane (BDCM), cholorodi-bromomethane (CDBM), and bromoform. Some of these DBPs are teratogenic to laboratory animals. Haloacetic acids, at concentrations higher than found in tap water, cause NTDs in rat embryos (Ahmed et al., 2005, Andrews et al., 2004; Hunter et al., 1996; Hunter et al., 2006). Ahmed et al., (2005) demonstrated that exposure to chloroacetonitrile (CAN), another disinfection by-product, caused degeneration of neurons affecting the development of fetal brain in CD-1 mice. Additionally, CAN crossed the placenta to induce oxidative stress and apoptosis in the fetal brain. Epidemiological data on reproductive effects of chlorination disinfectant by-products

indicate that these products can cause a variety of birth defects including: low birth weight, skeletal deformities, growth retardation, spontaneous abortions and NTDs but at relatively low rates (reviewed by Bove et al., 2002). Schenck et al., (2009) demonstrated a dose dependent correlation between the mutagenicity and chlorination of the drinking water. In general, it has been accepted that health risks from exposure to disinfection byproducts (DBPs) are less than from consuming untreated water.

3. Molecular mechanism associated with apoptosis in the formation of neural tube defects

Wlodarczyk et al., (1996) analyzed expression of genes regulating early embryogenesis, cell cycle and proliferation (*Emx-1*, *Emx-2, c-fos, c-jun, Creb, p53* and *bcl-2*) in the LM/Bc mouse embryos in response to VA. Expression of transcription factors that regulate morphogenic events (*Emx-1, Emx-2, c-fos, c-jun*, and *Creb*) were increased in response to VA exposure. Expression of genes that regulate cell proliferation, *p53* and *Bcl2*, was also increased in VA-treated embryos. Upregulation of *p53* results in inhibition of cell proliferation and induction of apoptosis. *Bcl2* prevents apoptosis induced by a variety of death stimuli (Thomadaki et al., 2007). Since *p53*/ *Bcl2* gene expression ratio was favored towards *Bcl2*, Wlodarczyk et al., (1996) speculated cell proliferation rather than apoptosis may be responsible for the NTDs in this strain. Neither levels of apoptosis nor inhibition of cell proliferation were analyzed directly, however. *Pax3* expression which has recently been associated with increased apoptosis and NTD formation was also down regulated (Chappell et al., 2009, Green et al., 2009, Burren et al., 2008, Morgan et al., 2008, Loeken 2006, Pani et al., 2002).

Zinc deficiency is associated with NTDs (Golalipour et al., 2006, Zhang et al., 2005, Srinivas et al., 2001). Okada et al., (2005) studied effect of VA on polycomb group genes in GD8 NMRI mouse embryos by microarray analysis. They noticed down regulation of genes of the polycomb group complex such *as Egr2, Zfp105* and *Zfp144* that are associated with zinc finger proteins in VA-treated embryos. Down regulation of zinc finger proteins is related to increased apoptosis (Herkert et al., 2010, Lademann et al., 2001, Przyborski et al., 1998). Jergil et al.,(2009) found an association between zinc finger proteins and NTDs. Okada (2005) also found down regulation of *Sirt-1, Eed, Ezh2, YY1* polycomb group genes in VA exposed embryos. Inhibition of these genes leads to apoptosis through HDACs pathway (Wang et al., 2010, Peck et al., 2010, He et al., 2010). Also *Sirt1*, a histone deacetylase, inhibits apoptosis by binding to *p53*. Many teratogens induce genetic damage in the embryo; and in response, expression of *p53* may be increased to prevent the genetic damage (Finnel et al., 2003).

Wlodarczyk et al., (1996) evaluated cell cycle gene expression changes (*Bcl-2, p53*, *Wee-1* and *Wnt-1*) in mouse embryos that are exposed to arsenic. Among the 4 genes that were analyzed, *Bcl2* and *p53* were up regulated that indicated a DNA damaging effect for arsenic. Upregulation of *p53* results in inhibition of cell proliferation and induction of apoptosis whereas upregulation of *Bcl2* results in inhibition of apoptosis. The authors proposed that arsenic inhibited cell proliferation rather than increasing apoptosis to cause NTDs since both the *Bcl2* and *p53* were overexpressed. Neither cell proliferation nor apoptosis levels were measured directly, however.

Eikel et al., (2006) showed an immediate inhibition of HDAC in response to VA in F9 embryonic carcinoma cells. They proposed that HDAC inhibition by VA may initiate a molecular pathway leading to NTD formation since HDAC inhibition stimulates the intrinsic apoptosis pathway through induction of pro-apoptotic genes (Zhang et al, 2006; Zhao et al., 2005).

Jergil et al., (2009) evaluated the gene expression profile in p19 murine embryonal carcinoma cells in response to VA using microarrays. These p19 cells are considered as a model system for early embryonic cells since both have high proliferation rates and pluripotency. VA-induced apoptosis and decreased cell viability in these cells. *Caspase 3* was also activated by VA that indicated induction of apoptosis. Expression levels of genes related to HDAC inhibition, G1-phase cell cycle arrest and apoptosis (*Btg1, Eif2ak3, Nme5, Plekhf1, Tradd*) were altered by VA in this study indicating VA can affect both cell proliferation as well as apoptosis.

Dawson et al., (2006) evaluated expression of proteins that regulate apoptosis in GD 9.5 embryos of CD-1 mice in response to VA. These proteins include the gene products of *p53, NF-κB, Pim-1, c-Myb, Bax* and *Bcl-2*. *p53* protein levels were increased in VA exposed embryos with open neural tubes compared to embryos with closed neural tubes of both VA exposed and control embryos. Expression of *NF-κB, c-Myb* and *Pim-1* proteins was decreased in the VA exposed embryos with open neural tubes compared to the VA-treated normal embryos and the control embryos. *Bcl-2* protein levels were decreased in both embryos that have open and closed neural tubes in the VA exposed

group compared to controls. There was no difference between the levels of pro-apoptotic protein, *Bax*, observed in VA exposed embryos and control embryos. The ratio of pro-apoptotic and anti-apoptotic proteins *Bax* and *Bcl2* was significantly higher in embryos with open neural tubes from VA-treated dams.

Dawson et al (2006) also evaluated the effect of folic acid or pantothenic acid supplementation on VA-induced NTDs in CD-1 mice. Folic acid reduced alterations in *p53, NF-κB, c-Myb,* and *Pim-1* protein levels caused by VA. Folic acid did not reduce alterations in pro-apoptotic protein *Bax* and the anti-apoptotic protein *Bcl-2* levels; but the ratio of *Bax* and *Bcl-2* was shown to be shifted towards anti-apoptotic pattern. Pantothenic acid did not affect VA altered *p53* protein levels but did reduce VA altered *NF-κB, c-Myb* and *Pim-1* protein levels. Expression levels of *Bax*, *Bcl-2* or their ratio of protein expression were not significantly affected by pantothenic acid in VA-exposed embryos. The authors suggested that folic acid and pantothenic acid have different mechanisms of actions in preventing the NTDs induced by same teratogen, VA.

Zhang et al., (2010) demonstrated that VA inhibits *Hoxa2* gene expression in CD-1 mouse embryos in culture. This effect was reversed when the embryos were grown in media with ascorbic acid. They proposed that ascorbic acid may be preventing neural tube defects in embryos exposed to VA by preventing the alteration of *Hoxa2* gene expression.

Bennette et al., (2000) demonstrated that VA affects different molecular pathways in LM/Bc and SWV embryos at three different time points including GD 8.75, GD 9 and GD 9.5. They showed that VA causes NTDs in the two strains by different molecular pathways or genes. In LM/Bc embryos, nerve growth factor *(Ngf), Trk, Ngf-R* and basic fibroblast growth factor (*Bfgf*) were up regulated at all these time points. These genes were not up regulated in SWV embryos; however, in SWV embryos, *Tgf-α, Tgf-ß1, Tgf-ß2, Tgf-ß3* were up regulated at GD 9. These genes remain unaltered in LM/Bc embryos with the exception of *Tgf-ß2* that was down regulated at GD 9. Bennette et al., (2000) performed both PCA and univariate analysis revealing that VA mainly affects principle components containing TGFs in LM/Bc embryos but not in SWV embryos. This indicates that VA disrupts regulation of the cell cycle in LM/Bc strain. The authors suggested that down regulation of TGFs may be the major pathway responsible for VA-induced NTDs, since TGFs protect neuroepithelial cells from apoptosis. The authors also proposed regulation of TGFs by VA in SWV may result in decreased cell proliferation rates leading to NTDs.

Together, the majority of these studies regarding teratogen-induced NTDs show that NTDs are related to alteration in apoptotic or cell proliferation rates. Teratogens alter the expression of the genes belong to Bcl-2, p53, NF-kB, Zinc finger proteins and TGF families. Alteration in the expression of these genes is associated with increased apoptosis or inhibition of cell proliferation. These studies indicate that different teratogens induce NTDs by different molecular pathways. Additionally, a single teratogen can cause NTDs by different mechanisms in different strains. Similarly,

intervention strategies reduce the same birth defect through different molecular mechanisms.

4. Nonspecific immune stimulation and birth defects

4 a. Role of nonspecific immune stimulation in preventing birth defects

Nonspecific stimulation of maternal immune system in mice reduces a wide variety of teratogen-induced fetal malformations. The immune stimulants shown to have broad-spectrum activity in reducing the birth defects (including NTDs) were Freund's complete adjuvant, Bacillus Calmette-Guerin (BCG), inert particles, allogeneic or zenogeneic lymphocytes, granulocyte macrophage colony-stimulating factor (GM-CSF), or interferon- γ (IFN-γ). The teratogens tested include various physical and chemical agents, and also diseases such as diabetes mellitus (Nomura et al., 1990; Holladay et al., 2000; Prater et al., 2004; Punareewattana and Holladay 2004; Toder et al., 1996; Laudermilch et al., 2005; Hrubec et al., 2006; Khaksary et al., 2006; Latorre et al., 2007).

Cleft palate, and palate shortening (Hrubec et al., 2009) as well as shortening of mandibular and maxillary lengths (Hrubec et al., 2006) are reduced by nonspecific immune stimulation of pregnant diabetic dams with Freund’s complete adjuvant (FCA) or GM-CSF. Punareewattana and Holladay (2004) demonstrated reduction in incidence of various birth defects including NTDs and eye defects in fetuses from diabetic ICR mice by maternal immune stimulation with FCA, GM-CSF or INF-γ. Cleft palate, digit defects and tail defects are reduced by maternal immune stimulation in ICR mice exposed to urethane or methyl nitrosourea (Nomura et al., 1990; Holladay et al., 2000; Prater et

al., 2004). Birth defects such as open eyes, exencephaly, and resorptions in ICR mice induced by hyperthermia are reduced by immune stimulation with rat splenocytes (Yitzhakie et al., 1999). Laudermilch et al., (2005) showed reduced incidence of syndactyly, polydactyly, and interdigital webbing in MNU-exposed C57BL/6N mice and CD-1 mice by maternal immune stimulation with IFNγ. Methylnitrosourea-induced endothelial and trophoblast placental damage was also diminished by maternal immune stimulation in both strains of mice. Maternal immune modulation reduces cyclophosphamide-induced fetal malformations such as resorptions and decreased embryonic weight in mice (Toder et al., 1996). Stress-triggered abortions (Clark et al., 1993) and spontaneous early embryonic resorptions that are induced by bacterial lipopolysaccharide (LPS) (Baines et al., 1996) in mice are prevented by immune stimulation. Gutierrez et al., (2009) reported reduced cardiac malformations with maternal immune stimulation using FCA in embryos from hyperglycemic mice. The protective mechanism of maternal immune stimulation is not well understood, however.

4 b. Proposed mechanisms by which maternal immune stimulation prevents birth defects

Nomura et al., (1990) proposed a mechanism whereby maternal immune stimulation activates macrophages. These activated macrophages then enter the fetus from the mother, kill and remove the pre- teratogenic cells that are then replaced with normal totipotent cells. However, later studies disproved this hypothesis by using flow cytometry and a cell–tracking probe to demonstrate that no maternal cells crossed the placenta to effect the protection (Holladay et al., 2002).

Sharova et al., (2003) suggested that prevention of teratogen-induced changes in maternal cytokine profile is a mechanism for the protective effect of maternal stimulation in reducing teratogenicity. These authors showed that maternal immune stimulation restored changes in Th1 and Th2 cytokine balance following urethane exposure. They observed that urethane favors the ratio towards Th1 cytokine profile (IFNγ and IL-2) whereas maternal immune stimulation shifts the ratio towards Th2 profile by up regulating the cytokine genes, IL-4, IL-10 and IL-13.

Teratogens, as mentioned in the previous sections, can affect the apoptotic process in developing embryos. Maternal immune stimulation prevents cyclophosphamide-induced apoptosis in mouse embryos (Toder et al., 1996; Torchinsky et al, 1995). This indicates involvement of apoptosis in the protective mechanism of maternal immune stimulation in reducing the teratogenicity. In later studies, Savion et al., (2003 & 2009) showed that this protective effect of maternal immune stimulation in reducing cyclophosphamide-induced apoptosis in embryos was mediated through the normalization of *p53* and *Bcl2*. Maternal immune stimulation also reduces hyperthermia-induced apoptosis in developing embryos that may be related to its protective effect (Yitzhakie et al., 1999).

Most teratogens cause malformations by altering expression of important cell cycle regulatory and/or apoptotic genes (Green et al., 2009, Eikel et al., 2005, Thomson et al., 2005, Wlodarczyk et al., 1996). Stimulation of the maternal immune system appears to normalize these alterations and reduce malformations (Sharova et al., 2002). Intervention

strategies such as antioxidants or folic acid that reduce birth defects, including NTDs, prevent the alteration in the expression pattern of genes that regulate apoptosis or cell cycle (Pani et al., 2002). Fetal gene expression normalized by maternal immune stimulation so far includes important regulatory genes of cell cycle such as *Tgf-β2, Tnf-α* from diabetes (Fein et al., 2001 & 2002; Sharova et al., 2003); *Bcl2α, Bcl2β, PkCα* and *p53* from urethane (Sharova et al., 2000); and *Tgf-β* and *Tnf-α* from cyclosphosphamide (Ivnitsky et al., 1998; Gorivodsky et al., 1999). Punareewattana et al., (2003) evaluated immune changes in pregnant dams resulting from maternal immune stimulation by microarray analysis of gene expression in the spleen of diabetic mice. Maternal immune stimulation normalized the expression of apoptotic, anti-apoptotic and cell proliferative genes. Sharova (2000) et al., studied protective effect of maternal immune stimulation in prevention of urethane-induced cleft palates. These authors analyzed the changes in gene expression of cell cycle and apoptotic genes due to maternal immune stimulation and urethane in heads of GD 14 embryos. They noticed down regulation of *Bcl2α, Bcl2β* and *PkCα* from urethane. These changes were reduced by maternal immune stimulation with IFNγ. In another study, Punareewattana et al., (2005) noticed up regulation of anti-apoptotic gene, *Bcl2*, and down regulation of apoptotic genes *R-ras* and *p-21* in the embryos from hyperglycemic dams that indicated decreased apoptosis in the embryo as a potential protective mechanism by which maternal immune stimulation acts to prevent birth defects.

Gutierrez et al., (2009) showed increased apoptotic cells in fetal myocardium from hyperglycemic mice. Maternal immune stimulation reduced apoptotic cells in the fetal

myocardium. Gutierrez et al., (2009) observed up regulation of *Bcl2*, an anti-apoptotic gene, in fetal myocardium of hyperglycemic mice. However, these authors reported that the increase in *Bcl2* in hyperglycemic fetuses was in contrast to other published studies where the expression of *Bcl2* was down regulated. Gutierrez et al (2009) also observed down regulation of pro-apoptotic gene *Casp9* in hyperglycemic embryos. Maternal immune stimulation did not alter the *Casp9* compared to hyperglycemic group. These authors proposed that increase in pro-apoptotic genes and decrease in anti-apoptotic genes may be a result of intracellular response in myocardial cells override the earlier extra cellular apoptotic signals.

Damage to the placental structure can expose the developing embryo to teratogens present in the maternal circulation. Intervention strategies that prevent birth defects may act by reducing the placental damage caused by teratogens. Sharova et al., (2003) noticed damage to the syncytiotrophoblast of placenta caused by urethane. Other changes to placenta caused by urethane include diffuse fibrosing villosis and reduced vascularization in the placenta. These changes were reduced by maternal immune stimulation with FCA or IFNγ. Prater et al., (2004) studied the protective mechanism by which maternal immune stimulation prevents methylnitrosourea (MNU)-induced limb defects. MNU caused necrosis of trophoblasts and endothelial cells of the labyrinthine layer and maternal immune stimulation with IFNγ reduced cell death in placental layers. Similar to Prater et al., (2004), Laudermilch et al (2005) noticed multifocal areas of necrosis, karyolysis and pyknosis, cellular fragmentation, hypereosinophilia, and collapse of vascular patency in labyrinthine layers of placenta in urethane exposed dams. These

changes were reduced with maternal immune stimulation. VA did not cause any histological changes in placenta in mice (Hrubec et al., 2006). This indicates placenta may not be involved in VA teratogencity. Punareewattana et al., (2005) also did not observe any changes in expression of placental genes from maternal immune stimulation in diabetic mice. This implies protective effect of immune stimulation is not be related to changes in the placenta.

Teratogens induce birth defects through different mechanisms depending on the type and timing of teratogen exposure. Maternal immune stimulation can prevent a wide array of birth defects. Thus, it appears that maternal immune stimulation may act through various mechanisms depending on the type of teratogen exposure and type of malformation. The predominant mechanism by which maternal immune stimulation appears to reduce fetal malformations is by preventing the changes in the genes or molecular pathways regulating cell proliferation and apoptosis in placenta and fetus.

Present literature suggests that teratogens may alter normal apoptotic processes in the embryo resulting in fetal malformations. This study will test the hypothesis that both VA and the teratogen present in tap water alter the location or amount of fetal apoptosis by changing apoptotic signaling in embryos resulting in formation of NTDs. The mechanism of protection by maternal immune stimulation is not well understood but believed to involve maternal modulation of developmental processes in embryo. This study will explore the role of apoptosis in the protection by maternal immune stimulation. These studies are expected to significantly increase our understanding of the role of apoptosis in

NTD formation and identify specific molecular mechanisms responsible for teratogen-induced NTDs. This research is of importance to human health as understanding the mechanisms of NTD induction will lead to successful intervention strategies.

III. MATERIALS AND METHODS

This project investigated the role of apoptosis in NTD formation induced by two teratogens: valproic acid (VA) and unknown contaminant present in tap water in a series of 5 experiments.

Study 1 identified the source of an unknown teratogen causing NTDs in control mice by testing multiple combinations of housing conditions, mouse strain, diet and water.

Study 2 characterized the teratogen in tap water to narrow down possible contaminants and aid in the identification of the unknown by analytical methods. This study determined whether the teratogen acted directly or was a metabolite, and also determined if the teratogen was a water disinfection byproduct.

Study 3 determined the role of apoptosis in the formation of NTDs from two teratogens, VA and tap water. The study also determined whether the protective effects of maternal immune stimulation are mediated by changes in apoptosis.

Study 4 profiled apoptotic pathway genes responsible for teratogen (VA and tap water) - induced apoptosis. The study also profiled apoptotic pathway genes that play a role in protective effects of maternal immune stimulation.

Study 5 determined which cellular signaling pathways were altered during teratogen-induced NTDs. Expression of key genes related to 18 different developmentally

important signaling pathways was analyzed. This study was carried out to elucidate if teratogens alter these signaling pathways to induce NTDs and also if the protective effect of maternal immune stimulation is related to changes in these signaling pathways.

Study 1. Neural tube defects in mice exposed to tap water

1 a. Animals

The following sources and strains of mice were used for to determine the cause of neural tube defects in control mice:

1. CD-1 strain raised in-house from brood stock obtained from Company A.
2. Purchased CD-1 mice were obtained from Company A at 5-8 weeks of age.
3. ICR strain, 6 weeks of age, purchased from Company B.
4. Time pregnant mice were purchased from laboratory animal providers as follows: CD-1 from Company A, Raleigh NC and Portage MI; ICR strain from both Company B, Dublin, VA and Company C, Germantown, NY; and Swiss Webster mice from Company C, Germantown, NY. All mice were bred at the vendor's facility and shipped on gestation day (GD) 10-11 after neural tube closure. Embryos were evaluated for NTDs immediately upon arrival in our facility on GD 11-12.
5. F2-DDI were derived from purchased CD-1 mice from Company A. Mice were provided only distilled de-ionized (DDI) water for two generations as described in Figure 1 to obtain F2-DDI mice.

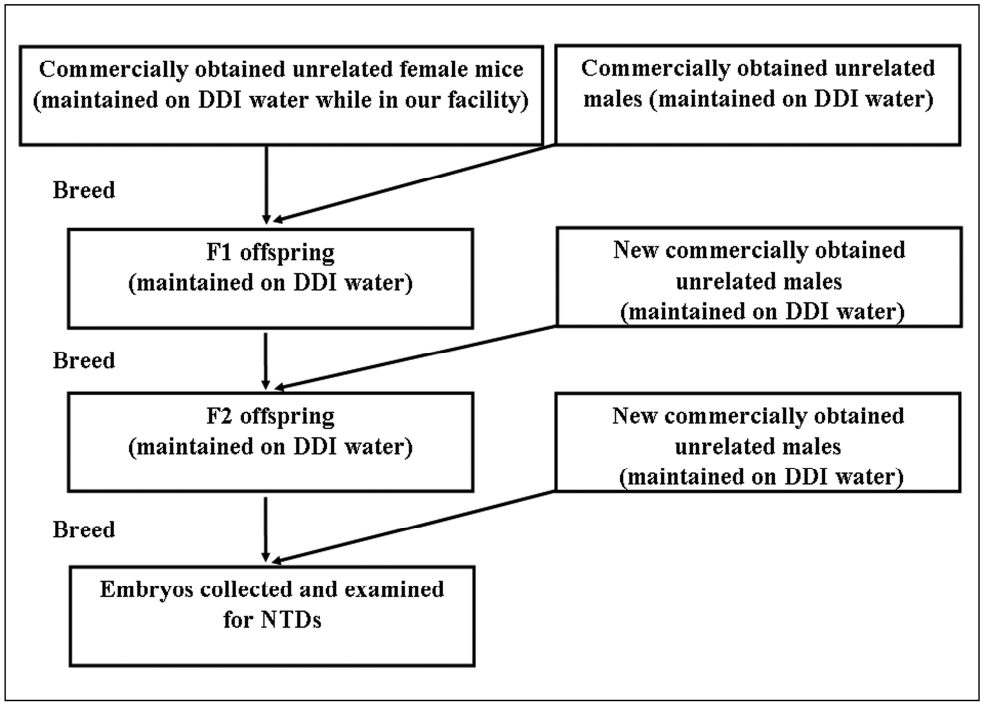

Figure 1. Breeding scheme of mice to obtain embryos from mice provided with distilled de-ionized (DDI) water for 2 generations (F2-DDI).

Mice that were raised or maintained in house were housed in conventional cages in a standard mouse room with 12 hours day length, 70-73^{o}F, 50±20% humidity and *ad libitum* access to feed and water. For breeding, 2 or 3 females were housed with a male of the same strain provided with the same experimental conditions. The females were checked for copulation plugs every morning. When a copulation plug was found, the female was separated and midpoint of the dark cycle was designated as gestation day (GD) 0. Mice were euthanized on GD 9.5 by an overdose of CO_2 and embryos were evaluated for NTDs under a stereozoom microscope (Olympus SZX7, Melville, NY). Embryos were staged by somite count, number of brachial arches and amount of limb bud growth to verify sufficient development for neural tube closure.

All procedures related to animal use were approved by and conducted in compliance with 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 International (AAALAC) accredited facility.

1 b. Experimental conditions to screen for possible risk factors

Purchased CD-1 mice were exposed to multiple combinations of housing, diet and water for 2 weeks in order to narrow down possible causes for the observed malformations. The feed was certified by the manufacturer (Teklad) using independent analysis to be free or within normal limits of the following potential contaminants.

- arsenic
- mercury
- aldrin
- thiodan
- heptachlor
- PCB's
- Mirex
- Diazinon
- malathion
- ethion
- cadmium
- selenium
- lindane
- dieldrin
- heptachlor epoxide
- Thimet
- Methoxychlor
- Disulfoton
- parathion
- trithion
- lead
- aflatoxin B1, B2, G1, G2
- chlordane
- endrin
- toxaphene
- Hexachlorobenzene
- DDT & related substances
- methyl parathion
- α, β, γ Benzene hexachloride

Bedding material was tested independently by the manufacturer (ALPHA-Dri, Shepherd Specialty Papers, Watertown, TN) for the following possible contaminants.

- Arsenic
- bismuth
- cadmium
- Lead
- organophosphate pesticides
- organochlorine pesticides
- PCBs

Experimental and sentinel mice were tested numerous times over a 7 month period for routine and comprehensive screens of known mouse pathogens by Rodent & Rabbit Serology Laboratory at BioReliance, Rockville, MD. The infectious agents that were tested include:

- Cilia-associated aespiratory bacillus
- *Clostridium piliforme* (Tyzzer's)
- Convict Creek virus (CCV)
- Ectromelia virus (Ectro)
- *Encephalitozoon cuniculi* (E. cun.)
- Epizootic diarrhea of infant mice
- Epstein-Barr related virus (EBV)
- *Helicobacter bilis*
- *Helicobacter hepaticus*
- *Helicobacter rodentium*
- *Helicobacter typhlonius*
- Hantaan virus
- *Helicobacter* spp
- K Virus (Mouse pneumonitis virus),
- Lymphocytic choriomeningitis virus
- Mouse adenovirus 1 & 2
- Mouse hepatitis virus (MHV)
- Mouse minute virus (MMV)
- Mouse parvovirus (MPV)
- Mouse reovirus
- Mouse reovirus type 3 (Reo3)
- Mouse thymic virus (MTV)
- Murine cytomegalovirus (MCMV)
- Murine norovirus (MNV)
- *Mycoplasma arthritidis*
- *Mycoplasma pulmonis*
- Parvovirus
- Pneumonia virus of mice (PVM)
- European hantavirus
- Respiratory synctial virus
- Rotavirus sendai virus
- Theiler's mouse encephalomyelitis virus

1 c. Association of source, strain and NTDs

The source and strain of mice was tested using CD-1 strain raised in house from brood stock obtained from Company A, CD-1 mice obtained from Company A and ICR mice obtained from Company B.

1 d. Association of housing and NTDs

Housing location was tested by raising mice at two different facilities of VA-MD Regional College of Veterinary Medicine in Blacksburg located approximately 2 miles apart.

Housing type was tested by maintaining newly purchased CD-1 mice in conventional cages (n= 92), in microisolator cages under sterile conditions in a HEPA filtered laminar flow hood (n=9), and under Biosafety Level 2 (BSL2) containment conditions in a separate facility (n=3).

1 e. Association of diet and NTDs

The effect of diet on NTDs was tested by providing newly purchased CD-1 mice four commercial rodent diets from three different vendors (Harlan, Purina and Ziegler). The commercial rodent diets that were tested included Harlan Teklad Rodent Diet 7013 (n=3), Madison, WI; Ziegler Rodent diets 18-6 (n=61) and NTP-2000 (n=9), Gardeners, PA and Purina 5001 (n=31), Richmond, IN, USA. All were complete diets suitable for growth and reproduction.

1 f. Association of water and NTDs

Three different sources of water were tested in newly purchased CD-1 mice including tap water from location A, south west VA (n=25), distilled-deionized (DDI) water (Milli-Q

system water at 18 Ohm resistance, Millipore Corporation, Billerica, MA) (n=30), and HPLC water (OmniSolv, EMD Chemicals Inc, Gibbstown, NJ) (n=26). Mice received fresh water once or twice a week. Mice that were maintained under sterile conditions received new autoclaved water once or twice a week.

1 g. F2 Generation: Association of water and NTDs

In our last experiment, F2-DDI mice were tested with five sources of water under controlled conditions. All mice were housed in conventional cages and were fed Zeigler NTP diet. The five sources of water were: municipal tap water from location 1 in southwest VA (n=25); municipal tap water from location 2 in northeast MD (n=23); municipal tap water from location 3 in central MD (n=21); DDI water (n=20); and HPLC water (n=8). Among these water sources, location 2 and 3 were from urban commercial water suppliers whereas location 1 was from a rural commercial water supplier. Freshwater from the five sources was provided to the mice once or twice a week.

1 h. Statistical analysis

Statistical analysis was conducted by the Laboratory for Study Design and Statistical Service at Virginia Tech. Dams were considered the treatment unit and the percentage of NTDs was calculated as the mean percentage of embryos with NTDs per litter. Associations between treatment variables and percentage of NTDs were assessed by Poisson regression using the Statistical Analysis System (version 9.1.3, SAS Institute, Cary, NC 27513) and statistical significance was set at $p \leq 0.05$.

Study 2. Characterization of teratogen present in tap water

2 a. Chemical characterization of teratogen present in tap water

This study determined if the teratogenic compound(s) was volatile or non-volatile. The results were helpful for further identification of the compound by chemical analysis.

Volatile and non-volatile fractions of tap water were prepared using distillation and solid phase extraction, respectively (Figure 2). Tap water and DDI water were collected and processed every week. Each week one liter of tap water sample was heated to vaporize the volatile compounds. The vapors, containing the volatile compounds, were passed through a condenser to cool and the condensate collected. The condensate was reconstituted back to 1 L with DDI to obtain the initial concentration in tap water. The non-volatile fraction was obtained by passing the remaining water sample after removal of volatile compounds through a C18 solid phase extraction column and the elute (~1 ml) was reconstituted back to original concentrations by adding DDI water (~999 ml).

F2-DDI mice were housed in conventional cages in a standard mouse room with 12 hours day length, 70-73^{o}F, 50±20% humidity and ad libitum access to Zeigler NTP diet 18-6 and DDI water. For the exposure study, 15 F2-DDI females were provided either volatile or non-volatile water fractions for 4 weeks prior to breeding. Fresh fractionated water was provided to the mice every week. Pregnant dams were continued on their water treatments during gestation and were euthanized by CO_2 inhalation on GD 9. Embryos were evaluated for the presence of NTDs.

Statistical analysis

Dams were considered the treatment unit and the percentage of NTDs was calculated as the mean percentage of embryos with NTDs per litter. Associations between treatment variables and percentage of NTDs were assessed by One way analysis of variance (ANOVA) (Statistix 8, Analytical Software, Tallahassee, FL). When a significant difference was observed ($p<0.05$), a Tukey's means comparison was used to determine differences between treatment groups.

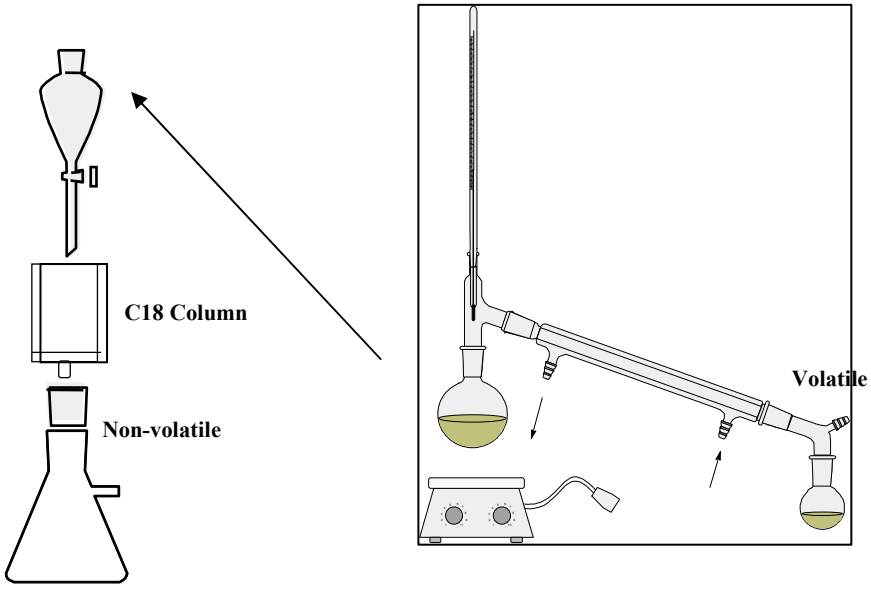

Figure 2. Separation of volatile and non-volatile fractions of water samples using distillation and solid phase extraction, respectively.

2 b. Metabolism study to determine whether teratogenicity is caused by a parent compound or its metabolite

The objective of this experiment was to demonstrate whether the parent compound or a metabolite was responsible for the fetal malformations. Cytochrome P450 enzymes are liver enzymes involved in metabolism of various xenobiotic compounds in order to eliminate them from the body (Anzenbacher et al., 2001, review). Cytochrome P450s may activate a chemical to a toxic metabolite or may inactivate a toxicant by metabolizing it to a non-toxic metabolite and/or increase the elimination of the compound from the body. If the parent compound is teratogenic, the teratogenicity should decrease when cytochrome P450 enzymes are induced as more parent compound is metabolized to a non-tertogenic form. If teratogenicity is caused by a metabolite, the number of malformations will increase when cytochrome P450 enzymes are induced as more teratogenic metabolites are formed. Cytochrome P450 enzyme inhibitors increase the teratogenicity if the parent compound is teratogenic and decrease the teratogenicity if teratogenicity is caused by a metabolite.

Administration of SKF 525-A dissolved in sterile water was used to inhibit cytochrome P450 enzymes. Initially, β-naphthoflavone in corn oil was selected to induce cytochrome P450 enzymes as suggested by previous studies (Brauze et al., 2002; Azoulay-Dupuis et al., 1988) but the compound did not dissolve in corn oil. β-naphthoflavone was then dissolved in DMSO; but when administered, 3 of the 5 mice injected died unexpectedly. Due to the unexpected results with β-naphthoflavone, phenobarbital dissolved in saline was selected as cytochrome P450 inducer.

F2-DDI mice bred in our lab were provided with either tap water or DDI water for 2 weeks prior to breeding. Each treatment group received either phenobarbital or SKF 525-A on gestational days 6, 7, 8 (spanning the time of neural tube closure) as described below.

Treatment groups:

- Phenobarbital (80 mg/kg/d in saline by IP injection) administered to F2 DDI dams provided with tap water (n=12).
- SKF 525-A (40 mg/kg/d in sterile water by IP injection) administered to F2 DDI dams provided with tap water (n=15).
- Normal Saline (0.1 ml by IP injection) administered to F2 DDI dams provided with tap water (n=11).
- Phenobarbital (80 mg/kg/d in saline by IP injection) administered to F2 DDI dams provided with DDI water (n=12).
- SKF 525-A (40 mg/kg/d in sterile water by IP injection) administered to F2 DDI dams provided with DDI water (n=11).

Animal housing conditions, diet and water were same as described in study 2a. For breeding, 2 or 3 females were added to a male. The females were checked for copulation plugs every morning. When a copulation plug was found, the female was separated and the midpoint of the dark cycle was designated as GD 0. Mice were euthanized on GD 9 by an over dosage of CO_2, and the embryos were evaluated for NTDs under stereozoom microscope (Olympus SZX7, Melville, NY).

Statistical analysis

Dams were considered the treatment unit and the percentage of NTDs was calculated as the mean percentage of embryos with NTDs per litter. NTD levels in embryos of mice consuming tap water were compared to DDI in each treatment group by student *t*-test. Statistical significance was set at $p \leq 0.05$.

2 c. Determination of whether the teratogen is a disinfection byproduct

Disinfection of drinking water results in numerous disinfection by-products (DBPs) such as haloacetic acids. Epidemiological as well as rodent studies have shown that DBPs are developmental toxicants and can cause NTDs (Bove et al., 2002). The appearance of NTDs in our mice in May 2006 correlated with increase in nitrates subsequent to a change in the water treatment from chlorination to chloramination. Drinking water facilities are increasingly changing the water treatment process from chlorination to chloramination in order to meet regulatory standards for drinking water. Chloramination results in low levels of haloacetic acids and other DBPs compared to chlorination; however, production and toxicity of novel DBPs resulting from chloramination are not well known. This experiment determined whether the unknown teratogen in tap water was a result of change in the water treatment.

Water treatment process (Chloramination):

Raw water is obtained from water sources (river, well, reservoir) and chlorine is added immediately to kill microorganisms (Figure 3). Water is then treated with flocculants to coagulate particulate matter and flows through sedimentation tanks where the coagulated

particles settle at the bottom of the tank. Water is then filtered to remove remaining particles. Chlorine is added again after filtration to kill remaining bacteria and the water is then stored until distributed. Ammonia is added to the water right before distribution to form stable chloramines that provide long-lasting disinfection in the distribution system. Chloramination allows treatment with less total chlorine because the long-lasting chloramines are more stable than chlorine.

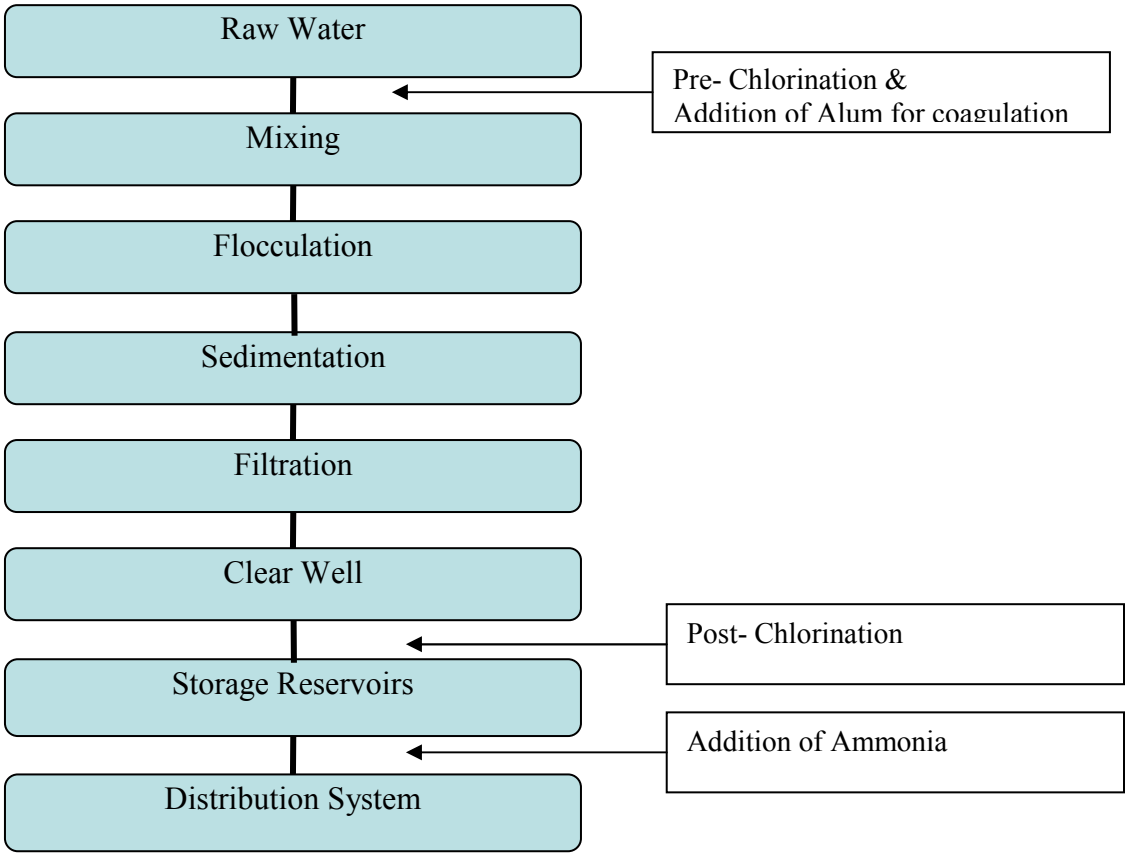

Figure 3. Flow chart showing different steps involved in water treatment process.

Mice were provided with water collected from different points during the treatment process. Treatment groups included:

- River water without any treatment collected right before entering treatment line (autoclaved to kill pathogens)
- River water after chlorinated treatment collected right before entering the distribution (autoclaved)
- Tap water collected post distribution (Chloraminated)
- Tap water collected post distribution (Chloraminated + Autoclaved)
- Well water collected post distribution (Chlorinated) from a separate municipal system in SW Virginia

Animal housing conditions, diet and water were the same as described in study 2a. River water and well water were autoclaved and refrigerated immediately after collection. Mice (n=15 per treatment) were provided with the above water types for 2 months prior to breeding. Pregnant dams were continued on their water treatments during gestation and were euthanized by CO_2 inhalation on GD 9. Embryos were evaluated for the presence of NTDs.

Satistical analysis

One way analysis of variance (ANOVA) (Statistix 8, Analytical Software, Tallahassee, FL) with the mother as the treatment unit was used to determine differences between the treatment groups. When a significant difference was observed ($p<0.05$), a Tukey's means comparison was used to determine differences between treatment groups.

Study 3. Apoptotic changes in embryos from teratogens and maternal immune stimulation

To determine if apoptosis plays a significant role in formation of NTDs from valproic acid (VA) and tap water, apoptotic percentages were quantified by flow cytometry and apoptotic regions were identified by TUNEL assay in GD 9 mouse embryonic heads exposed to teratogens. To determine whether maternal immune stimulation could prevent changes in apoptotic levels, apoptotic regions were assessed in embryos of IFNγ stimulated VA-treated mice.

Animal housing conditions, diet and water were same as described in study 2a. Mice from the various treatment groups were sacrificed on GD 9 and embryos were collected. The amount of apoptosis was determined by flow cytometry (study 3a) and the location of apoptotic pattern was determined by TUNEL assay (study 3b) in embryonic heads.

Valproic acid and maternal immune stimulation

To determine the apoptotic changes from VA and immune stimulant administration, the following 4 treatment groups of CD-1 mice (n=12 litters per group) were used:

- Control (0.1 ml saline by IP injection pre-breeding day -10 & day -5, and on GD 8)
- Valproic acid (0.1 ml saline by IP injection pre-breeding day -10 & day -5, and 500 mg/kg body wt. of VA by IP injection on gestation day (GD) 8)
- Immune stimulation with IFNγ (1000 IU by IP injection on pre-breeding day -10 & day -5, and 0.1 ml saline by IP injection on GD 8)

- Valproic acid + IFNγ (1000 IU of IFNγ by IP injection on pre-breeding day -10 & day -5, and 500 mg/kg body wt. of VA by IP injection on GD 8)

DDI and tap water

Apoptosis in embryos of tap water exposed dams was determined to identify whether apoptosis was involved in the formation of NTDs by the water teratogen.

The following groups were examined (n=12 litters/group)

- Control (F2-DDI mice provided with DDI water only)
- Tap water (F2-DDI mice provided with tap water for 4 weeks prior to breeding)

Stastical analysis

One way analysis of variance (ANOVA) (Statistix 8, Analytical Software, Tallahassee, FL) with the mother as the treatment unit was used to determine differences between the treatment groups. When a significant difference was observed ($p<0.05$), a Tukey's means comparison was used to determine differences between treatment groups.

3 a. Quantitative estimation of apoptotic changes by flow cytometry in the heads of mouse embryos exposed to VA with or without maternal immune stimulation and in embryos exposed to tap water

An assay to quantify apoptotic cells in early embryos was developed. The technique uses differential staining of disassociated embryonic cells to allow identification of apoptotic and dead cells. Apoptotic and dead cells both stain with Annexin V-FITC, since it binds to the exposed phospholipid phosphatidyl-serine of disintegrating cells. Dead cells will only stain with propidium iodide (PI) since this stain enters through leaks in the cell membrane of dead cells to bind DNA. Thus, single staining apoptotic cells can be differentiated from the double staining dead cells.

The percentage of apoptotic cells in the developing heads of GD 9 mouse embryos was quantified in each treatment group using flow cytometry. Embryos were isolated from the decidua and the heads were separated in Dulbecco's Modified Eagle Medium (DMEM). Heads were then incubated for 1 hour at 37^{o}C in 1 ml of Dispase II enzyme (2.4 U/ml) (Roche Diagnostics GmbH, Penzbeg, Germany) to disassociate the cells. After the incubation, cells were further disassociated by gently pressing the tissue with a plunger. Cells in the Dispase II solution were collected in a 5 ml centrifuge tube and centrifuged for 5 min at 1000 x g rpm. The supernatant was poured off, cells were then re-suspended in 1 ml DMEM and aliquoted in to 4 separate tubes (250 ml each). Annexin V-FITC (BD Biosciences Pharmingen, San Jone, CA) and PI (Sigma-Aldrich, St. Louis, MO) dyes were added to the 4 separate tubes and incubated for 5 min in the dark to stain the cells as follows:

- Negative control (No annexin V and no PI)
- Annexin V control (5 µl of Annexin V only)
- PI control (20 µl of PI only)
- Test (5 µl of Annexin V + 20 µl of PI)

The cells were then evaluated by flow cytometry to quantify apoptosis. Flow cytometry acquisition was performed on a BD FACSAria I (BD Bioscience, San Jose, CA, USA) and data were analyzed with FlowJo Software (TreeStar, Ashland, OR, USA). As the cells pass through the laser beam in flow cytomter, light is scattered. Cell size was measured by analyzing the light scattered in a forwarding direction. Granularity was measured by analyzing side scatter. Three different gates were used to isolate singlet cells and exclude aggregate cells. The first gate was drawn on a forward scatter pulse area (FSC-A) vs side scatter pulse area (SSC-A) plot and included all events except debris. The second gate was drawn on a forward scatter pulse width (FSC-W) vs a forward scatter pulse height (FSC-H) plot to exclude most aggregated cells. The third gate was drawn on a side scatter pulse width (SSC-W) vs side scatter pulse height (SSC-H) plot to exclude remaining aggregated cells missed in the second gate. On the final plot of Annexin V-FITC vs PI, a quadrant gate was drawn using the single color controls to determine placement. For each sample, 10,000 singlet cells were counted.

3 b. Localization of specific apoptotic regions in the heads of mouse embryos exposed to VA with or without maternal immune stimulation and in embryos exposed to tap water by Terminal Transferase dUTP Nick End Labeling (TUNEL) assay

Apoptotic cells were localized by visualizing fragmented DNA, one of the hallmarks of apoptosis. Fragmentation of DNA results in free 3'-hydroxyl ends. Terminal deoxynucleotidyl transferase (TdT) is used to add the fluorescein -2'deoxyuridine 5'-tri-phosphate (fluorescein-dUTP) to the free hydroxyl ends of DNA. The incorporated BrdUTP can then be detected by an anti-fluorescein antibody colour conjugate alkaline phosphatase that can be visualized by light microscopy.

Apoptotic cells were localized by TUNEL assay in the heads of mouse embryos from above mentioned treatment groups in study 3a. The assay was performed following the procedure of Kultima et al., (2004) using an In-situ Cell Death Detection Kit (Roche Molecular Biochemicals, Indianapolis, USA). Gestation day 9 embryo heads were fixed in 4% paraformaldehyde in phosphate buffer solution with 0.1% Tween-20 (PBS-T) overnight. Heads were then transferred to 100 % methanol and stored at -20^{o}C until the assay. Embryos were prepared for the assay by placing them in 70% methanol in PBS-T for 10 minutes followed by transfer to 100% PBS-T for an additional 10 minutes before permeabilization in 1% Triton X- 100 in PBS-T for 2 minutes on ice to allow the reagents to reach their target molecules. Embryos were transferred to depression slides and rinsed 3 times using PBS-T and 50 µl of TUNEL reaction mixture containing 10 µl of TdT enzyme solution and 40 µl of labeling solution (dUTP nucleotide mix) was added to the embryo heads in depression slides. The heads were incubated at 37^{o}C in a dark

humidified chamber for 1 hour. After incubation, slides were rinsed 3 times with PBS-T and covered with 50 µl of 3% bovine serum albumin (BSA) in PBS-T to block non-specific activity of endogenous alkaline phosphatase. BSA was washed off and then 50 µl Converter - AP (anti- fluorescein-alkaline phosphatase conjugate) was added and incubated at 37^{o}C in a dark humidified chamber for 30 minutes. The heads were then rinsed 3 times with PBS-T and covered with 50 µl of NBT (nitro blue tetrazolium chloride) /BCIP (5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt) substrate solution (200 µl NBT/BCIP in 10 ml 0.1 M Tris-HCl, pH 9.5 (20°C), 0.1 M NaCl, 0.05 M $MgCl_2$) and incubated in dark at room temperature for 20 minutes. The heads were rinsed 3 times with PBS-T and analyzed for apoptotic cells under light microscopy. Positive controls were prepared by pre-treating embryos with DNase to induce DNA strand brakes that would satin positive in the reaction. Negative controls were prepared by only adding TUNEL labeling solution that contained nucleotides (no TdT enzyme) instead of the standard TUNEL mixture. Negative controls ensured the staining did not result from nonspecific binding or other reagents such as Converter – AP and substrate solution.

Study 4. Molecular regulation of apoptosis by VA with or without maternal immune stimulation and by tap water

Analysis of pathway-focused genes provides insight regarding the overall mechanism of VA and tap water-induced teratogenicity and protection by immune stimulation. In this study, alterations in expression of apoptotic pathway genes were determined in response to VA with or without maternal immune stimulation and tap water. Gene families included in this study were TNF ligands and their receptors, TRAF, death domain and death effector domain family genes, IAPs (inhibitor of apoptosis proteins) family genes, Caspases, Bcl-2 family genes and p53 pathway and other DNA damage induced genes (Appendix 1 for complete list of genes).

4 a. Changes in apoptotic pathway gene expression in GD 8.5 and 9 mouse embryos from VA with and without maternal immune stimulation exposure

The following 4 groups of CD-1 mice were tested:

- Control (0.1 ml saline by IP injection pre-breeding day -10 & day -5 and on GD 8)
- Valproic acid (0.1 ml saline by IP injection pre-breeding day -10 & day -5 and 500 mg/ kg body wt. of VA by IP injection on gestation day (GD 8)
- Immune stimulation with IFNγ (1000 IU of IFNγ by IP injection on pre-breeding day -10 & day -5 and 0.1 ml saline by IP injection on GD 8)
- Valproic acid + IFNγ (1000 IU of IFNγ by IP injection on pre-breeding day -10 & day -5 and 500 mg/kg body wt. of VA by IP injection on GD 8)

Animal housing conditions, diet and water were same as described in study 2a. Mice were euthanized by carbon dioxide and embryonic heads were collected on days 8.5 and 9 of gestation (spanning the time of neural tube closure) from the above 4 groups of mice (n=3 for each treatment). Embryos from each dam were divided into two pools based on whether they had open or closed neural tubes. Embryonic heads were separated (Figure 4) and then were stored in RNAlater until RNA extraction (Figure 5). Pooling embryos belonging to the same litter (keeping embryos with open and closed neural tubes separate) was necessary, since the amount of RNA from one embryo was not sufficient for our RT² Profiler PCR Arrays experiments. RNA was isolated separately from each pool using RNeasy Mini, RNA isolation kit (QIAGEN, Valencia, CA) as described below.

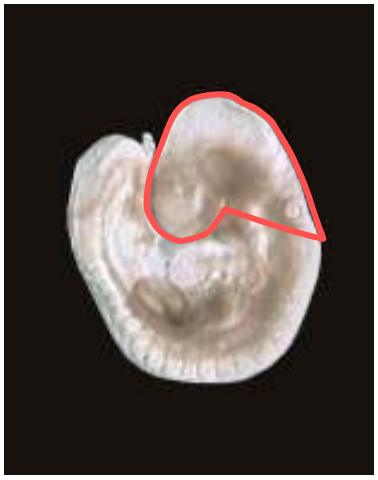

Figure 4. Removal of heads from the GD9 embryos for RNA. Embryonic heads were removed by cutting along the red line.

Isolation of RNA:

RNA was isolated as follows.

1. Pooled embryo heads from each litter in RNAlater were transferred to a collection tube containing 350 µL of lysis buffer (Buffer RLT in β- meracapto ethanol, 1:100).
2. Heads were homogenized immediately using a tissue homogenizer making sure there were no chunks of tissue remaining in solution. The homogenate was transferred to a capped test tube.
3. The homogenate was centrifuged for 4 min at 14,000 g at 20-25^{o}C. (To pellet tissue debris and film).
4. The supernatant was transferred into a new 2 mL microcentrifuge tube and 1 volume equivalent (~ 350 µL) of 70% ethanol was added to the supernatant and mixed well by pipetting.
5. The above supernatant (700 µL), including any precipitate that may have formed, was transferred into an RNeasy Mini Spin Column in a 2 mL collection tube and centrifuged at 10,000 g for 15 sec.
6. The flow-through was discarded and 350 µL Buffer RW1 was pipetted into the column that was then centrifuged at 10,000 g for 15 sec to wash. The flow-through was discarded again.
7. To eliminate the genomic DNA contamination, 80 µL of DNase I solution (10 µL of DNase I stock solution in 70 µL Buffer RDD) was added directly onto the RNeasy silica-gel membrane of the column and incubated at room temp for 15 min.

8. The column was washed with 350 µL Buffer RPE and centrifuged for 15 s at 10,000 g.
9. The flow-through and the collection tube were discarded and the column was transferred into a new 2 mL collection tube.
10. Buffer RPE (500 µL) was transferred onto the column and centrifuged for 15 sec at 10,000 g. The flow-through was discarded.
11. Another 500 µL of Buffer RPE was added onto the column and centrifuged for 2 min at 10,000 g to dry the silica gel membrane.
12. The column was removed from the collection tube without touching the flow-through and was placed in a new 2 mL collection tube. The old tube and flow-through were discarded. The column was then centrifuged at 14,000 g for 2 min.
13. To elute, the column was transferred into a new 1.5 mL collection tube. 15 µL RNase-free water was pipetted directly onto the membrane and centrifuged for 1 min at 10,000 g to elute.
14. Quantity (µg/ml) and quality (260/280 ratio) of RNA sample aliquot were measured using Eppendorf BioPhotometer.
15. The eluted RNA sample was stored at -20^{o}C until cDNA synthesis.

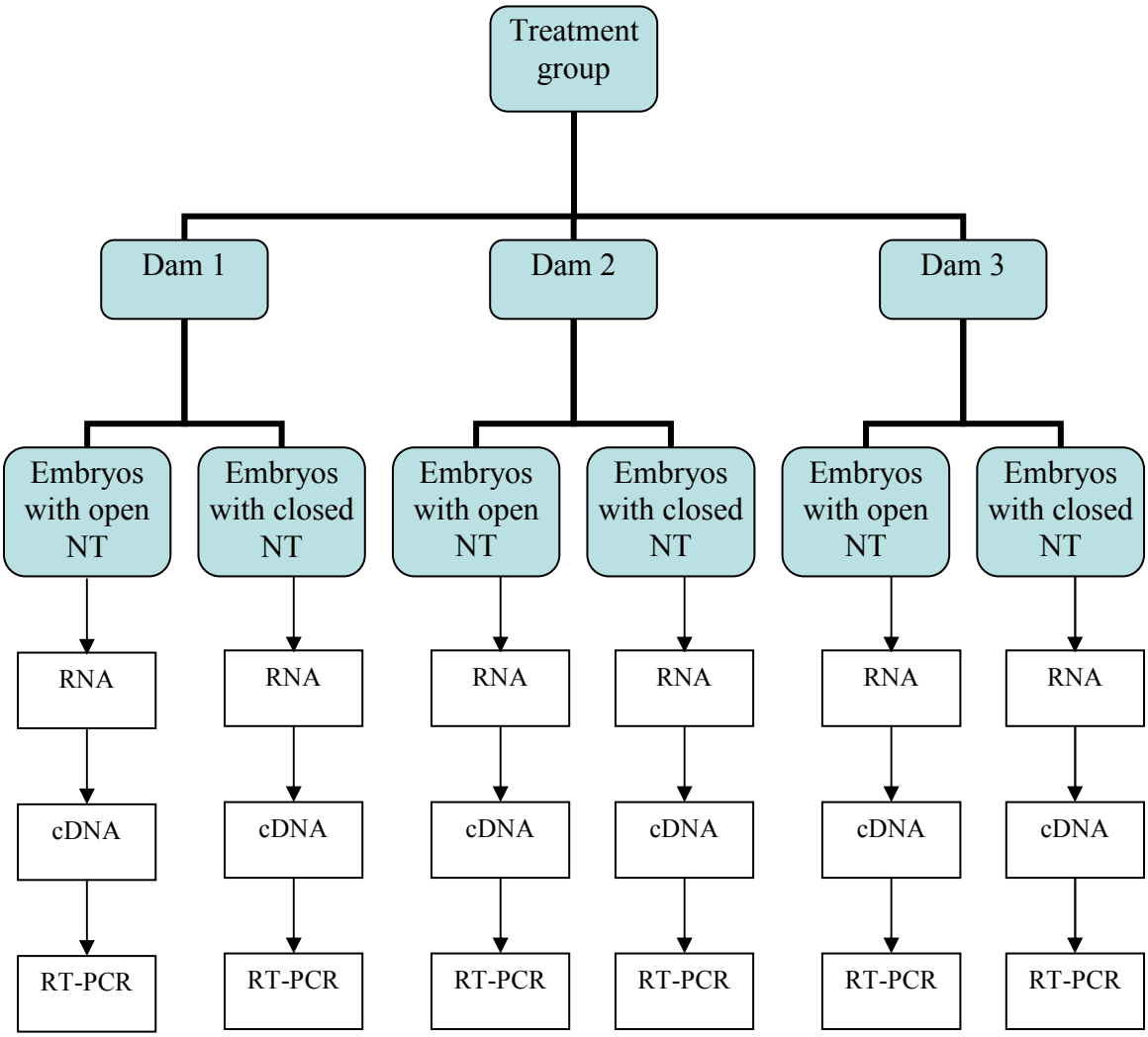

Figure 5. Schematic diagram showing pooling of the embryos, isolation of RNA, cDNA synthesis and Real-Time PCR from each treatment group.

cDNA synthesis:

cDNA was prepared from 1 µg total RNA of each pooled sample by using a RT^2 PCR array first strand kit (C-03; SuperArray Bioscience, Frederick, MD) according to the manufacturer's instructions. Briefly, 1 µg sample of extracted RNA was mixed with 2 µl of super array 5 X genomic DNA elimination mixture to eliminate genomic DNA contamination. RNase-free water was added to makeup the final volume of 10 µl. The

above mixture was incubated at 42^{o}C for 5 min and immediately chilled on ice. To the above mixture, equal amount of RT cocktail (containing 4 µl of 5X super array RT buffer, 1 µl of Primer and External Control Mix, 2 µl of super array RT enzyme mix 3, and 3 µl RNase-free water) was added. The mix was incubated at 42^{o}C for 15 min, and then heated at 95^{o}C for 5 min to inactivate the reverse transcriptase. The mix was then stored at -20^{o}C until analysis.

Real time PCR for gene expression

Real time PCR was performed using mouse apoptosis RT^{2} Profiler PCR array (PAMM-012, Super Array Biosciences Corporation, Frederick, MD). The expressions of 84 genes associated with apoptosis were analyzed according to the manufacturer instructions.

Each PCR array plate layout consisted of 96 wells (8 rows (A-H) X 12 columns (1-12)). Wells A1 through G12 contained a real-time PCR assay for apoptotic pathway genes (Figure 6). Wells H1 through H5 contained a housekeeping gene panel to normalize PCR Array data. The 5 housekeeping genes that were included in this PCR array were Gusb, Hprt1, Hsp90ab1, Gapdh and ß-actin. Well H6 contained the Genomic DNA Control (GDC) to test for genomic DNA contamination in each sample during each run. Wells H7 through H9 contained replicate Reverse Transcription Controls (RTC) to test for impurities in RNA sample that could affect reverse transcription reaction during cDNA synthesis. Wells H10 through H12 contained replicate Positive PCR Controls (PPC) to test for inhibitors of PCR amplification.

A total volume of 25 µl of PCR mixture, which included 12.5 µl of RT^2 Real-Time SYBR Green/ROX PCR master mix from SuperArray Bioscience (containing HotStart DNA polymerase, SYBR Green dye, and the ROX reference dye), 11.5 µl of double-distilled H_2O, and 1 µl of template cDNA, was loaded in each well of the PCR array.

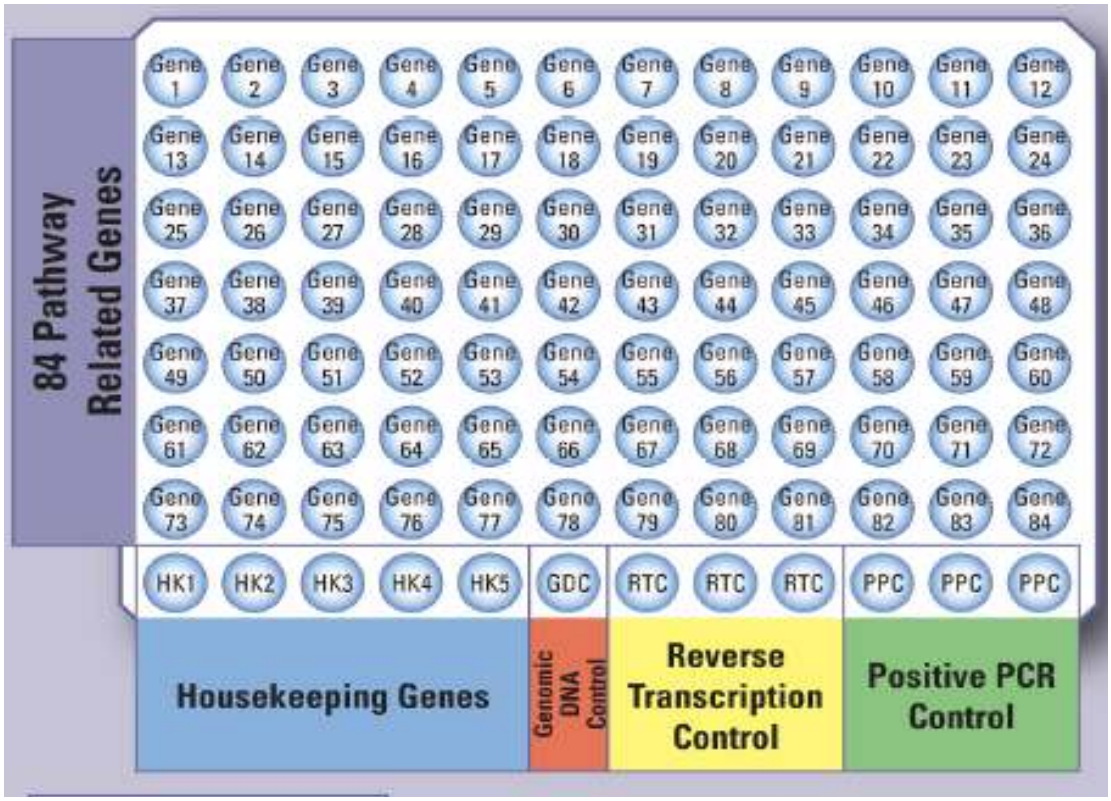

Figure 6. Layout of RT^2 Profiler PCR array. Wells A1 through G12 contain a real-time PCR assay for apoptotic pathway genes. Wells H1 through H5 contain a housekeeping gene panel to normalize PCR Array data. Well H6 contains the Genomic DNA Control (GDC).

PCR amplification was conducted using Bio-Rad iCYCLER iQ5 Real Time PCR instrument with an initial 10 minutes step at 95°C (required to activate the HotStart DNA polymerase) followed by 40 cycles of 95°C for 15 sec and 60°C for 1 minute (denaturation and annealing steps). The fluorescent signal from SYBR Green was

detected immediately after the extension step of each cycle, and the cycle at which the product was first detectable was recorded as the cycle threshold.

Statistical Analysis

Data were imported into an Excel database and analyzed using the comparative cycle threshold (ΔΔ Ct) method with normalization of the raw data to housekeeping genes using software from www.superarray.com website. The ΔΔ Ct was determined as follows: Δ Ct value of each gene on the PCR plate was calculated by subtracting Ct value of the gene of interest (GOI) with average the Ct value of housekeeping genes. To calculate ΔΔ Ct value of each gene of interest, the Δ Ct value of the gene of interest in the treatment group was subtracted from the same GOI of the control group.

Δ Ct of GOI = Ct of GOI- Average Ct of housekeeping genes

ΔΔ Ct = Δ Ct of GOI (treatment group) - Δ Ct (control group)

The fold change for each gene from treatment group to control group was calculated as $2^{-\Delta\Delta Ct}$. Fold change values less than one indicate a down-regulation and values greater than one indicate an up-regulation. The *p* values were calculated based on a Student's *t*-test of the replicate $2^{-\Delta Ct}$ values for each gene in the control group and treatment groups, and statistical significance was set at $p \leq 0.05$. Genes with significant changes more than 2-fold regulation (Fold-change greater than 2 or less than 0.5) were considered to be major contributors.

4 b. Changes in expression of apoptotic pathway genes in GD 9 embryos from tap water exposure

The following groups of F2-DDI mice were tested (n=3 litters for each treatment).

- Tap water (F2-DDI mice provided tap water for 8 weeks prior to breeding). (GD 9 only)
- DDI water (GD 9 only)

Mice were euthanized by carbon dioxide (CO_2) and embryonic heads were collected on 9 of gestation from above groups. Embryos from each litter were divided into open or closed neural tube group and stored in RNAlater (AM 7021, Ambion, Naugatuck, CT) as described for the VA study until RNA extraction. RNA was isolated using RNeasy Mini, RNA isolation kit (Qiagen) as described in study 4a.

Study 5. Molecular regulation of signal transduction pathways by VA with and without maternal immune stimulation and by tap water

The expression pattern of 84 key genes representative of 18 different signal transduction pathways that are important in development and apoptosis was analyzed. The pathways that were analyzed included Wnt pathway, TGF- β pathway, hedgehog pathway, PI3 Kinase / AKT Pathway, Jak/Src pathway, NFκB pathway and retinoic acid pathway (see the Appendix 2 for full list of genes). If any pathways were shown to be involved in VA induced teratogenicity or maternal immune stimulation protective effect, the specific pathway can be analyzed more comprehensively.

5 a. Changes in expression of signal transduction pathway genes in GD 8.5 and 9 mouse embryos from VA with and without maternal immune stimulation exposure

The following 4 groups of CD-1 mice were tested (n=3 litters per group):

- Control (0.1 ml saline by IP injection pre-breeding day -10 & day -5 and on GD 8)
- Valproic acid (0.1 ml saline by IP injection pre-breeding day -10 & day -5 and 500 mg/ kg body wt. of VA by IP injection on gestation day (GD 8)
- Immune stimulation with IFNγ (1000 IU of IFNγ by IP injection on pre-breeding day -10 & day -5 and 0.1 ml saline by IP injection on GD 8)
- Valproic acid + IFNγ (1000 IU of IFNγ by IP injection on pre-breeding day -10 & day -5 and 500 mg/kg body wt. of VA by IP injection on GD 8)

Mice were euthanized by CO_2 and embryonic heads were collected on 8.5 and 9:0 of gestation (spanning the time of neural tube closure) from the above 4 groups of mice.

Embryos from each litter were divided into open or closed neural tube group and were stored in RNAlater (AM 7021, Ambion, Naugatuck, CT) until RNA extraction. RNA was isolated using RNeasy Mini, RNA isolation kit (Qiagen) as described in study 4a. cDNA was prepared from 1 μg total RNA of each pooled sample by using a RT^2 PCR array first strand kit (C-03; SuperArray Bioscience, Frederick, MD) according to the manufacturer's instructions as described in study 4a. The real time PCR was performed using mouse apoptosis RT^2 Profiler PCR array (PAMM- 014, Super Array Biosciences Corporation, Frederick, MD). Real time PCR was performed using Signal Transduction Pathway Finder RT^2 Profiler PCR array (PAMM- 014; Super Array Biosciences Corporation, Frederick, MD) as described in study 4a.

5 b. Changes in expression of signal transduction pathway genes in GD 9 mouse embryos from tap water exposure

The following groups of F2-DDI mice were tested (n=3 litters per group):

- Tap water (F2-DDI mice provided tap water for 8 weeks prior to breeding, GD 9 only.
- DDI water, GD 9 only.

Mice were euthanized by carbon dioxide (CO_2) and embryonic heads were collected on day 9 of gestation from above groups. Embryos from each litter were divided into open or closed neural tube group and stored in RNAlater (AM 7021, Ambion, Naugatuck, CT) as described for the VA study until RNA extraction. RNA isolation, cDNA synthesis and Real Time PCR were performed as described in study 5a.

IV. RESULTS

Study 1. Neural tube defects in mice exposed to tap water

NTDs were observed in control CD-1 mice not exposed to an experimental teratogen. Embryos showed failure of neural fold closure at 3 distinct locations thus resulting in spina bifida, exencephaly, and a split face defect (Figure 7). Split face defects were observed in embryos with unfused neural folds at initiation site 3 (Figure 7D). Unfused neural folds in the midbrain region (initiation site 2) were observed in day 9 embryos (Figure 7 E&F). Some embryos exhibited both split face and unfused neural folds in the mid brain region (Figure 7G). Spina bifidia was also observed in some embryos with unfused neural folds rostral to initiation site 1 (Figure 7 H).

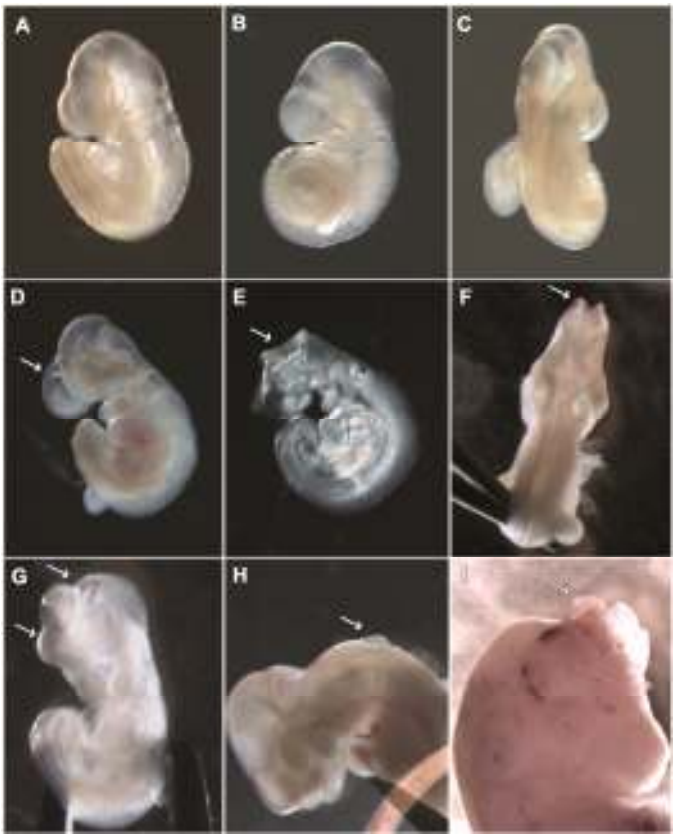

Figure 7. NTDs in late day 9 to day 13 mouse embryos caused by tap water. A-C. Normal day 9 embryos, 3 different views; D. Day 9 embryo with unfused neural folds at initiation site 3 resulting in a split face defect; E-F. Day 9 embryo with unfused neural folds in the midbrain region resulting in classic exencephaly; G. Day 9 embryo with unfused neural folds at both initiation sites 2 and 3 resulting in exencephaly and a split face; H. Day 10 embryo with unfused neural folds rostral to initiation site 1, resulting in spina bifida; and I. Day 13 embryo with classic exencephaly caused by failure of the neural folds to fuse in the midbrain region.

The source of the unknown teratogen that caused NTDs in control mice was identified by testing multiple combinations of housing, mouse strain, diet and water.

1 a. Screening for infectious and toxic exposure

Tests to determine the source of teratogen were conducted including analysis of the bedding and feed. The feed analysis demonstrated the feed to be free of heavy metals, organophosphates, and chlorinated hydrocarbons. The feed also tested negative for aflatoxin and fumonisin, two common mycotoxins in corn-based feeds. Bedding material was free of arsenic, bismuth, cadmium, lead, organophosphate pesticides, organochlorine pesticides, PCBs and other possible contaminants (see Materials and Methods section for detailed list of contaminants). Serological tests to identify possible infectious causes of NTDs in the mice were consistently negative (see Materials and Methods section for detailed list of serological tests). These analyses indicated that a source of NTDs from infectious agent or common toxicants was unlikely.

1 b. Association of housing and NTDs

Purchased mice held under all three housing conditions continued to produce embryos with NTDs (Figure 8). There was no significant difference in NTD rates observed among mice that were raised in conventional open mouse boxes, under sterile conditions in a HEPA filtered laminar flow hood, and in a separate facility under BSL2 containment conditions ($p \leq 0.05$). This indicates that NTDs were not resulting from housing conditions.

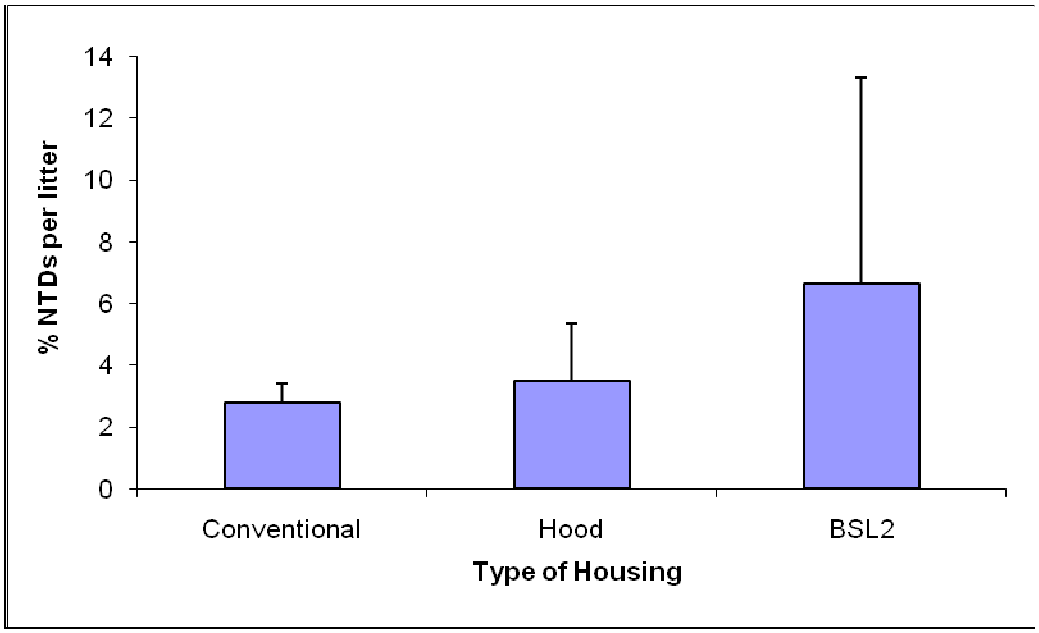

Figure 8. Association of type of housing and NTDs in CD-1 mice. There is no significant difference ($p \leq 0.05$) in NTD rates in mice housed in conventional open mouse boxes, under sterile conditions in a HEPA filtered laminar flow hood, and in a separate facility under BSL2 containment conditions. Values represent the mean percentages ± SE of fetuses affected with NTDs. (n=92 litters for conventional; n=9 litters for hood; n = 3 litters for BSL2).

1 c. NTDs in timed pregnant mice from commercial suppliers

To further test whether the NTDs resulted from unknown infectious or genetic causes localized to our facility, timed pregnant mice of different strains were shipped from suppliers on GD 10-11 after the time of neural tube closure. Embryos of timed pregnant mice were examined for NTDs within 1 hour upon arrival. Embryos from all 3 strains and from all suppliers exhibited a similar percentages of NTDs (Figure 9) with no significant differences (p=0.13) between strain or supplier. This indicates that NTDs were not just localized to a single strain or facility making an infectious or genetic cause unlikely.

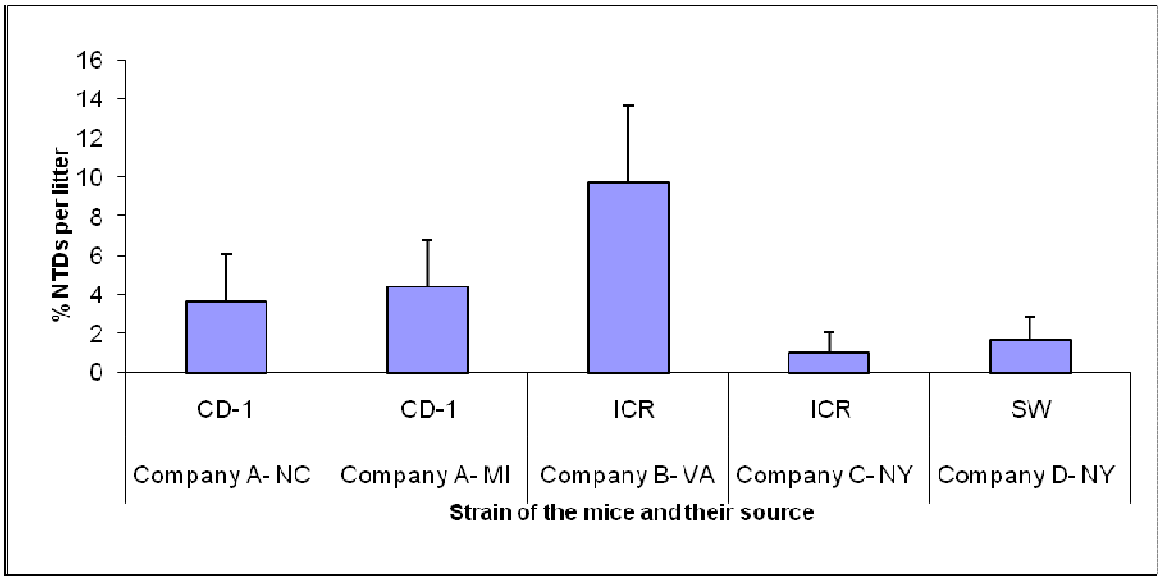

Figure 9. NTDs in timed pregnant mice from 3 different commercial suppliers in 4 different geographical locations. Values represent the mean percentages ± SE of fetuses affected with NTDs from 8-9 litters per group. No statistical significance difference is noted between the groups ($p \leq 0.05$).

1 d. Association of diet and NTDs

The percentage of NTD formation was compared in fetuses of newly purchased dams provided 4 different commercial rodent diets for 2 weeks prior to breeding. There was no significant effect of diet on the percentage of NTDs (p=0.7) (Figure 10).

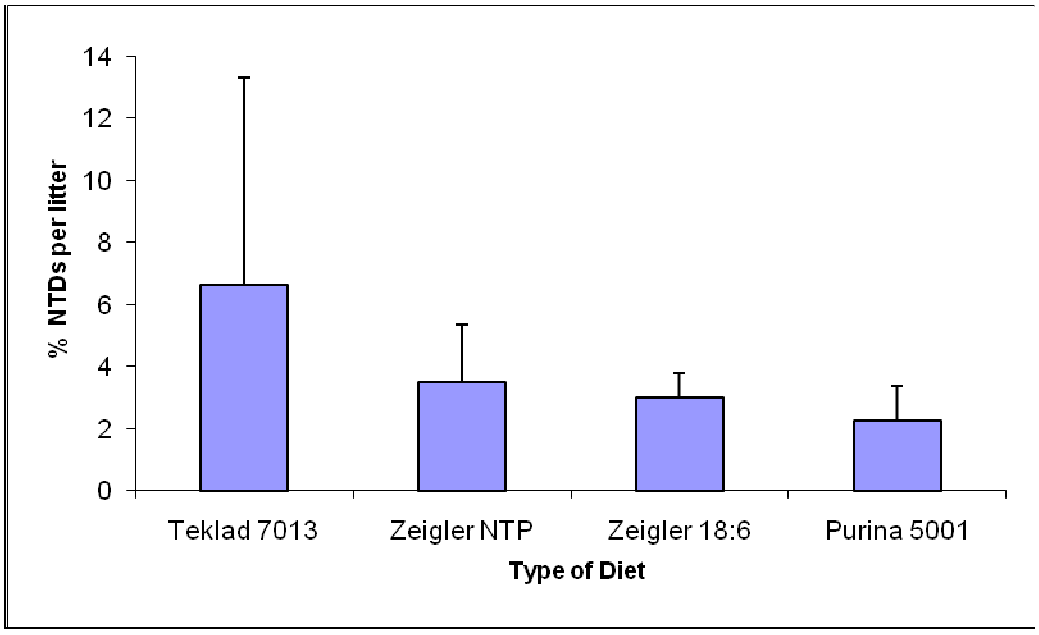

Figure 10. Association of diet and NTDs in CD-1 mice. There is no significant difference ($p \leq 0.05$) in NTD rates in mice fed Teklad 7013 diet, Ziegler diet NTP-2000, Zeigler 18-6 diet or Purina 5001. Values represent the mean percentages ± SE of fetuses affected with NTDs. (n=3 litters for Teklad 7013; n = 9 litters for Zeigler NTP-2000; n=61 litters for Zeigler 18:6; n=31 litters for Purina 5001).

1 e. Association of source of water and NTDs

NTD rates were compared in embryos from newly purchased mice provided 3 different sources of water for 2 weeks prior to breeding: tap water (location 1), DDI and HPLC water. Embryos from mice provided DDI (p=0.005) or HPLC water (p=0.001) had significantly lower percentage of NTDs compared to tap water (Figure 11) that indicated water as the source of the teratogen.

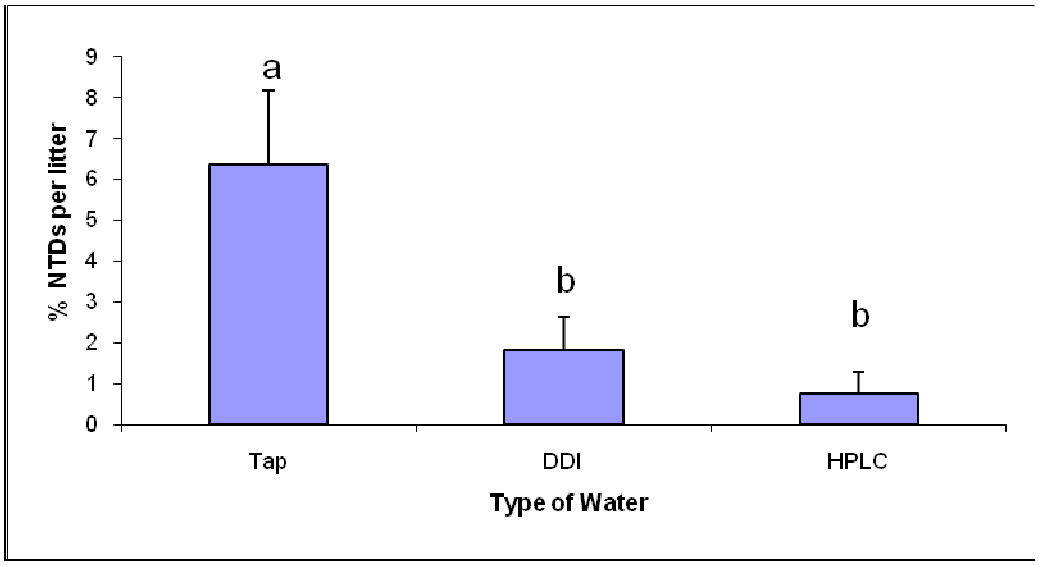

Figure 11. Association of type of water and neural tube defects in purchased CD-1 mice. NTD rates are significantly decreased in mice provided DDI or HPLC water for 2 weeks prior to breeding compared to tap water. Values represent the mean percentages ± SE of fetuses affected with NTDs. (n = 25 litters for Tap; n=30 litters for DDI; n=26 litters for HPLC water). Statistical significance is set at $p \leq 0.05$. Different litters indicate treatments are significantly different.

1 f. F2 Generation: Association of water and NTDs

Initial results indicated that tap water may be responsible for the NTDs in control mice. The NTDs were only reduced, not eliminated, with DDI or HPLC water, however. We hypothesized that a residual effect of teratogen, possibly due to a long half-life, may be responsible for the low levels of NTDs in purchased mice when provided DDI or HPLC water in our facility. To eliminate the possibility that purchased mice may have received prior teratogen exposure, mice were provided DDI for two generations. When F2-DDI mice (provided only DDI water) were bred, NTDs were completely eliminated in the offspring (Figure 12).

NTDs were not observed when F2-DDI mice were transferred to HPLC water for 2 weeks prior to breeding. When F2-DDI mice were provided tap water from Location 1, Location 2, or Location 3 for 2 weeks prior to breeding, NTDs were observed in all groups of tap water exposed mice (Figure 12). The comparisons, however, did not reach statistical significance compared to DDI water (*p*-values: 0.18, 0.13, 0.06 for Locations 1, 2, 3 respectively). When the tap water exposure length was increased to 8 weeks prior to breeding, all three groups of mice had significantly higher rates of NTDs than age matched F2-DDI controls (*p*-values: 0.003, 0.005, 0.007 for Locations 1, 2, 3 respectively). NTDs were completely eliminated in F2-DDI mice and reappeared when F2-DDI mice transferred to tap water confirming that the unknown teratogen was present tap water. Longer periods of exposure to tap water from all municipalities revealed a dose response effect. The same relative percentage of NTDs between the three locations was maintained at both exposure times.

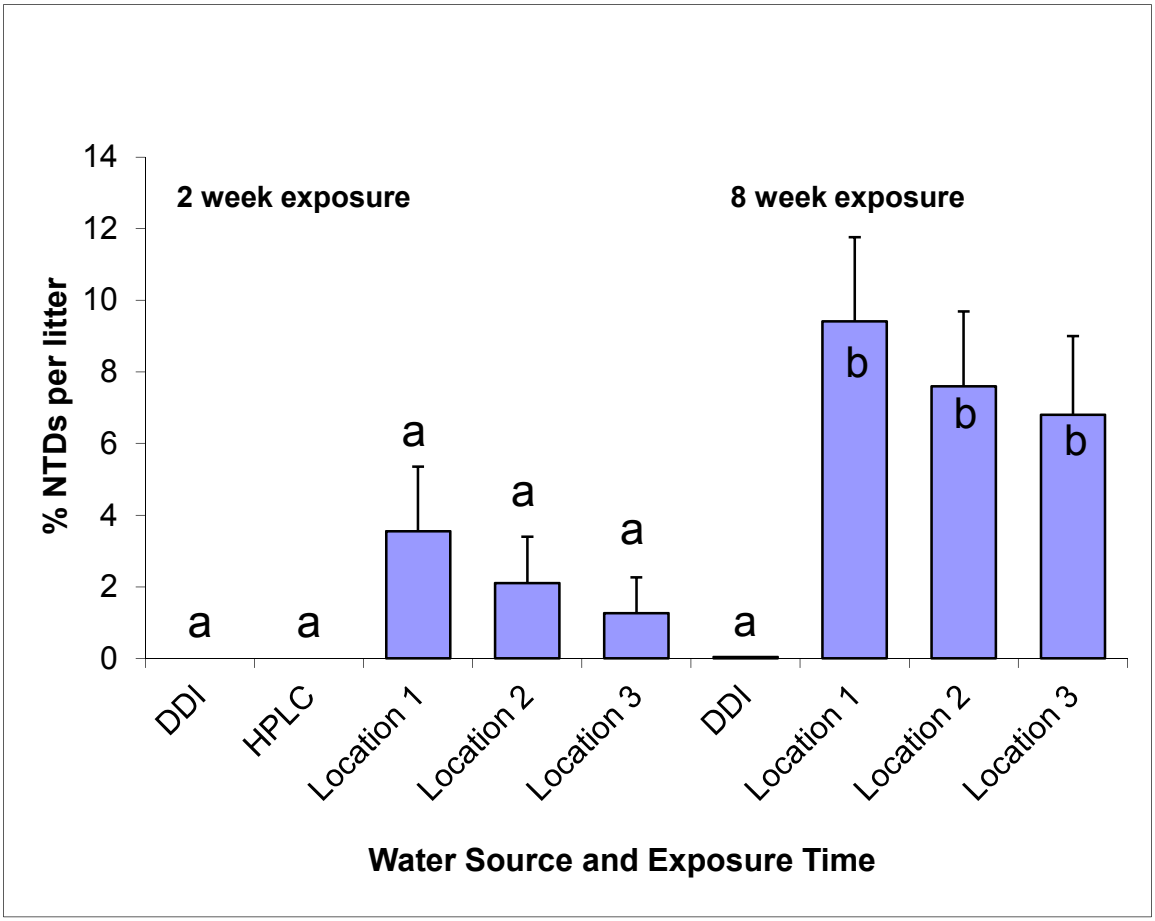

Figure 12. NTDs in offspring of dams maintained exclusively on DDI for 2 generations (F2-DDI) and switched to municipal tap water 2 or 8 weeks prior to breeding. Mice exposed to tap water for 8 weeks had higher percentages of NTDs in their embryos than mice exposed to DDI and tap water for 2 weeks. Values represent the mean percentages ± SE of fetuses affected with neural tube defects. n= 7-9 litters per group in the 2 week exposure, and 14-15 litters per group in the 8 week exposure. Statistical significance is set at $p \leq 0.05$. Different letters indicate statistically different groups.

Study 2. Characterization of teratogen present in tap water

The teratogen in the tap water was characterized in order to narrow down possible contaminants and help identify the teratogen by analytical methods. The study had three components. The first was to determine whether the teratogen was volatile or non-volatile. The second was to determine if the teratogen acted directly or was metabolized. The third was to determine whether the teratogen was introduced into tap water during the water treatment process.

2 a. Chemical characterization of teratogen present in tap water

Water was prepared to separate volatile and non-volatile compounds. Embryos from mice provided the volatile fraction of tap water had higher levels of NTDs (2.87±1.2 %) than the non-volatile fraction of tap water (0.67±0.6 %). Embryos from mice provided with either volatile or non-volatile fractions of DDI did not develop NTDs (Figure 13). These results indicated that the teratogen mainly partitioned into the volatile fraction.

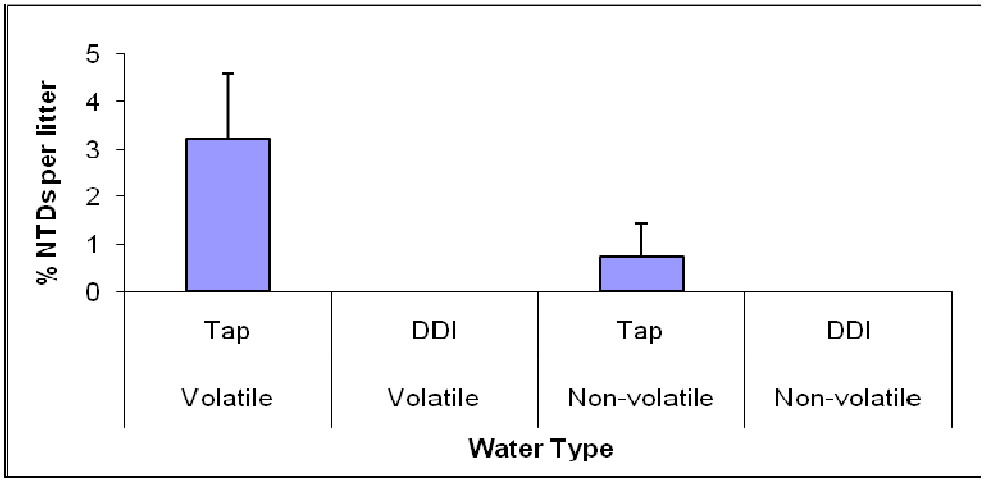

Figure 13. NTDs in embryos from mice provided volatile or non-volatile fractions of tap and DDI water. Values represent the mean percentages ± SE of fetuses affected with NTDs (n=11 litters for volatile fraction of tap water; n= 9 litters for volatile fraction of DDI water; n=10 litters for non-volatile fraction of tap water; n= 10 litters for non-volatile fraction of DDI water). No statistical significance difference is noted between the groups ($p \leq 0.05$).

2 b. Metabolism study to determine whether the teratogenicity is caused by a parent compound or its metabolite

Tap water exposed embryos treated with SKF-525A (cytochrome p450 inhibitor) exhibited NTDs at levels similar to tap water exposed embryos from saline injected mice (Figure 14). NTDs were also exhibited in embryos from both tap water and DDI exposed mice treated with phenobarbital (cytochrome p450 inducer) (Figure 14).

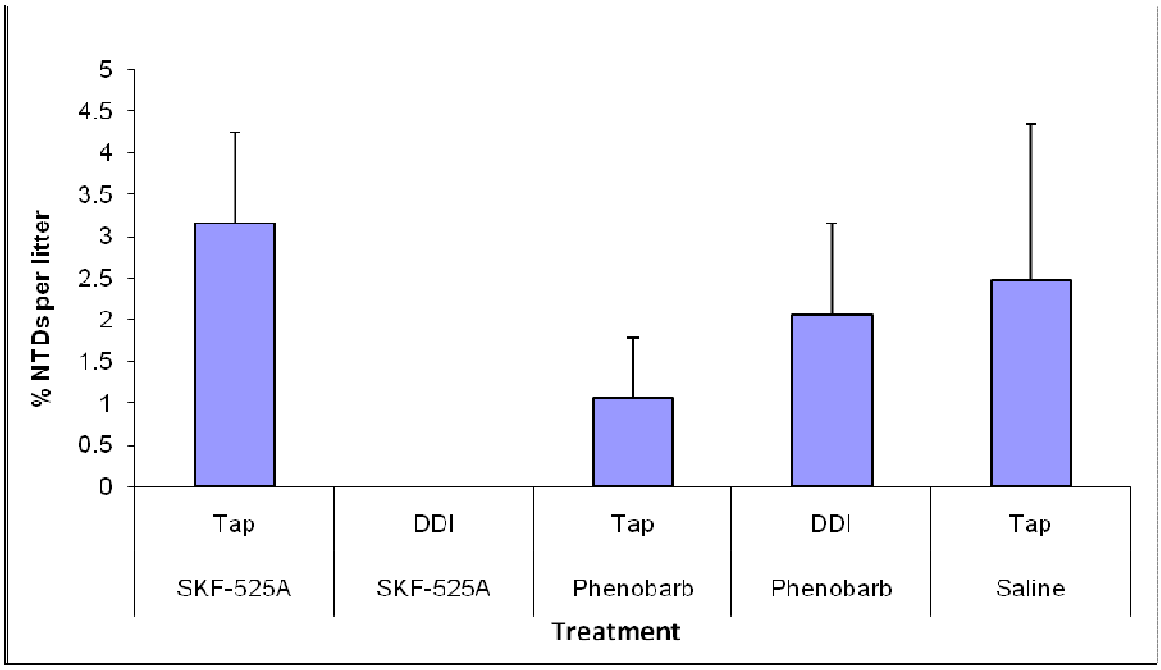

Figure 14. NTDs in embryos from mice injected with SKF-525A, phenobarbital or saline. Mice were provided either with tap water or DDI for 1 month prior to breeding. (n=15 litters for mice provided with tap water and injected with SKF-525A; n=11 litters for mice provided with DDI and injected with SKF 525-A; n=12 litters for mice provided with tap water and injected with phenobarbital; n=12 litters for mice provided with DDI and injected with phenobarbital; n=11 litters for mice provided with tap water and injected with saline). No statistical significance difference is noted between the groups ($p \leq 0.05$).

2 c. Determining if the teratogen is a disinfection byproduct

NTDs were not observed in embryos from mice provided DDI or well water treated with chlorine (Figure 15). NTDs were observed, and were significantly increased in mice provided any of the surface water types (autoclaved river water, autoclaved chlorinated water, chloraminated tap water, autoclaved chloraminated tap water) (Figure 15). The results indicated that was teratogen causing NTDs was not introduced into the water during the treatment process.

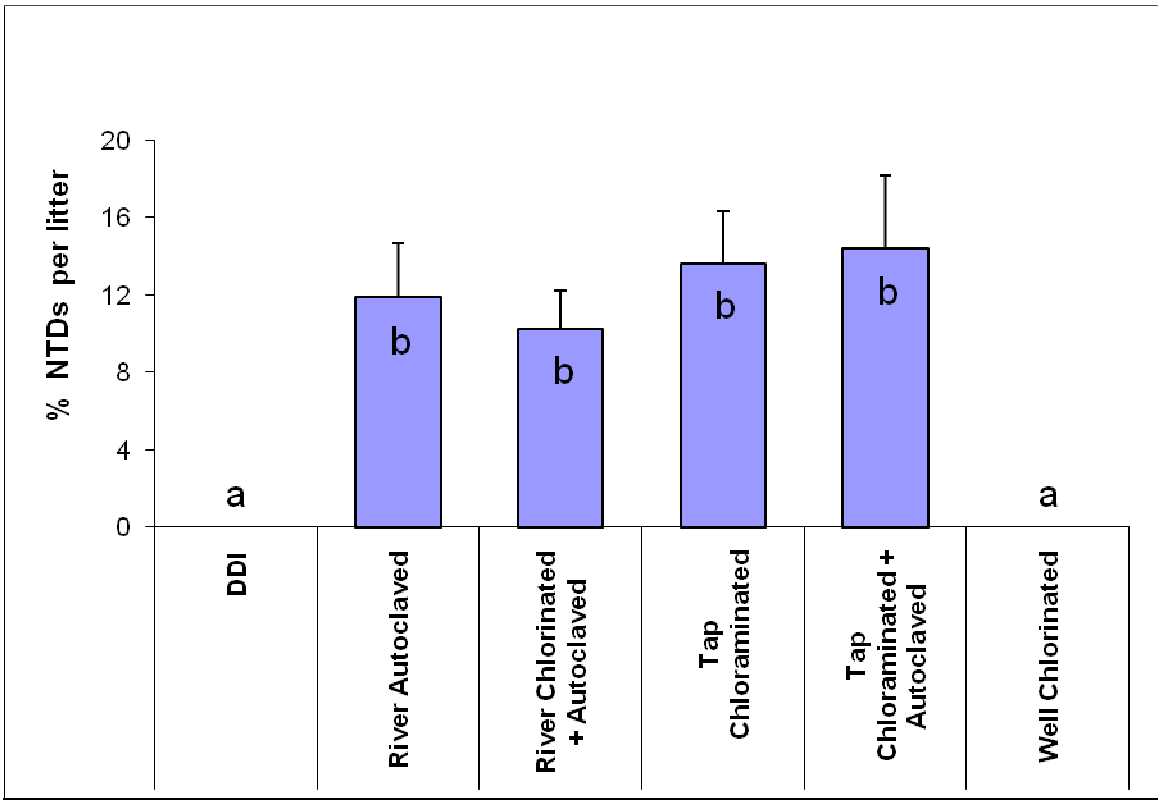

Figure 15. NTDs in embryos from F2-DDI mice provided with DDI water, autoclaved river water, autoclaved chlorinated water, chloraminated tap water, or chlorinated well water for 8 weeks prior to breeding. Statistical significance is set at $p \leq 0.05$. Different letters indicate groups are significantly different.

Study 3. Apoptotic changes in embryos from teratogens and maternal immune stimulation

3 a. Quantitative estimation of apoptotic changes by flow cytometry in the heads of mouse embryos exposed to VA with and without maternal immune stimulation and in embryos exposed to tap water

To determine if apoptosis played a significant role in formation of NTDs by VA or tap water, and also to determine whether maternal immune stimulation could prevent VA induced changes in apoptotic levels, flow cytometry and TUNEL assay were used to assess apoptotic changes in GD9 embryos.

Valproic acid and maternal immune stimulation

The percentage of apoptotic cells was higher in embryos with open neural tubes from VA-exposed mice compared to controls (saline injected). A significant increase in apoptosis was also observed in embryos with closed neural tubes from VA-exposed dams compared to controls (Figures 16 & 17). Therefore, VA caused apoptosis in heads of embryos with both open and closed neural tubes.

Embryos from mice treated with IFNγ+VA had lower levels of NTDs (21.2±4 %) than the embryos from the mice treated with VA (45.3±10.4 %). There was no significant difference in percentage of apoptotic cells between embryos from IFNγ and saline control groups, which indicated IFNγ did not affect apoptosis. There was no significant difference in the percentage of apoptotic cells between the IFNγ+VA-treated embryos with closed neural tubes compared to controls. Apoptosis was significantly increased in IFNγ+VA treated embryos with open neural tubes compared to controls (Figure 16 &

17). Maternal immune stimulation with IFNγ was able to prevent apoptosis in embryos with closed neural tubes but not in embryos with open neural tubes.

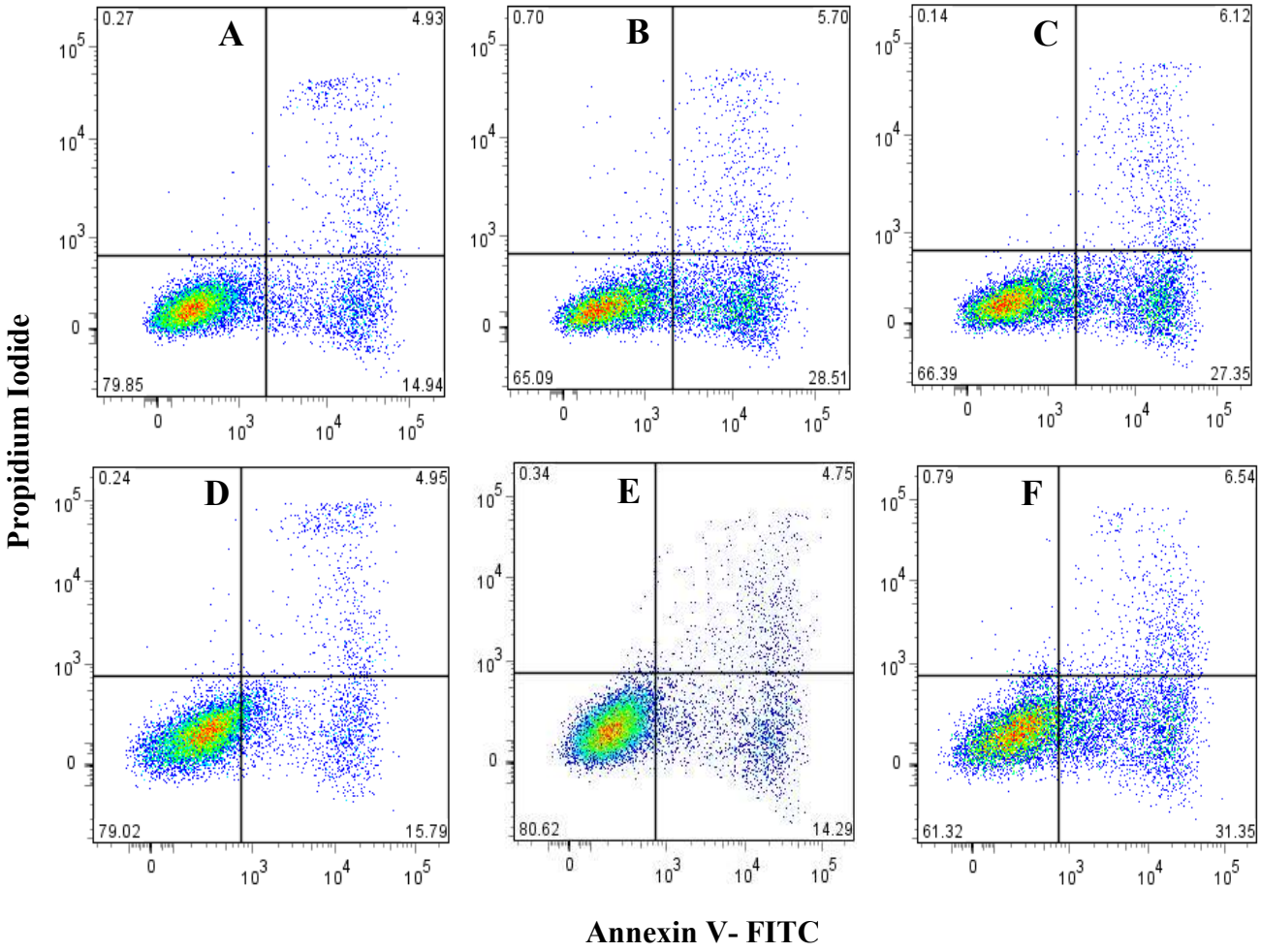

Figure 16. Apoptotic changes in the heads of GD 9 embryos exposed to VA with and without maternal immune stimulation. Histograms are from representatives of A: Embryos from control dams; B: Embryos with closed neural tubes from VA-exposed dams; C: Embryos with open neural tubes from VA-exposed dams; D: Embryos from IFNγ dams; E: Embryos with closed neural tubes from IFNγ +VA-exposed dams; F: Embryos with open neural tubes from IFNγ +VA-exposed dams. The X-axis represents Annexin-V FITC staining intensity and Y-axis represents Propidium Iodide (PI) staining intensity. In each histogram, lower left quadrant shows viable cells (stained negative for Annexin-V or PI), lower right quadrant shows apoptotic cells (stained positive for Annexin-V only) and upper right quadrant shows dead cells (stained positive for both Annexin-V & PI). Increased apoptotic cells can be observed in (lower right quadrant) embryos from VA-exposed dams (open and closed neural tubes) and embryos with open neural tubes from IFNγ +VA-exposed dams compared to embryos from control, IFNγ -exposed dams, and also embryos with closed neural tubes from IFNγ +VA-exposed dams.

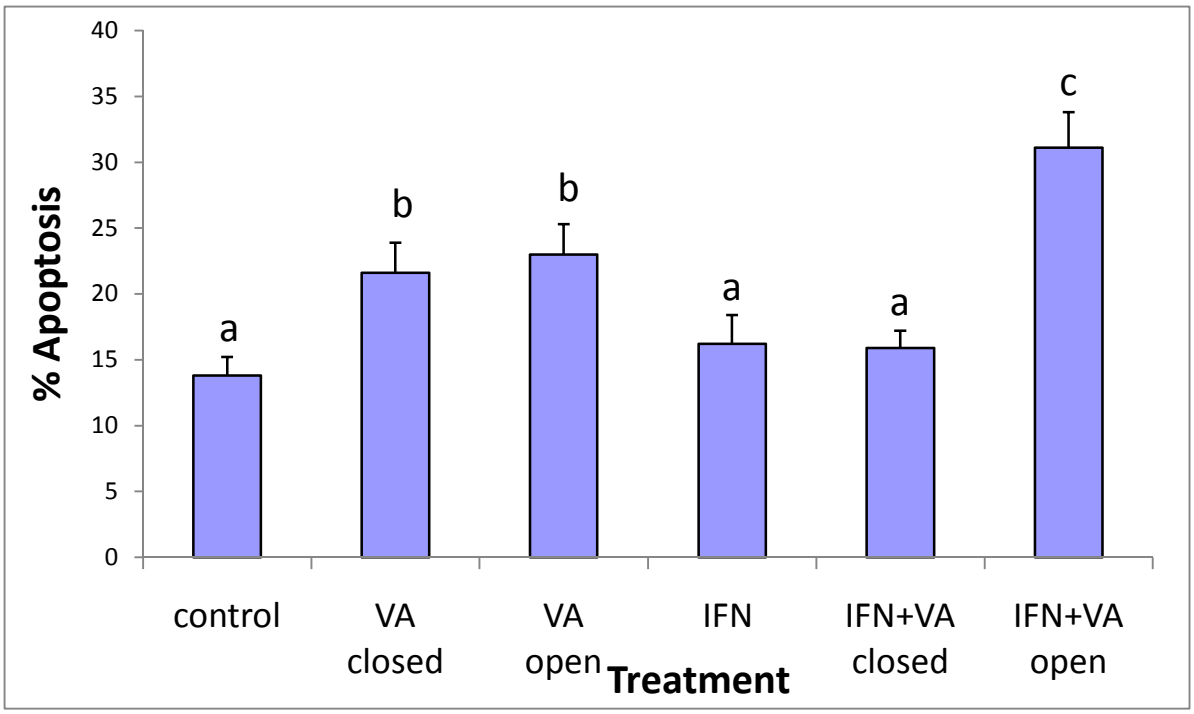

Figure 17. Percentage of apoptotic cells in embryo heads (GD 9) from control (n=10 litters), VA (n=10 litters), IFNγ (n=8 litters) and VA+ IFNγ (n=8 litters) exposed dams. Embryos from VA and VA+ IFNγ treatment groups are separated based on the open and closed neural tubes. Statistical significance is set at $p \leq 0.05$. Different letters indicate groups are significantly different.

DDI and tap water

The percentage of apoptotic cells in embryonic heads were compared between F2-DDI mice and F2-DDI mice provided tap water for 2 weeks. There was no significant difference in apoptotic percentages between embryos exposed to DDI or tap water (Figure 18). Apoptosis was not involved in teratogenicity observed in tap water-exposed mice.

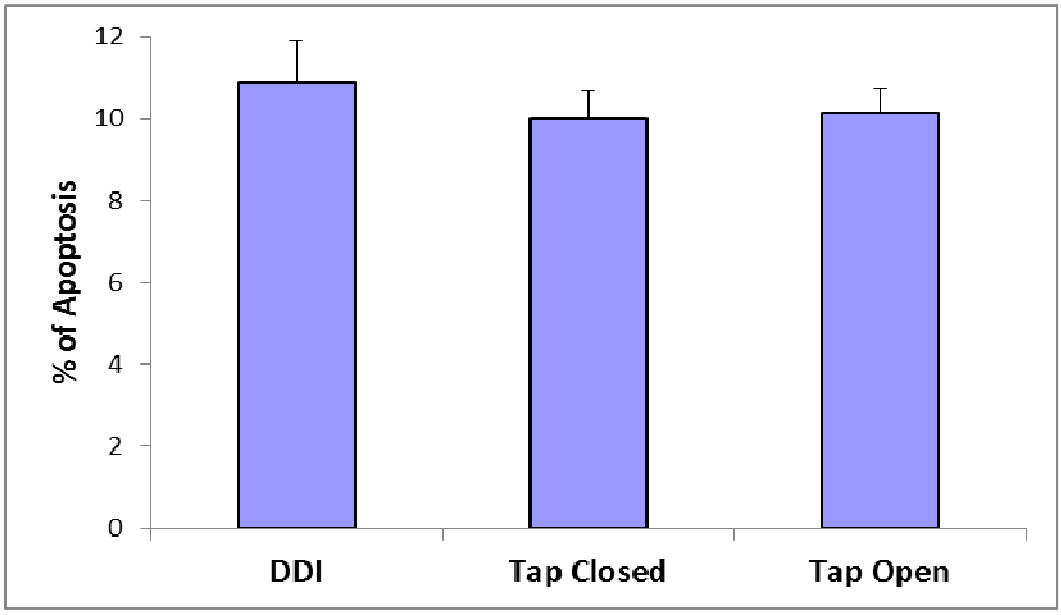

Figure 18. Percentage of apoptotic cells in embryo heads (GD 9) from DDI water (n=10 litters) and Tap water (n= 10) exposed embryos. Embryos from tap water exposure are separated based on the open and closed neural tubes. No statistical significance difference is noted between the groups ($p \leq 0.05$).

3 b. Localization of specific apoptotic regions in the heads of mouse embryos exposed to VA with or without maternal immune stimulation and in embryos exposed to tap water by Terminal Transferase dUTP Nick End Labeling (TUNEL) assay

Valproic acid and maternal immune stimulation

Embryos from saline injected control mice exhibited low amounts of apoptotic staining along the midline of the developing neural tube. Apoptotic staining was observed along the open neural folds of the embryos from VA-treated dams. The increased apoptosis observed in these embryos by flow cytometry was likely localized to the midline of the neural folds. Even though increased apoptosis was observed along the neural folds in VA-exposed embryos, it was more pronounced in midbrain/hind brain region (indicated by arrows in Figure 19). Embryos with closed neural tubes from the VA-treated dams also exhibited apoptosis along the neural folds, but to a lesser degree than in embryos with open neural tubes.

Embryos from dams treated with IFNγ only exhibited a low amount of apoptosis along the neural folds. Similar to embryos with open neural tubes from VA-exposed dams, embryos with open neural tubes from IFNγ +VA-treated dams exhibited increased apoptosis particularly in hind and mid brain regions along the neural folds (indicated by arrows in Figure 20). Embryos with closed neural tubes from IFNγ +VA dams showed low to moderate apoptosis (Figure 20).

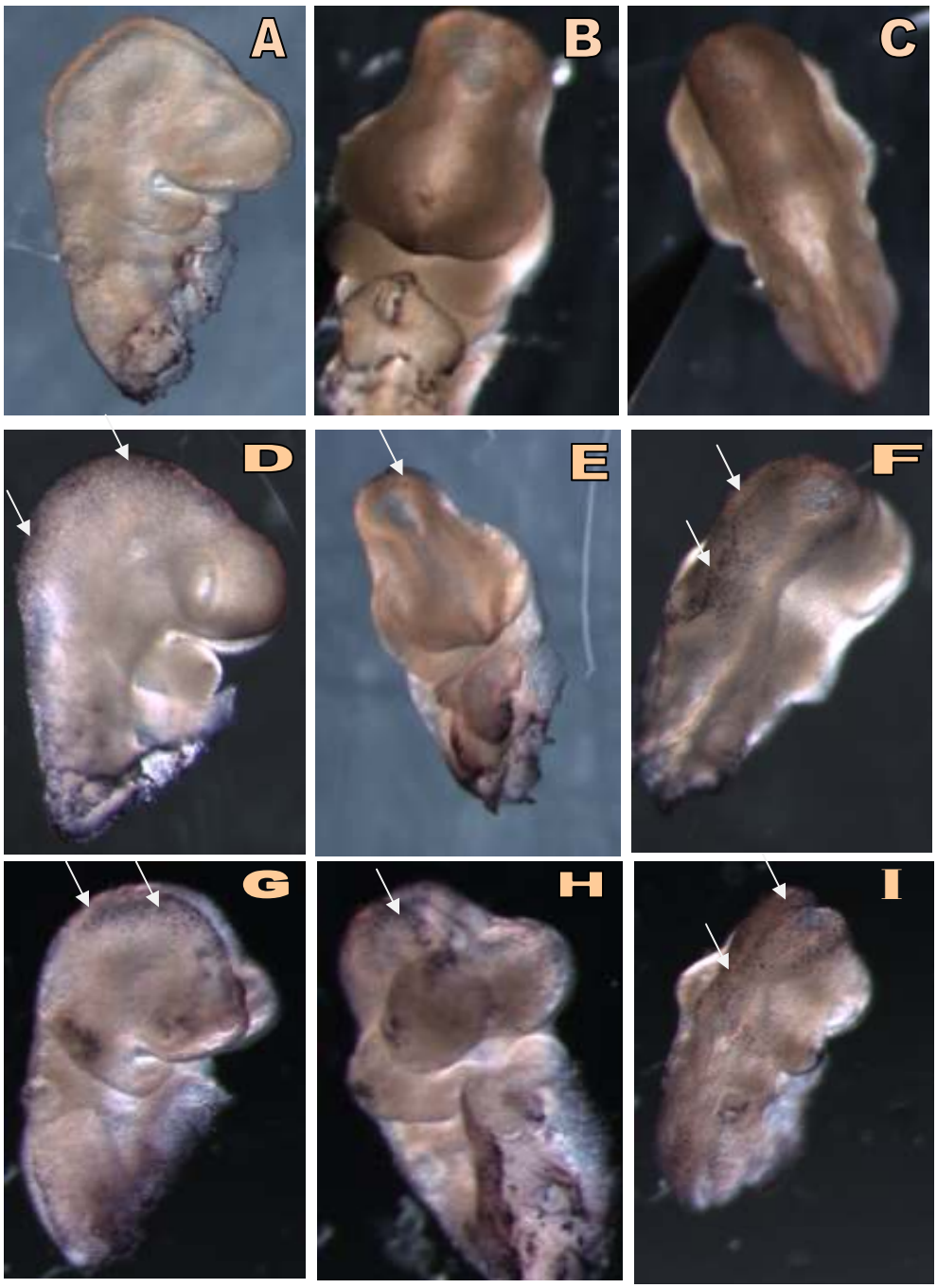

Figure 19. Identification of apoptotic regions in the heads of GD9 mouse embryos from VA-exposed dams by Terminal Transferase dUTP Nick End Labeling (TUNEL) assay in different views. A-C: Embryos from control group; D-F: Embryos with closed neural tubes from VA group; G-I: Embryos with open neural tubes from VA group. Purple stained apoptotic regions are identified by yellow arrows.

Figure 20. Identification of apoptotic regions in the heads of GD9 mouse embryos from IFNγ +VA group by Terminal Transferase dUTP Nick End Labeling (TUNEL) assay in different views. J-L: Embryos from IFNγ group; M-O: Embryos with closed neural tubes from IFNγ +VA dams; P-R: Embryos with open neural tubes from IFNγ +VA dams. Purple stained apoptotic regions are identified by white arrows.

DDI and tap water

Embryos from F2-DDI mice provided DDI showed low amounts of apoptotic staining along the midline of the neural tube. Similar to embryos from F2-DDI mice, only light apoptotic staining was observed in tap water-exposed embryos with NTDs (Figure 21). These results supported the flow cytometry results, which indicated tap water exposure did not increase apoptosis in GD 9 embryonic heads.

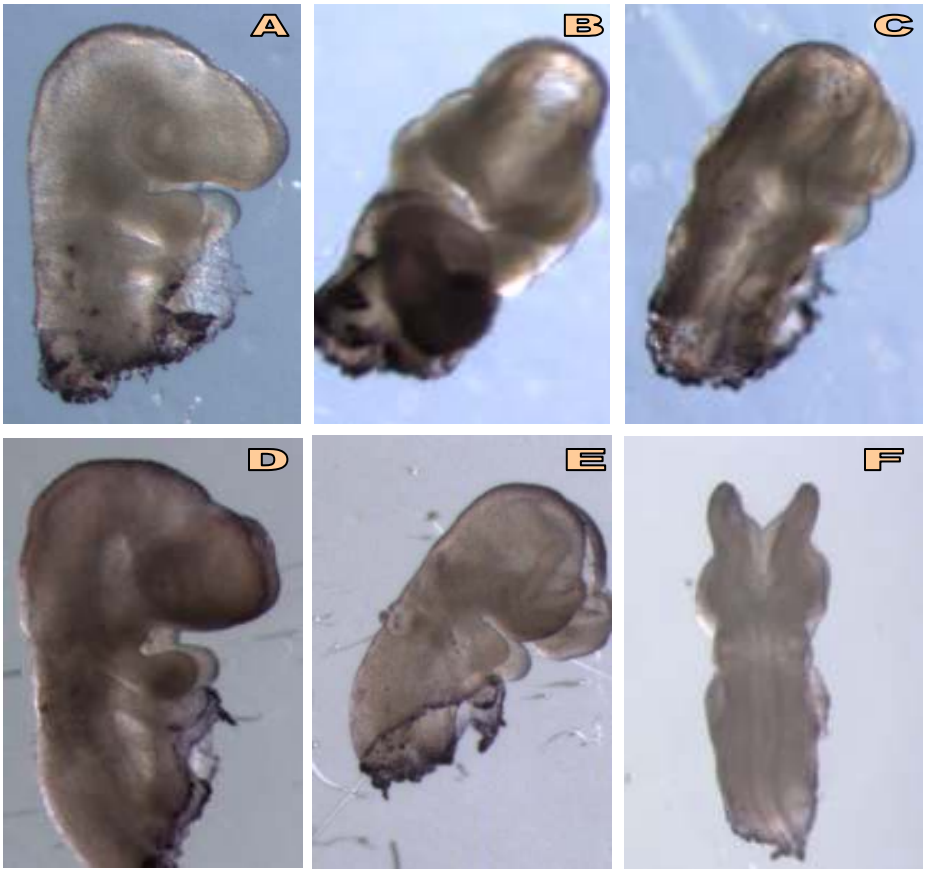

Figure 21. Identification of apoptotic regions in the heads of GD9 mouse embryos from tap water-exposed dams by Terminal Transferase dUTP Nick End Labeling (TUNEL) assay in different views. A-C: Embryos from F2-DDI mice provided with DDI; D-F: Embryos with open neural tubes from F2-DDI dams provided with tap water for 4 weeks prior breeding. Apoptotic regions (purple staining) are not observed in embryos from either group.

Study 4. Molecular regulation of apoptosis by VA with and without maternal immune stimulation and tap water

Changes in the relative expression of apoptotic pathway genes were determined in embryonic heads from dams treated by VA with and without immune stimulation and by tap water. This study was conducted to evaluate the molecular mechanisms responsible for alteration of apoptosis.

4 a. Changes in expression of apoptotic pathway genes in GD 8.5 and 9 embryos from VA with and without maternal immune stimulation exposure

Changes in gene expression of GD 8.5 and GD 9 embryos from VA-treated mice were compared to embryos from mice injected with saline. Changes in gene expression of GD 8.5 and GD 9 embryos from VA+ IFNγ-treated mice were compared to embryos from mice injected with IFNγ only. In our study, genes with significant changes more than 2-fold regulation (fold-change greater than 2 or less than 0.5) were considered as major contributors. Complete lists of results are presented in Appendices 3-9.

Gestation Day 8.5

Apoptosis inducing gene, *Cideb*, was the only gene significantly down regulated in embryos exposed to VA compared to saline injected controls. In VA+IFNγ- treated embryos, pro-apoptotic genes, *Nod1* and *Trp73* were significantly down regulated compared to embryos treated with IFNγ only (Table 1).

Table 1. Fold change and *p*-values of genes significantly altered in GD8.5 embryos from IFNγ +VA group compared to IFNγ only

Gene	Family	Function	Fold change	*p*-value
Nod1	CARD	Pro-apoptotic	0.25	0.002
Trp73	p53	Pro-apoptotic	0.16	0.005

Gestation day 9

Changes in gene expression of GD 9 embryos with open and closed neural tubes from VA-treated mice were compared to embryos from mice injected with saline. In GD9 embryos with open neural tube from VA-treated dams, apoptotic genes *Bad, Bak1, Bok, Casp9* and *Cideb*, and anti-apoptotic genes *Mcl1* and *Prdx2* were significantly up regulated (Table 2) compared to embryos from control mice (saline injected). Apoptotic genes *Apaf1, Casp8, Cradd, Dffa, Fadd, Ripk1* and *Traf2*, and anti-apoptotic genes *Akt1, Bcl2l1, Cflar, Dad1, Cd40*, were significantly down regulated (Table 2). These results indicate overall gene expression favored pro-apoptotic processes in GD 9 embryos with open neural tubes from VA-treated mice (Figure 22).

Table 2. Fold change and *p*-values of genes significantly altered ($p \leq 0.05$) in GD9 embryos with open neural tubes from VA-treated dams compared to control group.

Gene	Family	Function	Fold Regulation	*p*-value
Bak1	Bcl-2	Pro-apoptotic	213.7	0.008
Cideb	CIDE domain	Pro-apoptotic	14.32	0.000
Bad	Bcl-2	Pro-apoptotic	3.92	0.028
Casp9	p53	Pro-apoptotic	3.84	0.020
Bok	Bcl-2	Pro-apoptotic	2.36	0.000
Fadd	Death Effector Domain	Pro-apoptotic	0.46	0.000
Dffa	CIDE domain	Pro-apoptotic	0.39	0.006
Ripk1	CARD/Death domain	Pro-apoptotic	0.37	0.000
Cradd	CARD	Pro-apoptotic	0.34	0.019
Apaf1	p53	Pro-apoptotic	0.29	0.042
Traf2	TRAF	Pro-apoptotic	0.05	0.001
Casp8	Death Effector Domain	Pro-apoptotic	0.03	0.01
Prdx2	Anti-oxidant	Anti-apoptotic	77.35	0.000
Mcl1	Bcl-2	Anti-apoptotic	64.29	0.005
Akt1	p53	Anti-apoptotic	0.46	0.003
Cd40	TNF	Anti-apoptotic	0.29	0. 032
Cflar	Death Effector Domain	Anti-apoptotic	0.23	0.005
Bcl2l1	Bcl-2	Anti-apoptotic	0.15	0.000
Dad1		Anti-apoptotic	0.002	0.000

In GD9 embryos with closed neural tube from VA- treated dams, apoptotic genes *Bad*, *Bak1*, *Bok*, *Casp9* and *Cideb*, and anti-apoptotic genes *Mcl1* and *Prdx2*, were significantly up regulated compared to embryos from control (saline injected) mice. Apoptotic genes *Apaf1*, *Casp8*, *Cradd*, *Dffa*, *Fadd*, *Ripk1* and *Traf2*, and anti-apoptotic

genes *Akt1*, *Bcl2l1*, *Cflar*, *Dad1*, *Cd40*, were significantly down regulated (Table 3). The results revealed that both pro and anti-apoptotic gene expression levels in GD 9 embryos with closed neural tubes changed, but overall expression tended towards a pro-apoptotic pattern (Figure 23).

Table 3. Fold change and *p*-values of genes significantly altered ($p \leq 0.05$) in GD9 embryos with closed neural tube from VA-treated dams compared to control (saline injected) group.

Gene	Family	Function	Fold Change	*p*-value
Bak1	Bcl-2	Pro-apoptotic	206.98	0.004
Cideb	CIDE domain	Pro-apoptotic	15.56	0.000
Bad	Bcl-2	Pro-apoptotic	4.17	0.005
Casp9	p53	Pro-apoptotic	3.55	0.022
Bok	Bcl-2	Pro-apoptotic	2.13	0.017
Fadd	Death Effector Domain	Pro-apoptotic	0.46	0.000
Ripk1	CARD/Death domain	Pro-apoptotic	0.42	0.000
Dffa	CIDE domain	Pro-apoptotic	0.41	0.007
Apaf1	p53	Pro-apoptotic	0.29	0.042
Cradd	CARD	Pro-apoptotic	0.29	0.016
Traf2	TRAF	Pro-apoptotic	0.05	0.001
Casp8	Death Effector Domain	Pro-apoptotic	0.03	0.01
Prdx2	Anti-oxidant	Anti-apoptotic	78.43	0.000
Mcl1	Bcl-2	Anti-apoptotic	63.7	0.003
Akt1	p53	Anti-apoptotic	0.42	0.004
Cd40	TNF	Anti-apoptotic	0.35	0.039
Cflar	Death Effector Domain	Anti-apoptotic	0.22	0.005
Bcl2l1	Bcl-2	Anti-apoptotic	0.14	0.000
Dad1		Anti-apoptotic	0.002	0.000

Gene expression levels of embryos from VA+IFNγ embryos were compared to IFNγ only embryos. In embryos with open neural tubes from VA+IFNγ dams compared to IFNγ only embryos, apoptotic genes *Bad*, *Bak1*, *Casp9*, and *Cideb* were significantly over expressed, whereas *Casp8*, *Dffa*, *Fadd*, *Fas*, *Ripk1*, *Tnfrsf1a*, and *Traf2* were significantly under expressed. Among anti-apoptotic genes, *Prdx2* was significantly over expressed whereas *Akt1*, *Bcl2l1*, *Cflar*, *Cd40*, and *Dad* were significantly down regulated (Table 4). Among the pro-apoptotic genes, some of the genes were up-regulated and some genes were down regulated, whereas most anti-apoptotic genes were down regulated (Figure 24). This indicates an overall trend towards an increased apoptotic pattern.

Table 4. Fold change and *p*-values of genes significantly altered ($p \leq 0.05$) in GD9 embryos with open neural tubes from IFNγ +VA-treated dams compared to control group.

Gene Symbol	Family	Function	Fold Regulation	*p*-value
Bak1	Bcl-2	Pro-apoptotic	249	0.003
Cideb	CIDE domain	Pro-apoptotic	19.6	0.000
Bad	Bcl-2	Pro-apoptotic	3.89	0.024
Casp9	p53	Pro-apoptotic	3.63	0.012
Tnfrsf1a	TNF	Pro-apoptotic	0.44	0.02
Ripk1	CARD/Death domain	Pro-apoptotic	0.37	0.015
Dffa	CIDE domain	Pro-apoptotic	0.36	0.009
Cd40	TNF	Pro-apoptotic	0.26	0.04
Fas	Death Effector Domain	Pro-apoptotic	0.21	0.021
Fadd	Death Effector Domain	Pro-apoptotic	0.18	0.047
Traf2	TRAF	Pro-apoptotic	0.05	0.006
Casp8	Death Effector Domain	Pro-apoptotic	0.03	0.004
Prdx2	Anti-oxidant	Anti-apoptotic	62.25	0.000
Cflar	Death Effector Domain	Anti-apoptotic	0.26	0.045
Akt1	p53	Anti-apoptotic	0.21	0.022
Bcl2l1	Bcl-2	Anti-apoptotic	0.04	0.001
Dad1		Anti-apoptotic	0.002	0.002

In embryos with closed neural tubes from VA+IFNγ dams compared to IFNγ only embryos, only caspase 9 (pro-apoptotic gene) was over expressed significantly. Pro-apoptotic gene *Traf2* was significantly down regulated (Figure 25). Therefore, IFNγ prevented major alterations in genes related to apoptotic process.

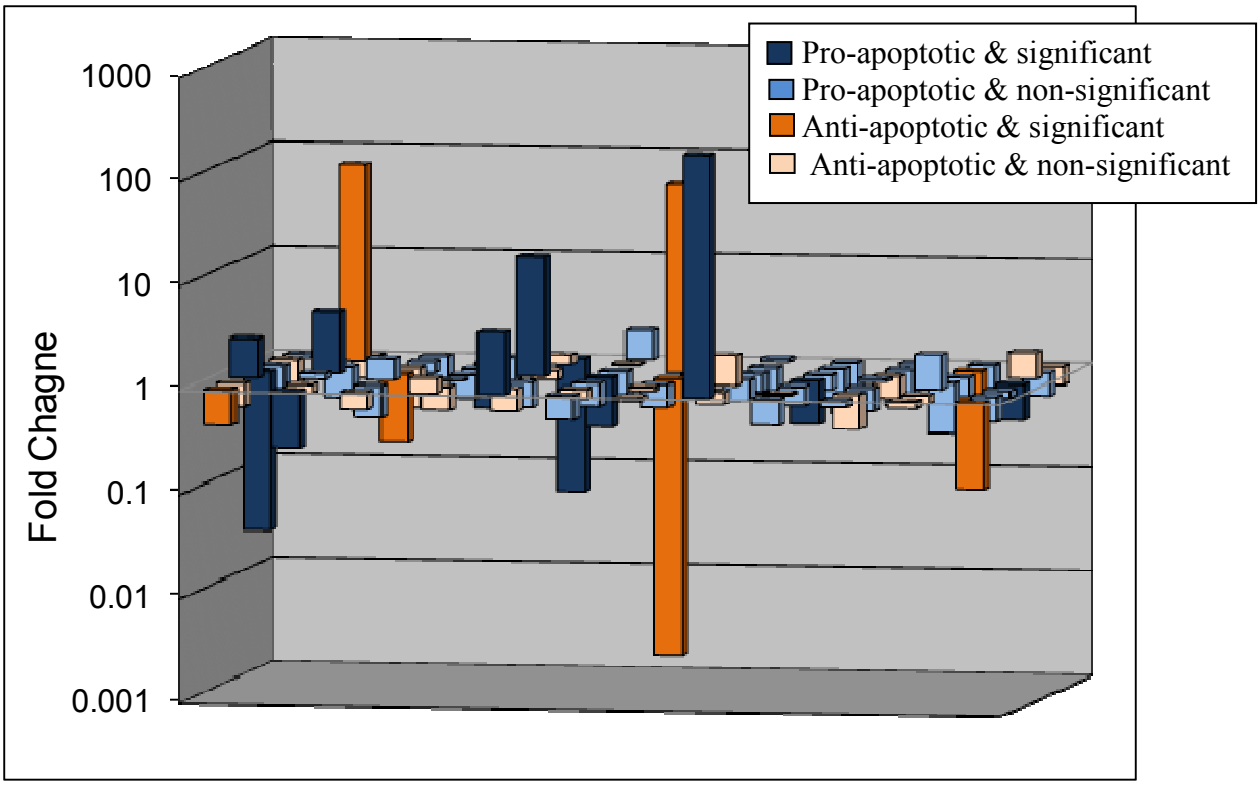

Figure 22. Fold change of genes related to apoptotic pathways significantly altered in GD9 embryos with open neural tube from VA-treated dams compared to control (saline injected) group. Statistical significance is set at $p \leq 0.05$.

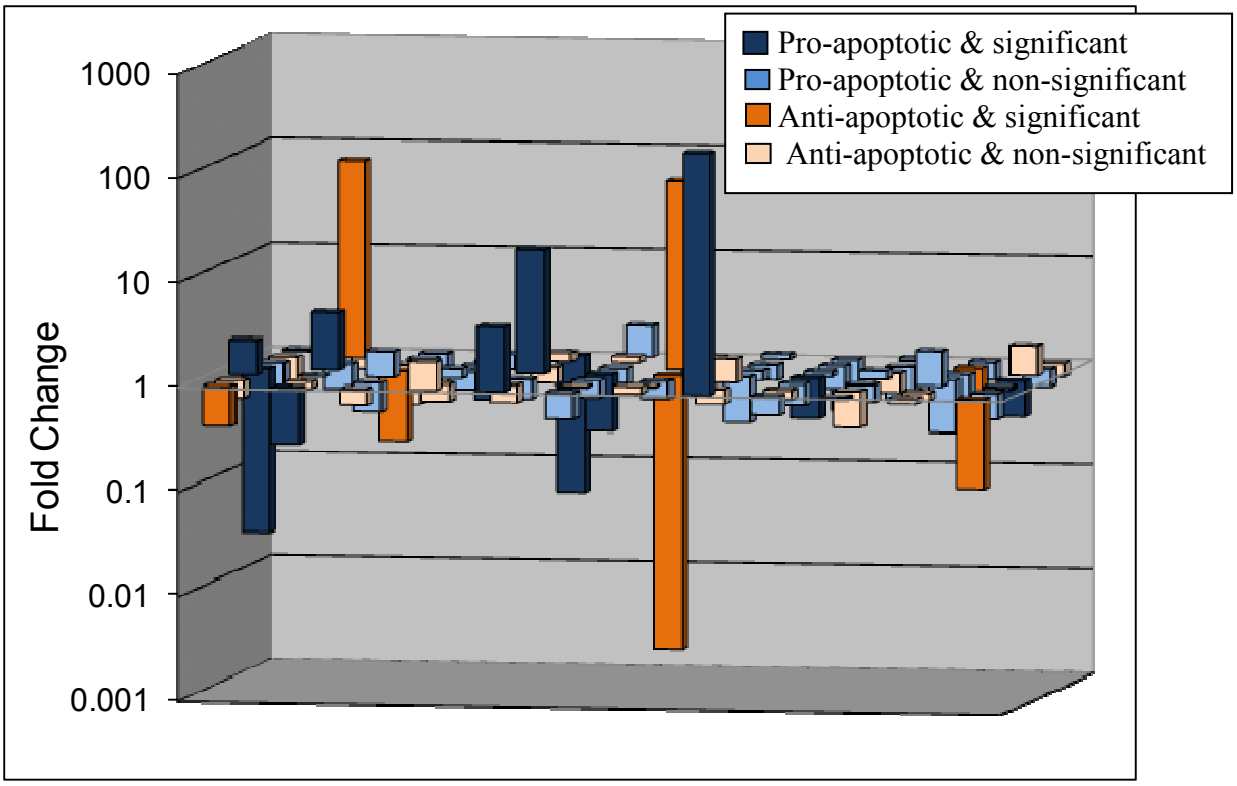

Figure 23. Fold change of genes related to apoptotic pathways significantly altered in GD9 embryos with closed neural tube from VA-treated dams compared to control (saline injected) group. Statistical significance is set at $p \leq 0.05$.

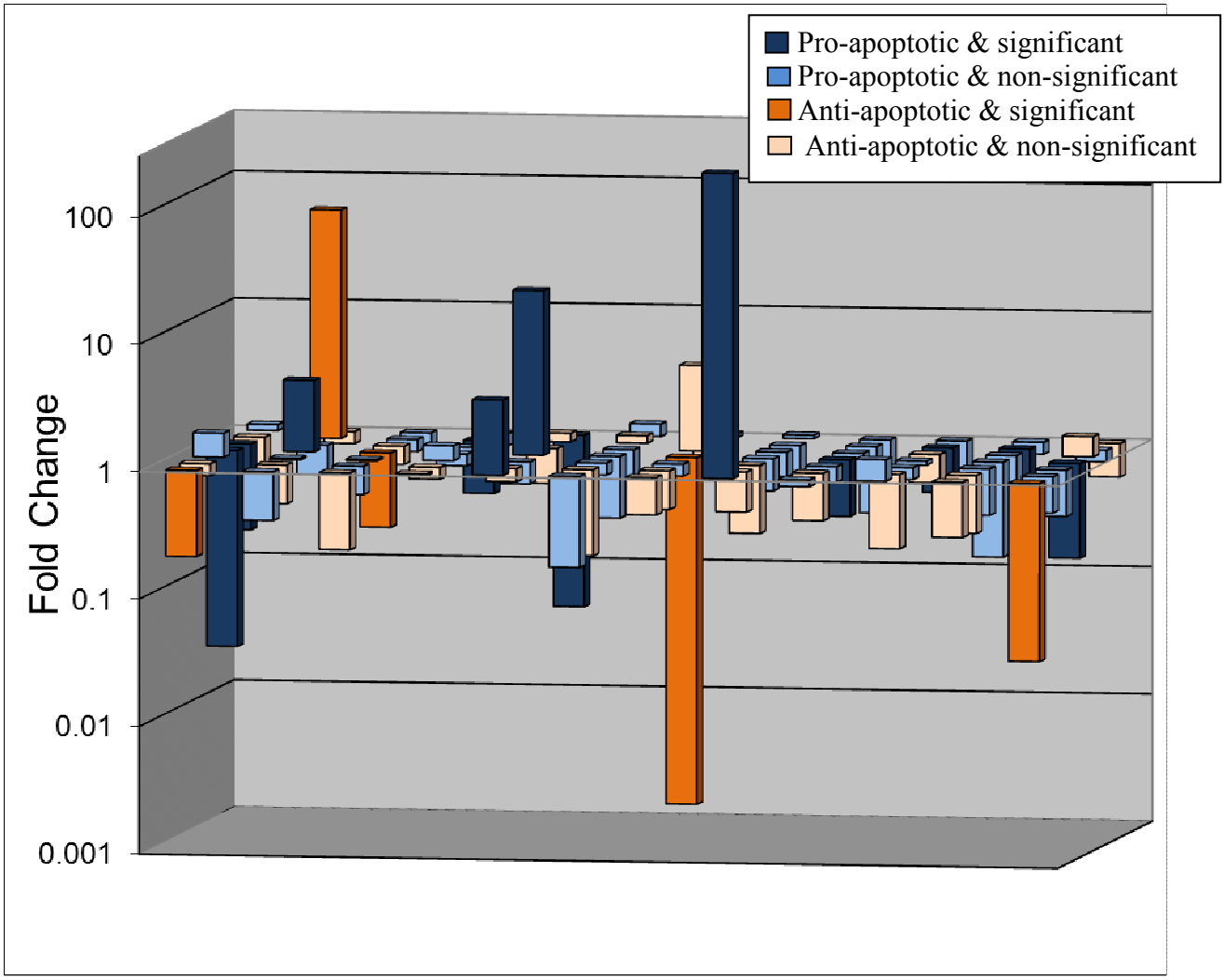

Figure 24. Fold change of genes related to apoptotic pathways significantly altered in GD9 embryos with open neural tube from IFNγ +VA-treated dams compared to control (IFNγ only) group. Statistical significance is set at $p \leq 0.05$.

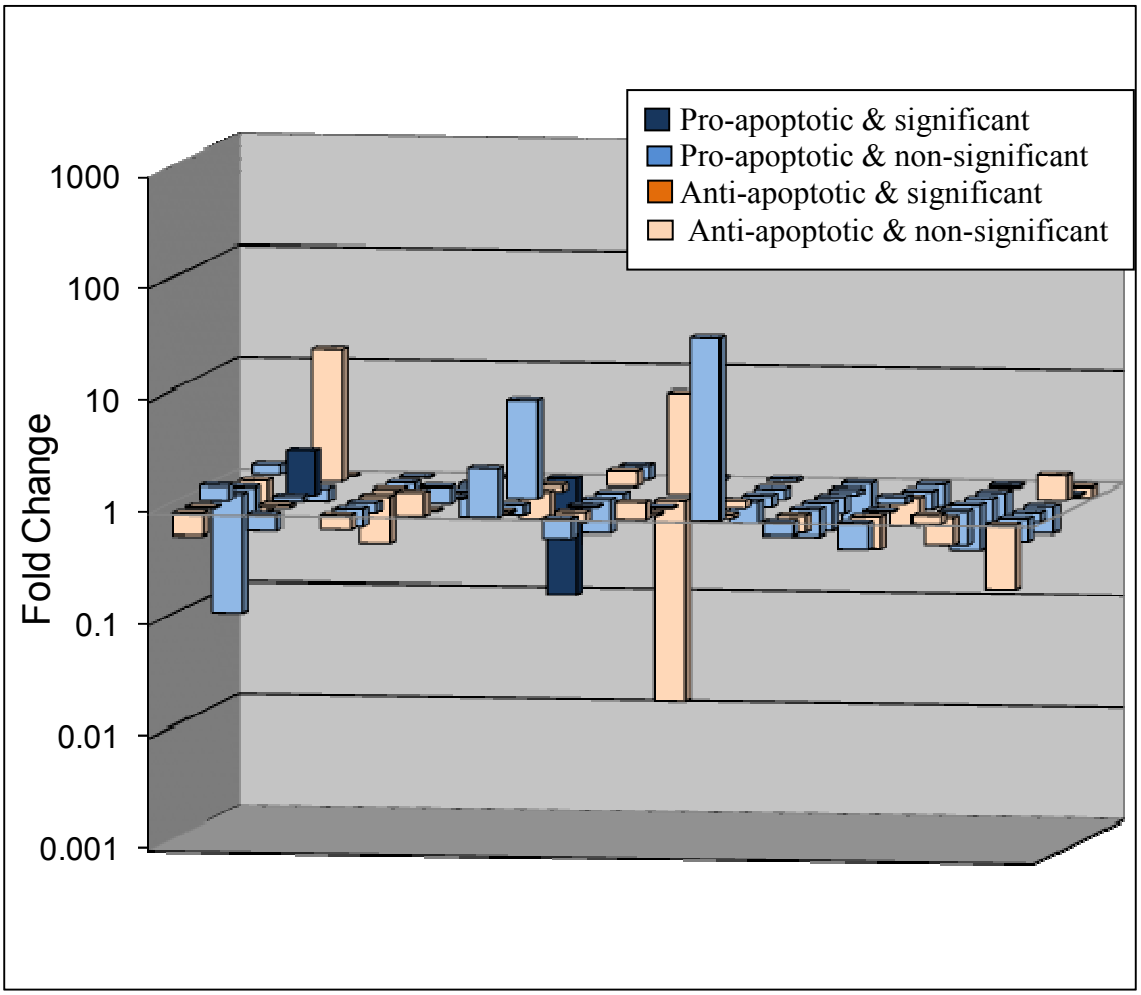

Figure 25. Fold change of genes related to apoptotic pathways significantly altered in GD9 embryos with closed neural tube from IFNγ +VA-treated dams compared to control (IFNγ only) group. Statistical significance is set at $p \leq 0.05$.

4 b. Changes in expression of apoptotic pathway genes in GD 9 embryos from tap water exposure

In embryos with open neural tubes from mice provided with tap water compared to DDI group, none of the genes were significantly altered. Pro-apoptotic genes, *Trp53inp1*, *card6*, and anti-apoptotic genes, *Atf5*, *Rnf7* were up regulated more than two-fold. Also pro-apoptotic gene, *Ltbr* and anti-apoptotic gene, *Bcl10* were down regulated more than two-fold. These results indicated that apoptotic pathways were not altered in tap water-exposed embryos.

Study 5. Molecular regulation of signal transduction pathways by VA with and without maternal immune stimulation and by tap water

Changes in the relative expression of signal transduction pathway genes were determined in embryonic heads from dams treated by VA with and without immune stimulation and by tap water. This study was conducted to evaluate the molecular mechanisms responsible for NTD formation in mice.

5 a. Changes in expression of signal transduction pathway genes in GD 8.5 and 9 embryos from VA with and without maternal immune stimulation exposure

Changes in gene expression of signal transduction pathways in GD 8.5 and GD 9 embryos from VA-treated mice were compared with embryos from mice injected with saline. Changes in gene expression of GD 8.5 and GD 9 embryos from VA+ IFNγ-treated mice were compared with embryos from mice injected with IFNγ. Genes with significant 2-fold change in their expression from treatment are discussed here. All other results are in Appendices 10-16.

Gestation day 8.5

In GD 8.5 embryos from mice exposed to VA compared to saline injected controls, *Tank* and *Vegfa* were significantly up regulated more than two-fold. In embryos from VA+IFNγ dams compared to IFNγ only embryos, none of the signal transduction genes were significantly changed.

Gestation day 9

Embryos from VA-treated mice were compared to embryos from saline injected dams. In GD9 embryos with closed neural tube from VA-treated dams, *Fgf4* (Wnt family), was significantly down regulated compared to embryos from control mice.

In GD9 embryos with open neural tubes from VA-treated dams, *Bmp2* (Hedgehog), *Wnt2*, *Fgf4* (Wnt family), *Hoxa1* (Retinoic acid) and *Hspb1* (Stress pathway) were significantly down regulated compared to controls (Table 5). None of the signal transduction genes were significantly up regulated compared to saline injected controls.

Table 5. Genes significantly ($p \leq 0.05$) altered in GD9 embryos with open neural tubes from VA-treated dams compared to control (saline injected) group.

Gene	Family	Fold change	*p*-value
Bmp2	Hedgehog	0.36	0.011
Wnt2	Wnt/hedgehog	0.02	0.000
Fgf4	Wnt	0.14	0.003
Hoxa1	Retinoic acid	0.05	0.005
Hspb1	Stress	0.17	0.028
Fn1	PI3/AKT	0.48	0.008

In GD 9 embryos with open neural tube from VA+IFNγ dams compared to IFNγ only embryos, *Il4ra* (NFκB Pathway) was significantly up regulated and *mdm2* (p53 pathway), *Cdh1*, *Wnt2* (Wnt pathway), and *Hoxa1* (Retinoic acid) were significantly down regulated (Table 6).

Table 6. Genes significantly altered ($p \leq 0.05$) in GD 9 embryos with open neural tube from IFNγ +VA-treated dams compared to IFNγ only group.

Gene	Family	Fold Change	*p*-value
Il4ra	NFκB Pathway	2.28	0.022
Birc3	NFκB Pathway	0.08	0.029
Cdh1	Wnt	0.5	0.009
Hoxa1	Retinoic acid	0.21	0.031
Mdm2	p53	0.01	0.019
Wnt2	Hedgehog/Wnt	0.045	0.034

In embryos with closed neural tubes from VA+IFNγ dams compared to IFNγ only embryos, none of the gene expression levels were significantly up regulated. Hoxa1 (retinoic acid pathway), Wnt2 (Wnt pathway), and Bmp2 (Hedgehog pathway) were significantly down regulated (Table 7).

Table 7. Genes significantly altered ($p \leq 0.05$) in GD 9 embryos with closed neural tubes from IFNγ +VA-treated dams compared to IFNγ only group.

Gene	Family	Fold Change	*p*-value
Bmp2	Hedgehog	0.46	0.000
Hoxa1	Retinoic acid	0.08	0.017
Wnt2	Hedgehog/Wnt	0.021	0.031

5 b. Changes in expression of signal transduction pathway genes in GD 9 embryos from tap water exposure

In embryos with open neural tubes from mice provided with tap water compared to DDI, none of the genes were significantly changed. Some of the genes were up or down regulated more than two-fold, but failed to reach significance. *Cdkn2a* (TGF- ß pathway), and *Tank* (NFkB pathway) were up regulated, whereas *Nab2* (mitogenic pathway), Rbp1 (retinoic acid pathway) *Wnt1* (hedgehog pathway) were down regulated (>2-fold), but expression levels did not reach significance (Table 8).

Table 8. Genes over-expressed or under-expressed in GD 9 embryos with open neural tube from dams provided with tap water for 2 months compared to embryos form F2-DDI mice.

Gene	Family	Fold Change	*p*-value
Cdkn2a	TGF-β	2.08	0.29
Tank	NFkB	5.13	0.52
Nab2	Mitogenic	0.27	0.17
Rbp1	Retinoic Acid	0.08	0.59
Tert	NFkB	0.47	0.29
Wnt1	Hedgehog	0.29	0.11

IV. DISCUSSION

Study 1. Neural tube defects in mice exposed to tap water

NTDs have multifactorial etiologies. To investigate the possible cause of NTDs in the CD-1 mouse colony maintained in our laboratory, we examined feed, water, housing location, bedding material, mouse strain and exposure to infectious agents. Each condition was evaluated for NTD production in the embryos of exposed mice to determine whether NTDs were increased or decreased.

Exposure to heavy metals such as arsenic and cadmium can induce neural tube defects in mice (Chaineau et al., 1990; Padmanabhan, 1987). Additionally, several epidemiological and animal studies have shown that fungal toxins such as fumonisin can induce neural tube defects (reviewed by Cabrera et al., 2004; Marasas et al., 2004). Most rodent diets contain corn that can become contaminated with mycotoxins such as aflotoxin and fumonisin. Fumonisin, produced by *Fusarium verticilloides*, reduces receptor mediated folate uptake (Stevens and Tang, 1997), thereby inducing NTDs (Cabrera et al., 2004; Marasas et al., 2004; Sadler et al., 2002). Heavy metals and fungal toxins were ruled out as likely causes of the NTDs by the negative feed and bedding analyses.

Maternal infectious diseases such as rubella, cytomegalovirus and *Toxoplasma gondii* directly affect development of the nervous system. Infectious agents can also elevate maternal core body temperature altering embryonic development and inducing NTDs (Shiota et al., 1988; Seller et al., 1987). The multiple negative serologic tests for

infectious diseases in our mice coupled with the fact that mice raised in different housing types and facilities all produced NTDs, make an infectious cause unlikely.

Spontaneous gene mutations may occur from inbreeding. Several single gene mutations can cause NTDs (Harris and Juriloff, 1999 & 2007). The presence of NTDs in embryos of timed pregnant mice obtained from commercial vendors indicated that the NTDs were not isolated to our facility nor were they localized to a single supplier or strain of mouse. Because of the presence of NTDs in different strains of mice from different suppliers, a direct genetic cause for these NTDs is unlikely.

In mice, it is known that maternal diet alters the risk of NTD development (Harris and Juriloff, 2005; Stoate et al., 2008). Lower dietary folate levels increase susceptibility to NTDs; however, we found that diet had no significant effect on NTDs in newly purchased mice, thus excluding diet as a possible cause of these unknown NTDs.

The percentage of NTDs was decreased but not completely eliminated when newly purchased mice were provided DDI or HPLC water for 2 weeks. This indicated water might be involved in the etiology of the NTDs (Figure 11). Since the timed pregnant mice obtained from commercial vendors exhibited NTDs (Figure 9), it was possible that the purchased mice were exposed to a teratogen prior to arrival. We hypothesized that the residual effect of teratogen, due to a long half-life, may be responsible for the low percentages of NTDs in purchased mice provided DDI or HPLC water after arriving in

our facility. This was confirmed when mice provided DDI water for 2 generations (F2-DDI) produced offspring without NTDs.

Since NTDs were not observed in F2-DDI mice or in F2-DDI mice transferred to HPLC water, but reappeared when the mice were exposed to a variety of tap waters from different municipalities, it was concluded that the cause of these unknown NTDs was exposure to tap water. The higher NTD rates in mice exposed for 8 weeks vs. 2 weeks, indicated a dose effect of some contaminant present in tap water. This dose response may account for the observed differences in NTD rates in the timed pregnant mice (Figure 12). The length of time of tap water exposure affected NTDs more so than strain or water source (i. e location). Different strains, however, may show variable vulnerability to teratogens because of their difference in genetic background (Burren et al., 2010; Ogawa et al., 2005; Lemos et al., 2009). Our data suggest that the teratogen apparently has wide geographic distribution as mice from different regions of the country exhibited NTDs.

In this study, we demonstrated an association between tap water and NTDs in mouse embryos. This was the first study to show fetal malformations from ambient exposure to tap water. Specific seasonal variation in NTD rates was not tested; however, we did not notice any fluctuations in percentages of NTDs throughout the 2 year project. Published literature indicates that many teratogenic compounds can be found in drinking water (discussed in literature review), but most are found at below what are believed to be toxic concentrations. The teratogen responsible for NTDs in our mice has not yet been identified.

Study 2. Characterization of teratogen present in tap water

Many toxic compounds are routinely found at low concentrations in tap water (Schwarzenbach et al., 2006). The US Geological Survey found in 2002 that 80% of the nation's rivers and streams were contaminated with wastewater contaminants. As many potential contaminants could be present in the water, chemical analysis to identify an unknown teratogen is a difficult task. Characterization of the teratogen in the tap water helps to narrow down the possible suspect contaminants and aids in identifying specific compound(s) by chemical analysis. To determine the chemical nature of the teratogen, tap water was fractioned chemically into volatile and non-volatile fractions and provided to F2-DDI mice. Additionally, this study also determined whether the teratogen acted directly or was a metabolite. Lastly, to identify the possible source of the teratogen and also to determine if the teratogen was a water disinfection byproduct, F2-DDI mice were exposed to municipal tap water with different water treatment regimens and from different locations within the water treatment plant.

According to U.S Environmental Protection Agency (US-EPA), one-fifth of the US water supplies contained volatile organic compounds. The U.S Geological Survey during 1985-2002, identified 55 volatile compounds present in domestic well waters; 1% of the samples contained high concentrations of volatile organic compounds known to cause adverse health effects (Rowe et al., 2007). In our study, embryos from mice provided the volatile fraction of tap water had higher percentages of NTDs, which indicated the predominant teratogen was a volatile compound. However, NTDs were still present in embryos from mice provided the non-volatile fraction. This may indicate either a mixture

of both volatile and non-volatile teratogens present in the water acting additively or synergistically to produce NTDs in mice, or retention of a small amount of compound in the non-volatile fraction because of low volatility.

The hepatic cytochrome P450 enzymes commonly play a major role in metabolism of xenobiotic compounds, converting them to metabolites that can be readily excreted from the body. Induction of cytochrome P450s can reduce the teratogenicity of compounds by converting the toxic parent compound to less toxic metabolites. For example, induction of cytochrome P450 enzymes using phenobarbital reduced the teratogenicity of cyclophosphamide (Gibson and Becker, 1968; Greenaway et al., 1982; Hales, 1981) by metabolizing the toxic cyclophospahmide into less toxic metabolites. In some instances however, teratogenicity increases when metabolites are more toxic than parent compound (Wells and Winn, 1996). Phenobarbital increased carbamazepine metabolism, resulting in greater teratogenicity from increased formation of a more toxic metabolite (Finnell et al., 1995). Increased teratogenicity of compounds such as phenytoin and cyclophosphamide was also observed with inhibition of cytochrome P450 enzymes by SKF525A (Greenaway et al., 1982).

In our study, neither SKF 525A nor phenobarbital altered the NTD levels in embryos exposed to tap water. These results indicated a number of possibilities. First, it is possible that neither SKF-525A nor phenobarbital inhibited or induced the specific cytochrome P450 enzymes involved in metabolism of this teratogen. Alternatively, cytochrome P450 enzymes are not big players in the metabolism of this teratogen. Lastly, it is possible that

both the parent compound and cytochrome P450 metabolite(s) were toxic and both induced NTDs. It was interesting to note that embryos from the F2-DDI mice pre-treated with phenobarbital exhibited NTDs. This might result from folate antagonistic properties of phenobarbital similar to other classes of anti-convulsants such as VA (Halwachs et al., 2009).

Teratogens could be produced in municipal water during the treatment processes used to make the water potable. Water is commonly treated with chlorine or chloramine to kill microorganisms. This treatment results in formation of a variety of disinfection byproducts. Adverse effects of water disinfection byproducts on pregnancy have been demonstrated in both laboratory animal studies as well as human epidemiological studies (Bove et al., 2002 review; Ahmed et al., 2005, Andrews et al., 2004; Hunter et al., 1996; Hunter et al., 2006). These adverse pregnancy outcomes include small for gestational age (SGA), low birth weight (Kramer et al., 1992; Munger et al., 1997), neural tube defects (Klotz and Pyrch, 1999; Bove et al. 1995; Dodds and King 2001; Klotz and Pyrch 1999), cardiac defects (Cedergren et al., 2002; Chisholm et al., 2008; Hwang et al., 2002) and cleft palates (Bove et al. 1995). In our study, NTD rates varied more with the source of water, not the treatment method. NTDs were not observed in embryos from mice exposed to chlorinated well water (ground water) but were significantly increased in embryos from mice exposed to all surface water sources including chlorinated surface water. This indicated that the teratogen may be a surface water pollutant. Surface waters are more frequently contaminated as they receive rain water runoff containing agricultural and man-made pollutants. Ground water is generally less contaminated than

the surface water since it is naturally filtered through layers of soil and rock. NTD rates were similar in mice provided autoclaved river water, chloraminated river water collected from the treatment plant and autoclaved and non-autoclaved chloraminated tap water. This indicated the teratogen causing NTDs was not introduced during the water treatment process and was a contaminant(s) in the surface water prior to water treatment. It is also interesting to note that neither autoclaving nor the water treatment process altered the teratogenicity of the compound.

Study 3. Detection of apoptotic changes in embryos from teratogens and maternal immune stimulation

Apoptosis is an essential component of normal development in the mammalian embryo. During embryonic development, cells proliferate rapidly and are vulnerable to teratogens. Exposure to teratogens can cause programmed cell death (Sulik et al., 1988). Many studies show increased apoptosis after exposure to various teratogens that indicated a correlation between increased apoptosis and teratogen-induced fetal malformations.

Arsenic-induced fetal malformations in the brain, optic system, somite segmentation and limb bud of rat embryos are related to increased apoptosis in those areas (Li et al., 1998). Singh et al., (2009) demonstrated increased apoptosis and fetal malformations in developing embryos exposed to salicylic acid and proposed a relation between apoptosis and teratogenesis. A positive correlation exists between homocysteine-induced apoptosis in heart and brain and teratogenesis in avian embryos (Li et al., 1999). Exposure to retinoic acid resulted in increased apoptosis in the neuroepithelium, spinal cord and heart of developing mice embryos that suggested apoptosis as a possible mechanism for retinoic acid induced NTDs and other fetal malformations (Sarkar and Sharma, 2002). Increased apoptosis also occurs in neural epithelium of nicotine-exposed embryos (Zhao et al., 2005). Increased apoptosis also is related to skeletal deformities in COX-2 transgenic mouse (Shim et al., 2010).

Cyclophosphamide induces a dose dependent increase in apoptotic cells in developing embryos as was demonstrated using flow cytometric analysis as well as TUNEL assay. Therefore, that altered apoptosis may be involved in the teratogenesis of malformations

(Singh et al., 2005; Savion et al., 2003; Torchinsky et al., 1995). Chen et al., (1994) found increased apoptosis in rat cultured embryos exposed to cyclophosphamide. This also suggests involvement of altered apoptosis in cyclophosphamide teratogenicity.

Correlations between infectious diseases, altered apoptosis and fetal malformations are also reported. Congenital eye malformations and NTDs from influenza B virus infection in both mouse and chicken embryos are related to increased apoptosis in neuroepithelium and surface ectoderm of the head regions (Chen et al., 2009). Teratogenicity of rubella virus is linked to increased apoptosis in humans (Lee and Bowden, 2000).

Increased apoptosis is a fundamental mechanism in hyperglycemia teratogenicity. Oyama et al., (2009) showed increased apoptosis in hindbrain region of mouse embryos from diabetic dams compared to controls. These apoptotic levels decreased when the diabetic dams were treated with folic acid. Jiang et al., (2008) showed an increase in apoptotic cells by TUNEL staining in cranial neural tube of embryos from diabetic mice. Apoptosis decreases in embryos from iNOS (inductible nitric oxide synthase)-treated diabetic mice, which suggests iNOS reduce NTDs by decreasing apoptosis in embryos from diabetic mice (Sugimura et al., 2009).

Altered apoptosis as a mechanism for VA induced teratogenicity has also been suggested but has not been clearly elucidated. VA causes oxidative stress that can induce cell death (Defoort et al., 2006). VA causes apoptosis in rat uterine and ovarian cells (Cansu et al., 2010). Inhibition of histone deacetylase (HDAC) increases sensitivity towards apoptotic

stimuli (Bai et al., 2010, Kisseberth et al., 2008, Pei et al., 2004). VA inhibits HDAC and induces apoptosis in various cell lines (Hubaux et al., 2010, Papi et al., 2010). Jergil et al., (2009) demonstrated a dose dependent increase in cell death in P19 embryonal carcinoma cells treated with VA. Evaluation of gene expression in these VA-treated carcinoma cells indicated approximately 200 altered genes; 30% of which were involved in NTD formation. Di Renzo et al., (2010) showed a relationship between axial skeletal malformations and increased apoptosis in somites caused by VA exposure in CD-1 mice.

From the studies just described, it is clear that increased apoptosis may result from teratogen exposure in developing embryos. It is also clear that the tissues or organs with increased apoptosis following teratogen exposure are prone to malformations. Several gene knockout models with altered apoptosis also exhibit fetal malformations that suggest a relationship between altered apoptosis and fetal malformations. There is, however, no clear evidence that altered apoptosis is directly responsible for the malformation.

Neural tube closure involves cell migration, proliferation, differentiation and apoptosis (Sabapathy et al., 1999). Disruption of these processes can cause NTDs. A certain degree of cell death occurs normally during formation of the neural tube in mice (Schlüter, 1973). Neural folds elevate dorsally and medially and appose each other in order to fuse. During fusion, the continuity between surface ectoderm and neuroepithelium is restructured by apoptosis so that the opposing neural folds can converge to form the neural tube (Copp, 2005). This tissue remodeling by apoptosis is a tightly controlled

process. Alteration of this process in several knockout mouse strains demonstrated neural tube closure defects associated with apoptosis related genes (Barrier et al., 2009; Hsu et al., 2008; Shirane et al., 2008; Wong et al., 2008). An insufficient number of cells resulting from increased apoptosis may affect morphogenetic processes that are critical to neural tube closure (Massa et al., 2009). Exuberant apoptosis in the neural folds could result in insufficient tissue for normal fold elevation and apposition resulting in NTDs. Thus, VA could prevent neural fold fusion by increasing apoptosis in neural folds.

In our study, flow cytometric analysis of cells from VA-treated embryonic heads with open neural tubes revealed increased apoptosis compared to saline injected controls. This correlated with increased apoptotic TUNEL staining along the neural folds of the VA exposed embryos compared to controls. The increased apoptosis was localized to neural folds, which suggested VA-induced apoptosis may lead to NTD formation. In contrast, Kultima et al., (2004) did not observe increased apoptosis in embryos along the neural folds from VA teratogenicity. Instead, a transverse band of apoptotic cells in forebrain region was observed. Apoptosis was measured 48 hours post exposure to VA as opposed to 12 hours in our study. At 48 hours post exposure, apoptotic cells might already have been removed from the neural tube area leading to the difference in results.

Interestingly apoptotic levels, measured by flow cytometry, were also increased in VA-exposed embryos with closed neural tubes. Therefore, VA also enhanced apoptosis in these embryos. These flow cytometry results were corroborated by TUNEL assay that revealed increased apoptosis along the midline of the neural fold of the VA embryos with

closed neural tubes. Dawson et al., (2006) observed changes in Bcl-2 family genes towards pro-apoptotic pattern in VA-exposed embryos with both open and closed neural tubes with no significant difference between these two groups. Although apoptosis was not measured by these authors, VA likely disrupts the apoptotic pattern in all exposed embryos.

Nonspecific stimulation of the maternal immune system in mice reduces a wide variety of teratogen-induced fetal malformations. The mechanism(s) by which broad-spectrum activity of immune stimulants reduces birth defects has not been completely elucidated, but embryos from immune stimulated dams are resistant to teratogen-induced apoptosis. Savion et al., (2003) demonstrated by flow cytometry and TUNEL assay that maternal immune stimulation prevented cyclophosphamide-induced apoptosis in the head region of developing mouse embryos. The authors suggested that immune stimulation prevented head malformations such as exencephaly, cleft palate and open eyes by regulating the apoptotic process. Similarly, Toder et al., (1996) showed a protective effect of immune stimulation with cyclophosphamide-induced limb defects. These authors demonstrated that cyclophosphamide-induced limb bud apoptosis. Maternal immune stimulation prevented cyclophosphamide-induced apoptosis thus reducing the malformations. Heat-shock also induces apoptosis causing NTDs and eye abnormalities. Maternal immune modulation prevents heatshock-induced alterations in apoptosis and also reduces these fetal malformations (Yitzhakie et al., 1999).

In our study, low TUNEL staining was observed in embryos from both saline injected controls and IFNγ only treated dams concomitant with low apoptotic levels in these two groups detected by flow cytometry. This suggests that IFNγ treatment by itself did not affect apoptosis. No significant difference in apoptotic levels was observed in embryos with closed neural tube from IFNγ +VA-treated dams, and apoptotic TUNEL staining in these embryos was similar to controls. This suggests embryos with closed neural tubes from immune stimulated dams were more resistant to teratogen-induced apoptosis. In embryos with NTDs from IFNγ +VA-treated dams, apoptotic levels were found to be significantly higher compared to embryos from control (IFNγ only) dams. Increased apoptotic TUNEL staining was visualized in the head regions along the neural fold edges in these embryos as well. This suggests that maternal immune stimulation failed to reduce apoptotic cells and also did not prevent NTDs in these embryos.

Increased apoptosis from VA exposure possibly resulted from a protective mechanism in the embryo that removes damaged cells and replaces them with new cells. If the ratio of damaged cells and live cells is too high, the beneficial effects of apoptosis become detrimental causing too many cells to be removed and disrupting normal organogenesis (Levy et al., 2001). In addition to induction of apoptosis, VA also inhibits cell proliferation rates (Voisard et al., 2010; Byun et al., 2009). Decreased cell proliferation rates together with increased apoptosis in embryos may reduce viable cells in VA-exposed embryos. Low number of viable cells in the neural folds could result in failure of neural tube closure thus causing NTDs. This idea was supported by microarray analysis of embryonic carcinoma cell lines (Jergil et al., 2009) and mouse embryos (Okada et al.,

2005) where both cell cycle arrest genes and apoptosis genes were altered from exposure to VA.

Decreased apoptosis in embryos with closed neural tubes from IFNγ +VA dams suggests that immune stimulation increases resistance to teratogen-induced apoptosis. This was supported by other studies that observed prevention of teratogen-induced apoptosis by immune stimulation (Toder et al., Yitzhakie et al., 1999). Increased apoptosis observed in embryos with open neural tubes from IFNγ +VA dams suggests immune stimulation could not prevent the teratogen-induced apoptosis in these embryos resulting in NTDs. This could have resulted from irreversible DNA damage induced by VA to the neuroepithelial cells in these embryos.

Overall, this study demonstrated increased apoptosis in embryos from VA-exposed dams by both flow cytometry and TUNEL assay. The increase in apoptosis was localized to midline of the neural tube closure in these embryos, which implicated involvement of altered apoptosis in teratogenicity of VA in inducing NTDs. Increased apoptosis in normal embryos form VA-exposed dams indicated that there may be other cellular processes such as cell proliferation and differentiation that might be affected in addition to apoptosis. Increased apoptosis together with inhibition of proliferation may have resulted in fewer cells available during neural tube closure that caused NTDs. Apoptotic changes in embryos with closed neural tubes from IFNγ +VA dams were similar to controls, which implied maternal immune stimulation could protect against teratogen-induced apoptosis. This protection may be related to decreased apoptosis in these

embryos. In the embryos that maternal immune stimulation failed to regulate apoptosis, exposure to VA resulted in NTDs.

Tap water-exposed embryos with open neural tubes had a similar apoptotic pattern compared to F2-DDI controls. Neural tube formation is a complicated process involving multiple cells and processes, such as cellular cytoskeleton, cell viability, apoptosis, cell cycle, cell proliferation and interactions between cell surface and extra cellular matrix. Alterations in any of these cellular functions or processes may lead to NTDs (Copp and Greene, 2010). Different teratogens can act through different mechanisms to cause a specific malformation (van Gelder et al., 2010). Unchanged apoptosis in embryos exposed to tap water suggests that apoptosis is not involved in this particular teratogenicity. It is possible that other cellular functions such as cell proliferation, differentiation, or migration might be involved other than apoptosis.

Study 4. Molecular regulation of apoptosis by VA with and without maternal immune stimulation and by tap water

Regulation of apoptosis is an essential component of normal development. Teratogens alter apoptosis by changing the expression of apoptotic regulatory genes. Analysis of single genes provides little information regarding the regulation of apoptosis. Conversely, analysis of a number of pathway-focused genes can provide insight in to the overall apoptotic mechanisms. In this study, expression of 84 key genes involved in apoptosis was determined in response to VA with and without maternal immune stimulation and to tap water exposure. The analysis included the TNF ligands and their receptors; members of the Bcl-2, caspases, IAP, TRAF, CARD, death domain, death effector domain, and CIDE families; as well as genes involved in the p53 and ATM pathways. TNF family members can send both survival and death signals to cells and play a major role in various cellular processes such as apoptosis, cell proliferation and differentiation (Kawasaki et al., 2002). p53 signaling regulates a variety of genes involved in apoptosis, inhibition of cell proliferation and growth arrest in response to cellular stress. Interaction between pro-apoptotic and anti-apoptotic members of the Bcl-2 family controls the mitochondrial apoptosis pathway (Jin and El-Deiry, 2005; Maddika et al., 2007). The balance between pro-apoptotic and anti-apoptotic signals is established upon activation of TRAF, CARD, death domain, death effector domain, and CIDE families that activate death stimuli. ATM (Ataxia Telangiectasia Mutated Protein) signaling plays a key role in regulating multiple signaling cascades in response to DNA strand breaks induced by damaging agents. Caspases, triggered by apoptosis signaling, activate a number of proteins in the cell that causes cell death and removal of cell debris. In our study, genes

that were significantly altered more than 2-fold were considered as major contributors and are discussed here.

In GD 8.5 embryos exposed to VA, no apoptotic genes were significantly up regulated more than two-fold compared to controls. CIDE proteins including *Cideb* activate apoptosis in mammalian cell lines (Inohara et al., 1998). In our study; however, the CIDE domain family gene, *Cideb*, that induces apoptosis, was significantly down regulated in GD 8.5 embryos exposed to VA compared to controls. p53 genes are involved in ethyl carbamate teratogenicity (Sharova et al., 2000). Similarly, congenital eye malformations induced by teratogens are related to increased apoptosis resulting from p53 dysregulation (Wubah et al., 1996; Charlap et al., 2003). Loeken et al., (2006) suggested a role for p53 induced apoptosis in diabetes-induced NTDs by demonstrating inhibition of *Pax3* by excess glucose that is needed to check p53 dependent apoptosis. In our study, the p53 pathway gene, *Trp63*, which induces apoptosis, was up-regulated significantly but was not altered more than two fold (1.51 fold). This suggests that p53 pathway may only be partially involved in VA-induced teratogenicity.

In GD 9 embryos, both apoptotic and anti-apoptotic gene expressions were affected by VA. Bcl-2, p53, and death effector domain family genes were predominantly affected in GD9 embryos from VA-treated dams compared to embryos from control mice (saline injected). Pro-apoptotic Bcl-2 family genes, *Bad*, *Bak1* and *Bok,* were up regulated in embryos from VA-treated dams. This indicates involvement of Bcl-2 family genes in teratogenesis of VA. Chemical and physical agents induce death stimuli by causing

oxidative stress and DNA damage. Death stimuli induced by chemicals leads to activation of pro-apoptotic Bcl-2 family proteins such as *Bax*, *Bid* and *Bak* (Reviewed by Torchinsky et al., 2005). *Bok* is a pro-apoptotic family gene that promotes chemotherapeutic-induced apoptosis in growing cells (Rodriguez et al., 2006) and is an essential for p53 induced apoptosis (Yakovlev et al., 2004). The results from our study indicated increased apoptosis in embryos from VA-treated dams could be related to up regulation of Bcl-2 family genes such as *Bad*, *Bak1* and *Bok*.

Expression of the Bcl-2 family apoptotic genes, *Bad, Bak1, Bok,* were normalized in embryos with closed neural tube from VA+IFNγ dams. Therefore, immune stimulation prevented the change in pro-apoptotic regulatory molecules belonging to Bcl-2 family. Similar results were obtained by Savion et al., (2009), with cyclophosphamide teratogenicity. Cyclophosphamide induced expression of *Bcl2,* whereas maternal immune stimulation normalized the expression.

In our study, expression of an anti-apoptotic gene in the Bcl-2 family, *Bcl2l,* was decreased by VA exposure. This is similar to results obtained by Dawson et al., (2006), who observed decreased expression of anti-apoptotic gene, *Bcl2*, in VA-exposed embryos with both open and closed neural tubes. Increased ratio of pro-apoptotic and anti-apoptotic genes of Bcl-2 family is associated with increased apoptosis (Dawson et al., 2006). Our results suggest that down regulation of anti-apoptotic Bcl-2 gene, *Bcl2l1*, together with up regulation of pro-apoptotic Bcl-2 family genes shifted the ratio towards

activation of pro-apoptotic signaling resulting in increased apoptosis in VA-exposed embryos.

Maternal immune stimulation normalized the expression levels of Bcl-2 family genes, which indicated these genes play a major role in the protective effect of immune modulation of VA-induced NTDs. Sharova et al., (2000) found similar results where maternal immune stimulation normalized decreased expression of anti-apoptotic Bcl-2 family genes in embryos exposed to urethane. In embryos with open neural tube from VA+IFNγ dams, apoptotic genes, *Bad, Bak1, Casp9, Cideb* were significantly over expressed compared to IFNγ only embryos similar to the VA-exposed embryos. These results were corroborated by the flow cytometric as well as TUNEL staining results that demonstrated higher percentages of apoptosis in VA+IFNγ embryos with open neural tubes. Taken together, these results indicate immune stimulation failed to reduce the expression of these apoptotic genes possibly resulting in the excessive apoptosis observed in this group.

Induction of *Casp9*, a positive regulator of apoptosis, is a major cellular event in stress conditions (reviewed by Torchinsky et al., 2005). Teratogens such as hyperthermia, cyclophosphamide, staurosporin (Little and Mirkes, 2002) and arsenic (Singh et al., 2010) induce cell death in GD 9 mouse embryos by induction of *Casp9*. The neuroepithelium is one of the most sensitive tissues prone to teratogen-induced apoptosis (Mirkes and Little, 1998. Our results are in agreement with the above studies. The over

expression of *Casp9* was observed in VA-exposed embryos with open and closed neural tubes that indicates a role of *Casp9* in the VA teratogenesis.

The apoptotic gene, *Casp9*, was also up regulated in GD9 embryos with closed neural tube from VA+IFNγ dams; however, expression levels were low compared to VA-exposed embryos. This indicates immune stimulation partially normalized the expression of *Casp9* towards control embryos. *Casp9* levels were up regulated in embryos with open neural tube from VA+IFNγ group. Expression levels in these embryos were similar to embryos from VA group, which indicates immune stimulation failed to reduce the expression of *Casp9* in these embryos.

Akt negatively regulates apoptosis by suppressing a variety of pro-apoptotic molecules such as *Bad* and *Casp9* (Kim et al., 2001). In our study, *Akt1* under expressed in VA-exposed embryos and also in embryos with open neural tube from IFNγ +VA dams, which suggests the effect of VA on negative regulators of apoptosis. In IFNγ +VA embryos with closed neural tubes, immune stimulation normalized the expression of *Akt1*. Chen et al., (2006) showed that VA induces apoptosis in cell lines by reducing the expression of *Akt1* thereby activating caspase 9. Our results indicate *Akt1* is a potential target for VA teratogenicity through caspase 9. Immune stimulation was able to prevent the effect of VA on this negative regulator of apoptosis.

Anti-apoptotic genes, *Cflar* and *Dad1*, were significantly down regulated by VA and may be a direct effect of the teratogen. Pro-apoptotic genes such as *Cradd*, *Fadd, Ripk1* and

Casp8 were also down regulated in VA-exposed embryos. This may result from activation of a survival mechanism in an effort to save the cells from teratogen-induced apoptosis. For example, activation of anti-apoptotic gene, *Mcl-1*, down regulates the pro-apoptotic gene, Casp8 (Chen et al., 2010).

Overall, our results indicated that VA initiated both death and survival signaling in the embryos. Up regulation of the apoptotic genes, *Bad, Bak1, Bok, Casp9* and *Cideb*, and down regulation of anti-apoptotic genes, *Akt1, Bcl2l1, Cflar* and *Dad1*, shifted the balance towards death signaling, thereby causing increased apoptosis in exposed embryos and NTDs. Our flow data as well as TUNEL assay supported these results demonstrating VA increased apoptosis in embryonic heads compared to controls. Studies indicate that teratogens affect both pro and anti-apoptotic regulators in developing embryos (Gutierrez et al., 2009; Toder et al., 2002). Once apoptosis is activated in a cell, negative regulators of apoptosis may also be activated to counteract the ongoing apoptotic process and to protect the cell. Survival or death of a particular cell following exposure to teratogen depends on the interaction of pro and anti-apoptotic regulators (Toder et al., 2002).

Maternal immune stimulation normalized changes in the expression of apoptotic initiating molecules induced by VA. Thus, the protective effect of immune stimulation may act by normalizing the teratogen effects on apoptotic regulatory mechanism, making the embryos resistant to VA-induced apoptosis and preventing NTDs. This is supported by the fact that pro-apoptotic genes in immune stimulated embryos with open neural

tubes were not normalized. Excessive apoptosis resulting from the altered apoptotic genes may lead to NTDs in these embryos.

In embryos from tap water-exposed dams, none of the apoptotic genes were significantly different compared to controls. Our flow cytometry and TUNEL results corroborated these findings with no increase in apoptosis observed in embryos exposed to tap water. Together, these results suggest that apoptosis may not be involved in the teratogenicity associated with tap water.

Study 5. Molecular regulation of signal transduction pathways by VA with and without maternal immune stimulation and by tap water

Signaling pathways can regulate many normal and abnormal cellular mechanisms and are important for development. The relative expressions of genes involved in regulating signal transduction were determined in VA (with and without immune stimulation) and in tap water-exposed embryos. Alterations in gene expression of signal transduction pathways were used to identify possible pathways involved in the formation of NTDs. The pathways that were analyzed included Wnt pathway, TGF- β pathway, hedgehog pathway, PI3 Kinase / AKT Pathway, Jak / Src pathway, NFκB pathway and retinoic acid pathway. Neural tube closure involves several morphogenic mechanisms such as cell proliferation, dorsoventral (DV) patterning, cell migration, cell fate specification and cell shaping (Copp et al., 1988, Padmanabhan, 2006). Numerous cell signaling molecules and pathways control cellular processes during normal development. Wnt, TGF- β, hedgehog pathway, PI3 Kinase / AKT, Jak / Src and NFκB pathways are the most prominent regulatory signals controls these biological processes. Wnt and Hedgehog family signaling molecules play a part in multiple developmental processes during embryogenesis such as cell proliferation, cell fate specification and cell differentiation (Logan and Nusse, 2004). AKT signaling pathway molecules participate in several biological responses such as inhibition of apoptosis and stimulation of cell proliferation.

Hedgehog or BMP/Wnt signaling pathway genes such as *Fgf4*, *Bmp2*, *Wnt2* were significantly down regulated in GD9 embryos with open neural tube from VA-treated dams. Only *Fgf4* was significantly down regulated in VA-exposed embryos with closed

neural tubes. Fibroblast growth promoter (Fgf) family genes promote cell proliferation in several cell lines (Tsuboi et al., 1990; Boilly et al., 2000; LaValle et al., 1998). Kosaka et al., (2006) demonstrated the ability of *Fgf4* to induce neural progenitor cell proliferation. Down regulation of *Fgf* in a cancer line following VA exposure was observed by Zgouras et al., (2004). In our study, down regulation of *Fgf4* was observed in VA-exposed embryos with both open and closed neural tubes; however, the down regulation of *Fgf4* was more pronounced in embryos with open neural tubes. This effect on *Fgf4* implied the possible arrest of cell proliferation by VA.

Immune stimulation with IFNγ normalized the expression of *Fgf4* in embryos with closed neural tubes, which indicates a role of *Fgf4* in immune stimulation protective mechanism. Immune stimulation also normalized the expression of *Fgf4* in embryos with open neural tubes. This suggests involvement of multiple molecular pathways including apoptotic pathways along with *Fgf4* in formation of VA-induced NTDs and also in protective mechanism of maternal immune stimulation.

Bone morphogenetic proteins (BMPs) play a major role in dorso-ventral (DV) patterning (Barth et al., 1999; Liem et al., 2000; Kishigami and Mishina, 2005), whereas sonic hedgehog determines the ventral patterning during neural tube formation (Briscoe et al., 1999). In our study, *Bmp2* was significantly down regulated in VA-exposed embryos with open neural tubes but not in embryos with closed neural tubes. Castranio and Mishina et al., 2009 observed failure of neural tube closure in *Bmp2* chimeras that indicated *Bmp2* is essential for the neural tube closure. Reduced expression of *Bmp2* in

embryonic and/or maternal tissues results in NTDs (Singh et al., 2008). Our results supported this idea as *Bmp2* in embryos with open neural tube was down regulated in VA-exposed dams that suggests role of *Bmp2* in VA teratogenesis. Maternal immune stimulation failed to normalize the expression of *Bmp2* in embryos with open as well as closed neural tubes from IFNγ +VA dams. Therefore, *Bmp2*, was not involved in the reduction of NTDs by maternal immune stimulation

Wnt signaling genes play a major role in intercellular signaling during embryonic development. Involvement of Wnts in CNS patterning and regulation of asymmetric cell divisions is seen in various gene knockouts (Wodarz and Nusse, 1998). Various studies on cancer cell lines demonstrate a decrease in cell proliferation and an increase in apoptosis resulting from inhibition of *Wnt2* expression (Shi et al., 2007; You et al., 2004; Pu et al., 2009; Wang et al., 2010). Expression of *Wnt2* in VA-exposed embryos with open neural tubes was decreased indicating role of *Wnt2* in VA teratogenicity. Maternal immune stimulation failed to normalize the expression of *Wnt2* in embryos with open as well as closed neural tubes from IFNγ +VA dams. This suggests that *Wnt2* was not involved in the reduction of NTDs by maternal immune stimulation.

Hox group genes, key regulators in early embryonic development, play a major role in patterning of the embryo. Disruption of these genes leads to malformations (Stodgell et al., 2006; Carpenter et al., 1993). In our study, expression of *Hoxa1* was reduced in embryos with open neural tubes. Recently Zhang et al., (2010) observed inhibition of *Hoxa2* in VA-exposed embryos. The authors also demonstrated that ascorbic acid

normalized *Hoxa2* expression and reduced VA-induced NTDs. These results imply that VA may disrupt Hox genes. In contrast to our study, Stodgell et al., (2006) observed over expression of *Hoxa1* in rat embryos exposed to VA. Whether this is a species difference or is due to measuring genes from whole embryos opposed to just embryonic heads as in our study is not known. Maternal immune stimulation failed to normalize the expression of *Hoxa1* in embryos with open as well as closed neural tubes from IFNγ+VA dams. Therefore, *Hoxa1* was not involved in the reduction of NTDs by maternal immune stimulation.

Fibronectins (Fn) promote cell migration and cytoskeletal organization (Hynes and Yamada, 1982; Darribere et al., 2000). Lack of fibronectin in developing embryos causes defects in the developing neural tube (George et al., 1993). In our study, VA significantly reduced the expression of *Fn1* in embryos with open neural tubes but not in embryos with closed neural tubes that indicated a role of *Fn1* in VA-induced teratogenesis. Maternal immune stimulation normalized the expression of *Fn1* in embryos with open as well as closed neural tubes indicated that *Fn1* may be involved in maternal immune stimulation protective mechanism.

Hspb1 is not essential for the mammalian development but can act together with other regulators to protect the embryo from stress-induced apoptosis (Huang et al., 2007). Down regulation of *Hspb1* was observed in our VA-exposed embryos with open neural tubes but not in embryos with closed neural tubes, which indicates a lack of cellular protective mechanism to prevent VA-induced apoptosis. Maternal immune stimulation

normalized the expression of *Hspb1* in embryos with closed neural tubes, which indicates that *Hspb1* may be involved in maternal immune stimulation protective mechanism. Maternal immune stimulation also normalized the expression of *Hspb1* in embryos with open neural tubes. Our studies showed that VA-induced alterations in apoptotic pathways were not normalized in these embryos. This indicates normalization of both pathways could be required for protection by immune stimulation.

In summary, VA altered the hedgehog (*Bmp2*), Wnt (*Fgf4, wnt2*), retinoic acid (*Hoxa1*) and AKT (*Fn1*) signaling pathways in embryos with open neural tubes but not in embryos with closed neural tubes except *Fn1*. These signaling pathways play roles in various morphogenic events during organogenesis including neural tube formation. The results from of our study indicate a role of these signaling pathways in formation of NTDs in addition to the apoptotic pathways. Maternal immune stimulation normalized the expression of *Fn1* and *Hspb1* in embryos with open as well as closed neural tubes, which indicated *Hspb1* and *Fn1* may be involved in maternal immune stimulation protective mechanism. Maternal immune stimulation failed to normalize the expression of *Bmp2*, *Wnt2* and *Hoxa1* in embryos with open as well as closed neural tubes from IFNγ+VA dams. This indicated that *Wnt2*, *Bmp2* and *Hoxa1* were not involved in the reduction of NTDs by maternal immune stimulation.

In embryos exposed to tap water, none of the signal transduction genes were significantly altered compared to controls. Expressions of the genes related to NFkB (*Tank and Tert*), Tgf- β pathway (*Cdkn2a*), mitogenic pathway (*Nab2*), retinoic acid pathway (*Rbp1*) and

hedgehog pathway (*Wnt1*) were altered more than two-fold, however, expression levels did not reach significance. As discussed above, all of these pathways play a role in embryonic development including neural tube formation. As only biological triplicates were used in this study, further analysis with larger sample size may give more insight into the molecular mechanisms behind the tap water teratogenicity. NTDs can result from functional change in the single gene or multiple genes (Copp and Greene, 2007). As this assay only screened candidate genes related to signaling pathways that are important in development, more detailed analysis of the pathways that showed a trend may be required to identify the specific signaling pathways that play a role in teratogenesis of the contaminat in the tap water.

V. CONCLUSIONS

VA-treated embryonic heads with open neural tubes had increased apoptosis localized to neural folds. Increased apoptosis from VA exposure possibly resulted from a protective mechanism in the embryo that removes damaged cells and replaces them with new cells. In addition to induction of apoptosis, VA also inhibits cell proliferation rates. Exuberant VA-induced apoptosis together with inhibition of cell proliferation in the neural fold could create insufficient tissue for normal fold elevation and apposition thus resulting in NTDs. Increased apoptosis in VA-exposed embryos with closed neural tubes suggests that other cellular processes such as cell proliferation and differentiation were not affected in these embryos. Apoptotic levels in embryos with closed neural tubes from IFNγ+VA dams were similar to controls indicating resistance to teratogen-induced apoptosis and protection against teratogens. In IFN+VA-exposed embryos with open neural tubes, maternal immune stimulation failed to regulate apoptosis, resulting in NTDs. This could have resulted from irreversible DNA damage induced by VA to the neuroepithelial cells in these embryos.

VA affected both apoptotic and anti-apoptotic gene expressions in GD 9 embryos. Bcl-2, p53, and death effector domain family genes were predominantly affected. VA down regulated anti-apoptotic Bcl-2 family genes and up regulated pro-apoptotic Bcl-2 family genes thereby shifting the ratio towards activation of pro-apoptotic signaling and increased apoptosis in neural folds. Expression of the Bcl-2 family apoptotic genes were normalized in embryos with closed NT from IFNγ+VA dams but not in embryos with open neural tubes, which indicates Bcl-2 family genes play a major role in the protective

effect of immune modulation of VA-induced NTDs. VA up regulated *caspase 9* and IFNγ+VA reduced the up regulation of *caspase 9.* VA down regulated *Akt1* and IFNγ+VA decreased the down regulation of *Akt1.* This indicated that VA affects regulators of apoptosis that belong to p53 family and immune stimulation normalizes this effect.

VA altered signal transduction genes relating to the hedgehog (*Bmp2*), Wnt (*Fgf4, wnt2*), retinoic acid (*Hoxa1*) and AKT (*Fn1*) families in embryos with open neural tubes but not in embryos with closed neural tubes. These results suggest that in addition to apoptotic pathways, VA also disrupted signaling pathways for various morphogenic events during organogenesis, including neural tube formation. Maternal immune stimulation normalized the expression of *Fn1* and *Hspb1* and thus may mediate protection through these signaling pathways.

In tap water-exposed embryos, neither flow cytometry nor TUNEL assay revealed any change in apoptosis compared to controls indicating apoptosis is not involved in teratogenicity from tap water exposure. These results were consistent with the PCR analysis that detected no changes in apoptotic pathway genes with exposure to tap water. This suggests that alteration of apoptosis is not a mechanism for teratogenicity resulting from exposure to the contaminant in tap water. Also, none of the pathways were significantly altered in tap water-exposed embryos.

Overall, these results suggest that teratogens may alter several biological processes including apoptosis to induce fetal malformations. Resistance to teratogen-induced apoptosis in embryos resulting from maternal immune stimulation may be involved in a protective mechanism.

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VII. APPENDIX

APPENDIX A. Functional Gene Grouping- Apoptotic pathways

TNF Ligand Family: Fasl (Tnfsf6), Tnf, Tnfsf10, Tnfsf12, Cd40lg, Cd70.

TNF Receptor Family: Fas (Tnfrsf6), Ltbr, Tnfrsf10b, Tnfrsf11b, Tnfrsf1a, Cd40.

Bcl-2 Family: Bad (Bbc2), Bag1, Bag3, Bak1, Bax, Bcl2, Bcl2l1, Bcl2l2, Bcl2l10, Bid, Bnip2, Bnip3, Bnip3l, Bok, Mcl1.

Caspase Family: Casp1, Casp2, Casp3, Casp4, Casp6, Casp7, Casp8, Casp9, Casp12, Casp14.

IAP Family: Birc1a, Birc1b, Birc2, Birc3, Birc4, Birc5.

TRAF Family: Traf1, Traf2, Traf3.

CARD Family: Apaf1, Bcl10, Birc3, Birc4, Nod1, Card6, Card10, Casp1, Casp2, Casp4, Casp9, Cradd, Nol3, Pycard (Asc), Ripk1.

Death Domain Family: Cradd, Dapk1, Fadd, Fas (Tnfrsf6), Ripk1, Tnfrsf10b (TRAIL-R), Tnfrsf11b, Tnfrsf1a.

Death Effector Domain Family: Casp8, Cflar (Cash), Fadd.

CIDE Domain Family: Cidea, Cideb, Dffa, Dffb.

p53 and DNA Damage-Induced Apoptosis: Akt1, Apaf1, Bad (Bbc2), Bax, Bcl2, Bcl2l1, Bid, Casp3, Casp6, Casp7, Casp9, Trp53 (p53), Trp53bp2, Trp53inp1, Trp63, Trp73.

Anti-Apoptosis: Akt1, Api5, Atf5, Bag1, Bag3, Bcl2, Bcl2l1, Bcl2l10, Bcl2l2, Birc1a, Birc1b, Birc2, Birc3, Birc4, Birc5, Bnip2, Bnip3, Casp2, Cflar, Dad1, Tsc22d3, Fas (Tnfrsf6), Hells, Il10, Lhx4, Mcl1, Nfkb1, Nme5, Pak7 (Arc), Pim2, Polb, Prdx2, Rnf7, Sphk2, Tnf, Cd40lg (CD40L), Zc3hc1 (Nipa)

APPENDIX B. Functional gene grouping- Signal transduction pathways

Mitogenic Pathway: Egr1 (egr-1), Fos (c-fos), Jun (c-jun), Nab2.

Wnt Pathway: Birc5, Ccnd1 (cyclin D1), Cdh1, Fgf4, Jun (c-jun), Lef1, Myc (c-myc), Pparg, Tcf7, Vegfa, Wisp1.

Hedgehog Pathway: Bmp2, Bmp4, En1 (engrailed), Foxa2 (forkhead box A2 / HNF3B), Hhip, Ptch1 (patched 1), Wnt1, Wnt2.

TGF-β Pathway: Cdkn1a (p21Waf1, p21Cip1), Cdkn1b (p27), Cdkn2a (p16Ink4), Cdkn2b (p15 Ink2b).

Survival Pathway:
PI3 Kinase / AKT Pathway: Bcl2 (Bcl-2), Ccnd1, Fn1 (fibronectin), Jun (c-jun), Mmp7 (matrilysin), Myc (c-myc).

Jak / Src Pathway: Bcl2 (Bcl-2), Bcl-XL.

NFκB Pathway: Birc1a, Birc2 (c-IAP2), Birc3 (c-IAP1), Tert.

p53 Pathway: Bax, Cdkn1a (p21Waf1, p21Cip1), Ei24 (Pig3), Fas (Tnfrsf6), Gadd45a (gadd45), Igfbp3, Mdm2.

Stress Pathway: Atf2, Fos (c-fos), Hsf1 (tcf5), Hspb1 (Hsp25), Myc (c-myc), Trp53 (p53).

NFκB Pathway: Ccl20, Cxcl1, Icam1, Ikbkb, Il1a, Il2, Lta (TNFb), Nfkbia, Nos2 (iNOS), Tank, Tnf (TNFa), Vcam1.

NFAT Pathway: Cd5, Fasl (Tnfsf6), Il2.

CREB Pathway: Cyp19a1, Egr1 (egr-1), Fos (c-fos).
Jak-Stat Pathway: Cxcl9 (MIG), Il4ra, Irf1, Mmp10 (stromelysin-2), Nos2 (iNOS).
Estrogen Pathway: Bcl2 (Bcl-2), Brca1, Greb1, Igfbp4, Nrip1.

Androgen Pathway: Cdk2, Cdkn1a (p21Waf1, p21Cip1), Tmepai.

Calcium and Protein Kinase C Pathway: Csf2 (GM-CSF), Fos (c-fos), Il2, Il2ra (IL-2 R), Jun (c-jun), Myc (c-myc), Odc1, Tfrc.

Phospholipase C Pathway: Bcl2 (Bcl-2), Egr1, Fos (c-fos), Icam1, Jun (c-jun), Nos2 (iNOS), Ptgs2 (cox-2), Vcam1.

Insulin Pathway: Cebpb (C/EBP), Fasn (fatty acid synthase), Gys1 (GS, glycogen synthase), Hk2 (hexokinase II), Lep (Ob).

LDL Pathway: Ccl2, Csf2 (GM-CSF), Sele, Selp (P-selectin), Vcam1.
Retinoic Acid Pathway: En1 (engrailed), Hoxa1, Rbp1 (CRBPI).

APPENDIX C. Fold change of genes related to apoptotic pathways in GD 8.5 embryos from VA treated dams compared to control group

Gene	Fold Change	*p*-value
Akt1	0.906	0.171
Apaf1	1.168	0.040
Api5	0.906	0.528
Atf5	0.671	0.067
Bad	0.687	0.013
Bag1	1.116	0.391
Bag3	0.719	0.095
Bak1	1.017	0.884
Bax	0.994	0.969
Bcl10	0.994	0.936
Bcl2	0.994	0.865
Bcl2l1	1.065	0.578
Bcl2l10	0.826	0.284
Bcl2l2	1.438	0.147
Bid	1.090	0.462
Naip1	0.826	0.284
Naip2	1.017	0.877
Birc2	1.168	0.392
Birc3	0.927	0.690
Xiap	1.406	0.260
Birc5	1.065	0.790
Bnip2	0.885	0.354
Bnip3	1.406	0.265
Bnip3l	1.168	0.336
Bok	0.753	0.089
Card10	0.994	0.866
Nod1	1.855	0.103
Card6	1.017	0.899
Casp1	0.687	0.303
Casp12	0.927	0.808
Casp14	0.826	0.284
Casp2	1.065	0.581
Casp3	1.252	0.211
Casp4	0.826	0.284
Casp6	0.885	0.117
Casp7	1.017	0.803
Casp8	1.090	0.489
Casp9	0.826	0.152
Cflar	1.142	0.242
Cidea	0.826	0.284
Cideb	0.206	0.012
Cradd	0.971	0.893
Dad1	0.736	0.042

Gene	Fold Change	*p*-value
Dapk1	0.906	0.503
Dffa	0.994	0.967
Dffb	1.342	0.221
Tsc22d3	1.196	0.290
Fadd	0.736	0.109
Fas	0.807	0.472
Fasl	1.168	0.608
Hells	1.116	0.630
Il10	0.826	0.284
Lhx4	0.807	0.612
Ltbr	1.090	0.730
Mcl1	1.374	0.460
Nfkb1	1.017	0.866
Nme5	0.994	0.999
Nol3	0.703	0.136
Pak7	1.065	0.773
Pim2	0.885	0.300
Polb	0.885	0.293
Prdx2	0.927	0.486
Pycard	0.789	0.289
Ripk1	1.406	0.046
Rnf7	1.988	0.111
Sphk2	0.949	0.357
Tnf	0.826	0.284
Tnfrsf10b	1.252	0.188
Tnfrsf11b	1.224	0.646
Tnfrsf1a	1.090	0.462
Cd40	0.994	0.925
Tnfsf10	0.826	0.284
Tnfsf12	0.641	0.052
Cd40lg	0.826	0.284
Cd70	0.826	0.284
Traf1	0.789	0.119
Traf2	0.906	0.249
Traf3	1.116	0.554
Trp53	0.885	0.214
Trp53bp2	1.142	0.358
Trp53inp1	1.090	0.414
Trp63	1.507	0.039
Trp73	0.826	0.284
Zc3hc1	0.687	0.022

APPENDIX D. Fold change of genes related to apoptotic pathways in GD 8.5 embryos from IFNγ+VA-treated dams compared to IFNγ only control group

Gene	Fold Change	*p*-value
Akt1	1.643	0.218
Apaf1	1.304	0.502
Api5	1.274	0.168
Atf5	1.059	0.695
Bad	0.554	0.400
Bag1	1.643	0.000
Bag3	1.304	0.055
Bak1	0.110	0.350
Bax	0.965	0.661
Bcl10	1.643	0.103
Bcl2	1.533	0.375
Bcl2l1	2.861	0.341
Bcl2l10	0.401	0.089
Bcl2l2	1.162	0.674
Bid	1.605	0.097
Naip1	0.401	0.089
Naip2	0.401	0.089
Birc2	1.011	0.909
Birc3	0.732	0.339
Xiap	0.802	0.279
Birc5	0.667	0.278
Bnip2	1.274	0.299
Bnip3	1.217	0.735
Bnip3l	1.398	0.277
Bok	1.245	0.427
Card10	0.802	0.231
Nod1	0.252	0.002
Card6	0.901	0.571
Casp1	0.401	0.089
Casp12	0.401	0.089
Casp14	0.401	0.089
Casp2	1.245	0.747
Casp3	0.802	0.462
Casp4	0.401	0.089
Casp6	1.274	0.079
Casp7	1.304	0.405
Casp8	3.605	0.360
Casp9	0.802	0.885
Cflar	1.430	0.426
Cidea	0.401	0.089
Cideb	0.608	0.448
Cradd	1.464	0.384
Dad1	2.996	0.376

Gene	Fold Change	*p*-value
Dapk1	0.652	0.482
Dffa	1.334	0.505
Dffb	1.304	0.146
Tsc22d3	1.398	0.051
Fadd	1.605	0.344
Fas	0.440	0.139
Fasl	0.699	0.949
Hells	1.109	0.701
Il10	0.517	0.139
Lhx4	0.840	0.635
Ltbr	1.681	0.359
Mcl1	0.341	0.132
Nfkb1	1.274	0.327
Nme5	0.943	0.904
Nol3	0.988	0.922
Pak7	0.608	0.067
Pim2	0.622	0.188
Polb	1.011	0.849
Prdx2	0.236	0.298
Pycard	0.732	0.217
Ripk1	1.217	0.547
Rnf7	0.784	0.098
Sphk2	0.965	0.898
Tnf	0.401	0.089
Tnfrsf10b	1.189	0.319
Tnfrsf11b	0.637	0.342
Tnfrsf1a	1.761	0.181
Cd40	1.398	0.361
Tnfsf10	0.401	0.089
Tnfsf12	0.766	0.300
Cd40lg	0.401	0.089
Cd70	0.401	0.089
Traf1	0.105	0.061
Traf2	2.378	0.382
Traf3	0.965	0.946
Trp53	1.059	0.552
Trp53bp2	0.901	0.511
Trp53inp1	0.110	0.272
Trp63	0.667	0.895
Trp73	0.155	0.004
Zc3hc1	0.098	0.661

APPENDIX E. Fold change of genes related to apoptotic pathways in GD9 embryos with open neural tubes from VA-treated dams compared to control group

Gene	Fold Change	*p*-value
Akt1	0.423	0.004
Apaf1	0.292	0.042
Api5	0.703	0.079
Atf5	1.857	0.001
Bad	4.169	0.005
Bag1	0.558	0.032
Bag3	1.170	0.371
Bak1	206.978	0.003
Bax	0.656	0.025
Bcl10	0.950	0.677
Bcl2	0.886	0.592
Bcl2l1	0.139	0.000
Bcl2l10	0.687	0.277
Bcl2l2	0.866	0.687
Bid	0.533	0.045
Naip1	0.687	0.277
Naip2	0.687	0.277
Birc2	0.972	0.805
Birc3	1.018	0.737
Xiap	0.720	0.225
Birc5	0.808	0.432
Bnip2	0.453	0.094
Bnip3	0.846	0.528
Bnip3l	0.571	0.029
Bok	2.133	0.016
Card10	0.995	0.967
Nod1	1.773	0.085
Card6	1.225	0.514
Casp1	0.656	0.267
Casp12	0.687	0.277
Casp14	0.687	0.277
Casp2	1.693	0.181
Casp3	0.627	0.220
Casp4	0.687	0.277
Casp6	2.183	0.914
Casp7	0.950	0.886
Casp8	0.027	0.010
Casp9	3.547	0.022
Cflar	0.216	0.004
Cidea	0.687	0.277
Cideb	15.562	0.000
Cradd	0.285	0.015
Dad1	0.002	0.000

Gene	Fold Change	*p*-value
Dapk1	0.368	0.147
Dffa	0.413	0.006
Dffb	1.197	0.121
Tsc22d3	0.313	0.085
Fadd	0.464	0.000
Fas	0.184	0.074
Fasl	0.585	0.095
Hells	0.423	0.104
Il10	0.687	0.277
Lhx4	0.703	0.625
Ltbr	0.687	0.058
Mcl1	63.704	0.002
Nfkb1	0.808	0.184
Nme5	0.907	0.509
Nol3	0.656	0.217
Pak7	0.545	0.169
Pim2	1.945	0.055
Polb	0.585	0.163
Prdx2	78.43	0.000
Pycard	0.907	0.618
Ripk1	0.423	0.000
Rnf7	1.225	0.249
Sphk2	1.143	0.328
Tnf	0.687	0.277
Tnfrsf10b	0.720	0.078
Tnfrsf11b	0.612	0.146
Tnfrsf1a	0.486	0.097
Cd40	0.351	0.038
Tnfsf10	0.687	0.277
Tnfsf12	1.042	0.804
Cd40lg	0.687	0.277
Cd70	0.687	0.277
Traf1	0.720	0.309
Traf2	0.049	0.001
Traf3	1.990	0.025
Trp53	1.018	0.987
Trp53bp2	1.117	0.292
Trp53inp1	0.328	0.102
Trp63	1.066	0.570
Trp73	0.687	0.277
Zc3hc1	0.754	0.049

APPENDIX F. Fold change of genes related to apoptotic pathways in GD9 embryos with closed neural tubes from VA-treated dams compared to control group

Gene	Fold Change	*p*-value
Akt1	0.458	0.002
Apaf1	0.288	0.042
Api5	0.678	0.097
Atf5	1.388	0.112
Bad	3.926	0.027
Bag1	0.590	0.042
Bag3	0.915	0.757
Bak1	213.782	0.008
Bax	0.563	0.011
Bcl10	0.854	0.395
Bcl2	0.854	0.560
Bcl2l1	0.147	0.000
Bcl2l10	0.604	0.192
Bcl2l2	0.835	0.575
Bid	0.502	0.036
Naip1	0.604	0.192
Naip2	0.604	0.192
Birc2	0.816	0.524
Birc3	1.076	0.844
Xiap	0.761	0.263
Birc5	0.959	0.916
Bnip2	0.458	0.094
Bnip3	0.835	0.524
Bnip3l	0.577	0.037
Bok	2.362	0.000
Card10	1.127	0.711
Nod1	1.594	0.089
Card6	1.028	0.966
Casp1	0.577	0.195
Casp12	0.604	0.192
Casp14	0.604	0.192
Casp2	1.963	0.056
Casp3	0.662	0.239
Casp4	0.604	0.192
Casp6	2.153	0.931
Casp7	0.854	0.680
Casp8	0.03	0.010
Casp9	3.837	0.020
Cflar	0.229	0.004
Cidea	0.604	0.192
Cideb	14.320	0.000
Cradd	0.339	0.018
Dad1	0.002	0.000

Gene	Fold Change	*p*-value
Dapk1	0.632	0.185
Dffa	0.389	0.005
Dffb	1.076	0.521
Tsc22d3	0.331	0.091
Fadd	0.458	0.000
Fas	0.181	0.075
Fasl	0.514	0.055
Hells	0.417	0.102
Il10	0.604	0.192
Lhx4	0.816	0.974
Ltbr	0.551	0.045
Mcl1	64.296	0.004
Nfkb1	0.678	0.034
Nme5	0.727	0.203
Nol3	0.618	0.363
Pak7	0.662	0.258
Pim2	1.790	0.096
Polb	0.538	0.139
Prdx2	77.350	0.000
Pycard	0.797	0.502
Ripk1	0.372	0.000
Rnf7	1.236	0.211
Sphk2	1.028	0.823
Tnf	0.604	0.192
Tnfrsf10b	0.563	0.012
Tnfrsf11b	0.538	0.091
Tnfrsf1a	0.447	0.083
Cd40	0.288	0.032
Tnfsf10	0.604	0.192
Tnfsf12	0.915	0.499
Cd40lg	0.604	0.192
Cd70	0.604	0.192
Traf1	0.604	0.192
Traf2	0.053	0.001
Traf3	1.918	0.060
Trp53	0.959	0.799
Trp53bp2	1.076	0.610
Trp53inp1	0.323	0.099
Trp63	0.835	0.412
Trp73	0.604	0.192
Zc3hc1	0.662	0.027

APPENDIX G. Fold change of genes related to apoptotic pathways in GD9 embryos with closed neural tubes from IFNγ+VA-treated dams compared to IFNγ only control group

Gene	Fold Change	*p*-value
Akt1	0.627	0.344
Apaf1	0.703	0.549
Api5	0.737	0.316
Atf5	1.654	0.066
Bad	2.751	0.150
Bag1	0.672	0.132
Bag3	1.440	0.156
Bak1	42.029	0.117
Bax	0.754	0.302
Bcl10	0.585	0.325
Bcl2	1.197	0.779
Bcl2l1	0.272	0.123
Bcl2l10	0.972	0.790
Bcl2l2	1.066	0.746
Bid	0.687	0.278
Naip1	0.972	0.790
Naip2	0.950	0.751
Birc2	0.808	0.356
Birc3	0.950	0.757
Xiap	0.928	0.690
Birc5	0.703	0.273
Bnip2	0.521	0.199
Bnip3	0.585	0.180
Bnip3l	0.641	0.176
Bok	1.344	0.313
Card10	1.117	0.699
Nod1	0.808	0.713
Card6	1.407	0.105
Casp1	0.808	0.533
Casp12	0.972	0.790
Casp14	0.972	0.790
Casp2	1.170	0.475
Casp3	0.571	0.244
Casp4	0.972	0.790
Casp6	0.509	0.246
Casp7	0.827	0.399
Casp8	0.086	0.053
Casp9	2.508	0.042
Cflar	0.386	0.117
Cidea	0.972	0.790
Cideb	7.603	0.121
Cradd	0.497	0.157
Dad1	0.016	0.143

Gene	Fold Change	*p*-value
Dapk1	0.641	0.609
Dffa	0.558	0.078
Dffb	1.170	0.067
Tsc22d3	0.395	0.161
Fadd	0.571	0.266
Fas	0.509	0.514
Fasl	0.772	0.380
Hells	0.772	0.466
Il10	0.972	0.790
Lhx4	0.571	0.160
Ltbr	0.720	0.214
Mcl1	7.963	0.125
Nfkb1	0.79	0.051
Nme5	0.703	0.270
Nol3	0.545	0.023
Pak7	0.612	0.222
Pim2	1.654	0.157
Polb	0.533	0.081
Prdx2	15.562	0.121
Pycard	0.79	0.401
Ripk1	0.509	0.047
Rnf7	0.886	0.874
Sphk2	1.375	0.111
Tnf	0.972	0.790
Tnfrsf10b	0.808	0.081
Tnfrsf11b	0.907	0.668
Tnfrsf1a	0.687	0.247
Cd40	0.404	0.092
Tnfsf10	0.972	0.790
Tnfsf12	1.225	0.399
Cd40lg	0.972	0.790
Cd70	0.972	0.790
Traf1	0.972	0.790
Traf2	0.094	0.039
Traf3	1.344	0.031
Trp53	1.066	0.717
Trp53bp2	1.066	0.619
Trp53inp1	0.386	0.161
Trp63	0.641	0.197
Trp73	0.972	0.790
Zc3hc1	0.808	0.388

APPENDIX H. Fold change of genes related to apoptotic pathways in GD9 embryos with open neural tubes from IFNγ+VA-treated dams compared to IFNγ only control group

Gene	Fold Change	*p*-value
Akt1	0.211	0.021
Apaf1	0.413	0.205
Api5	0.248	0.180
Atf5	0.972	0.681
Bad	3.890	0.024
Bag1	0.193	0.069
Bag3	0.509	0.819
Bak1	248.999	0.003
Bax	0.886	0.996
Bcl10	1.474	0.383
Bcl2	0.368	0.390
Bcl2l1	0.040	0.001
Bcl2l10	0.808	0.505
Bcl2l2	0.497	0.386
Bid	0.598	0.142
Naip1	0.808	0.505
Naip2	0.79	0.471
Birc2	0.211	0.185
Birc3	0.497	0.483
Xiap	0.486	0.198
Birc5	0.423	0.155
Bnip2	0.260	0.101
Bnip3	0.351	0.282
Bnip3l	0.521	0.073
Bok	1.544	0.240
Card10	0.972	0.826
Nod1	0.972	0.896
Card6	1.313	0.284
Casp1	0.672	0.411
Casp12	0.808	0.505
Casp14	0.808	0.505
Casp2	0.292	0.934
Casp3	0.754	0.431
Casp4	0.808	0.505
Casp6	0.433	0.251
Casp7	0.433	0.418
Casp8	0.029	0.004
Casp9	3.630	0.012
Cflar	0.260	0.045
Cidea	0.808	0.505
Cideb	19.607	0.000
Cradd	0.328	0.061
Dad1	0.001	0.002

Gene	Fold Change	*p*-value
Dapk1	0.558	0.237
Dffa	0.360	0.009
Dffb	0.928	0.531
Tsc22d3	0.180	0.084
Fadd	0.180	0.046
Fas	0.211	0.021
Fasl	0.585	0.139
Hells	0.720	0.422
Il10	0.808	0.505
Lhx4	0.533	0.248
Ltbr	0.509	0.046
Mcl1	4.680	0.155
Nfkb1	0.641	0.013
Nme5	0.720	0.245
Nol3	0.598	0.133
Pak7	0.377	0.111
Pim2	1.440	0.289
Polb	0.533	0.082
Prdx2	62.249	0.000
Pycard	0.808	0.396
Ripk1	0.386	0.015
Rnf7	1.170	0.274
Sphk2	1.143	0.571
Tnf	0.808	0.505
Tnfrsf10b	0.627	0.006
Tnfrsf11b	0.754	0.403
Tnfrsf1a	0.443	0.019
Cd40	0.260	0.040
Tnfsf10	0.808	0.505
Tnfsf12	1.117	0.687
Cd40lg	0.808	0.505
Cd70	0.808	0.505
Traf1	0.808	0.505
Traf2	0.045	0.005
Traf3	1.254	0.287
Trp53	1.066	0.704
Trp53bp2	1.066	0.561
Trp53inp1	0.266	0.124
Trp63	0.453	0.106
Trp73	0.808	0.505
Zc3hc1	0.545	0.110

APPENDIX I. Fold change of genes related to apoptotic pathways in GD9 embryos with open neural tubes from tap water exposed dams compared to control group

Gene	Fold Change	*p*-value
Akt1	1.168	0.799
Apaf1	0.826	0.889
Api5	0.807	0.758
Atf5	5.247	0.561
Bad	0.971	0.955
Bag1	0.736	0.638
Bag3	0.971	0.877
Bak1	1.252	0.846
Bax	0.753	0.274
Bcl10	0.376	0.445
Bcl2	0.703	0.628
Bcl2l1	0.584	0.621
Bcl2l10	1.224	0.610
Bcl2l2	1.041	0.878
Bid	1.142	0.636
Naip1	1.224	0.610
Naip2	0.906	0.866
Birc2	0.687	0.815
Birc3	0.927	0.798
Xiap	0.865	0.899
Birc5	0.826	0.546
Bnip2	0.687	0.476
Bnip3	0.753	0.598
Bnip3l	0.906	0.925
Bok	1.017	0.937
Card10	1.281	0.474
Nod1	0.865	0.660
Card6	3.462	0.369
Casp1	1.252	0.446
Casp12	1.578	0.268
Casp14	0.949	0.969
Casp2	0.771	0.723
Casp3	0.703	0.380
Casp4	1.065	0.791
Casp6	0.719	0.264
Casp7	0.807	0.728
Casp8	1.065	0.881
Casp9	0.771	0.665
Cflar	1.065	0.741
Cidea	1.224	0.610
Cideb	1.142	0.882
Cradd	1.065	0.774
Dad1	0.719	0.934

Gene	Fold Change	*p*-value
Dapk1	1.041	0.928
Dffa	1.041	0.846
Dffb	0.807	0.415
Tsc22d3	0.865	0.731
Fadd	0.826	0.932
Fas	1.041	0.910
Fasl	1.342	0.414
Hells	1.142	0.985
Il10	1.224	0.610
Lhx4	1.281	0.555
Ltbr	0.242	0.592
Mcl1	1.406	0.933
Nfkb1	0.736	0.208
Nme5	1.116	0.950
Nol3	1.281	0.518
Pak7	0.736	0.454
Pim2	1.281	0.810
Polb	1.017	0.815
Prdx2	0.545	0.576
Pycard	0.807	0.668
Ripk1	0.703	0.401
Rnf7	2.685	0.386
Sphk2	0.807	0.443
Tnf	1.224	0.610
Tnfrsf10b	0.845	0.533
Tnfrsf11b	0.771	0.562
Tnfrsf1a	0.807	0.464
Cd40	0.885	0.741
Tnfsf10	1.224	0.610
Tnfsf12	1.017	0.979
Cd40lg	1.224	0.610
Cd70	1.224	0.610
Traf1	1.116	0.724
Traf2	0.906	0.909
Traf3	0.571	0.899
Trp53	1.041	0.688
Trp53bp2	1.116	0.486
Trp53inp1	4.784	0.223
Trp63	1.017	0.875
Trp73	1.224	0.610
Zc3hc1	0.771	0.493

APPENDIX J. Fold change of genes related to signal transduction pathways in GD 8.5 embryos from VA-treated dams compared to control group

Gene	Fold Change	*p*-value
Atf2	0.821	0.171
Bax	1.430	0.050
Bcl2	2.118	0.202
Naip1	0.683	0.009
Birc2	1.304	0.029
Birc3	0.529	0.105
Birc5	1.109	0.740
Bmp2	0.840	0.392
Bmp4	1.059	0.630
Brca1	1.430	0.093
Ccl2	0.608	0.145
Ccl20	0.683	0.009
Ccnd1	2.324	0.089
Cd5	0.420	0.189
Cdh1	0.374	0.082
Cdk2	0.471	0.129
Cdkn1a	1.189	0.102
Cdkn1b	1.109	0.448
Cdkn2a	0.567	0.356
Cdkn2b	1.887	0.030
Cebpb	0.637	0.007
Csf2	0.683	0.009
Cxcl1	0.715	0.053
Cxcl9	0.683	0.009
Cyp19a1	0.683	0.009
Egr1	0.318	0.106
Ei24	1.802	0.027
En1	1.189	0.255
Fas	0.860	0.633
Fasl	0.471	0.382
Fasn	7.551	0.111
Fgf4	0.965	0.794
Fn1	1.109	0.498
Fos	0.505	0.126
Foxa2	0.943	0.615
Gadd45a	1.011	0.956
Greb1	1.245	0.120
Gys1	0.988	0.976
Hhip	1.084	0.649
Hk2	0.988	0.896
Hoxa1	0.567	0.146
Hsf1	0.784	0.227
Hspb1	0.318	0.119

Gene	Fold Change	*p*-value
Icam1	1.162	0.439
Igfbp3	1.135	0.351
Igfbp4	1.189	0.509
Ikbkb	1.011	0.897
Il1a	0.471	0.231
Il2	0.683	0.009
Il2ra	0.683	0.009
Il4ra	5.722	0.448
Irf1	1.304	0.094
Jun	1.189	0.025
Lef1	0.567	0.150
Lep	0.683	0.009
Lta	0.860	0.592
Mdm2	0.191	0.109
Mmp10	0.683	0.009
Mmp7	0.683	0.009
Myc	0.880	0.608
Nab2	1.334	0.207
Nfkbia	0.542	0.100
Nos2	0.392	0.241
Nrip1	0.554	0.213
Odc1	1.059	0.433
Pparg	2.219	0.129
Ptch1	1.533	0.057
Ptgs2	1.274	0.585
Rbp1	1.245	0.346
Sele	1.084	0.589
Selp	0.450	0.234
Tank	47.945	0.045
Tcf7	0.483	0.056
Tert	1.189	0.360
Tfrc	1.533	0.068
Pmepa1	2.434	0.756
Tnf	0.840	0.710
Trp53	1.245	0.072
Vcam1	0.494	0.150
Vegfa	2.669	0.036
Wisp1	0.283	0.115
Wnt1	1.217	0.111
Wnt2	0.440	0.516

APPENDIX K. Fold change of genes related to signal transduction pathways in GD 8.5 embryos from IFNγ+VA-treated dams compared to IFNγ only control group

Gene	Fold Change	*p*-value
Atf2	0.762	0.159
Bax	1.422	0.116
Bcl2	2.011	0.267
Naip1	0.855	0.850
Birc2	0.917	0.408
Birc3	0.619	0.365
Birc5	1.422	0.437
Bmp2	0.960	0.896
Bmp4	1.455	0.196
Brca1	0.982	0.838
Ccl2	1.327	0.459
Ccl20	0.855	0.850
Ccnd1	1.560	0.445
Cd5	0.458	0.235
Cdh1	0.648	0.657
Cdk2	0.577	0.346
Cdkn1a	1.238	0.266
Cdkn1b	1.389	0.463
Cdkn2a	2.364	0.404
Cdkn2b	1.965	0.105
Cebpb	0.780	0.109
Csf2	0.855	0.850
Cxcl1	0.855	0.850
Cxcl9	2.156	0.391
Cyp19a1	1.21	0.488
Egr1	0.564	0.388
Ei24	1.103	0.729
En1	1.21	0.212
Fas	1.078	0.949
Fasl	0.381	0.395
Fasn	2.310	0.797
Fgf4	1.155	0.615
Fn1	0.917	0.555
Fos	1.455	0.451
Foxa2	1.053	0.767
Gadd45a	1.596	0.323
Greb1	1.005	0.919
Gys1	0.817	0.300
Hhip	0.917	0.559
Hk2	1.029	0.795
Hoxa1	0.982	0.899
Hsf1	0.711	0.131
Hspb1	0.564	0.528

Gene	Fold Change	*p*-value
Icam1	0.780	0.140
Igfbp3	1.21	0.107
Igfbp4	1.238	0.465
Ikbkb	0.836	0.433
Il1a	0.591	0.569
Il2	0.896	0.815
Il2ra	1.029	0.586
Il4ra	2.911	0.817
Irf1	1.389	0.266
Jun	0.896	0.143
Lef1	0.648	0.244
Lep	1.489	0.429
Lta	1.560	0.116
Mdm2	0.526	0.403
Mmp10	0.855	0.850
Mmp7	1.965	0.396
Myc	0.780	0.231
Nab2	1.103	0.428
Nfkbia	0.648	0.244
Nos2	1.524	0.402
Nrip1	0.437	0.170
Odc1	1.005	0.860
Pparg	2.979	0.334
Ptch1	1.155	0.503
Ptgs2	2.011	0.116
Rbp1	0.128	0.978
Sele	4.311	0.375
Selp	1.524	0.412
Tank	36.971	0.117
Tcf7	0.679	0.391
Tert	0.938	0.731
Tfrc	1.389	0.069
Pmepa1	3.193	0.657
Tnf	1.422	0.290
Trp53	1.155	0.476
Vcam1	0.648	0.813
Vegfa	1.455	0.497
Wisp1	0.347	0.17
Wnt1	1.21	0.451
Wnt2	0.855	0.892

APPENDIX L. Fold change of genes related to signal transduction pathways in GD9 embryos with closed neural tubes from VA-treated dams compared to control group

Gene	Fold Change	*p*-value
Atf2	0.911	0.677
Bax	1.148	0.625
Bcl2	0.850	0.763
Naip1	0.850	0.685
Birc2	0.775	0.228
Birc3	0.775	0.709
Birc5	1.447	0.182
Bmp2	0.535	0.200
Bmp4	0.740	0.113
Brca1	1.071	0.940
Ccl2	0.870	0.535
Ccl20	0.850	0.685
Ccnd1	1.023	0.877
Cd5	0.707	0.568
Cdh1	0.615	0.670
Cdk2	0.850	0.942
Cdkn1a	0.977	0.970
Cdkn1b	0.850	0.307
Cdkn2a	0.337	0.740
Cdkn2b	0.831	0.527
Cebpb	1	0.953
Csf2	0.850	0.685
Cxcl1	0.812	0.547
Cxcl9	0.850	0.685
Cyp19a1	0.870	0.789
Egr1	0.793	0.971
Ei24	0.911	0.721
En1	0.740	0.162
Fas	0.933	0.984
Fasl	0.396	0.355
Fasn	0.757	0.647
Fgf4	0.353	0.029
Fn1	0.561	0.113
Fos	0.574	0.751
Foxa2	0.587	0.140
Gadd45a	0.659	0.208
Greb1	0.775	0.156
Gys1	0.911	0.224
Hhip	0.954	0.895
Hk2	0.890	0.696
Hoxa1	0.198	0.386
Hsf1	0.870	0.963
Hspb1	0.267	0.935

Gene	Fold Change	*p*-value
Icam1	0.850	0.454
Igfbp3	0.723	0.077
Igfbp4	0.831	0.533
Ikbkb	1	0.813
Il1a	0.587	0.363
Il2	0.850	0.685
Il2ra	0.850	0.685
Il4ra	0.850	0.743
Irf1	0.933	0.635
Jun	0.691	0.065
Lef1	1	0.876
Lep	0.850	0.685
Lta	0.812	0.558
Mdm2	0.775	0.898
Mmp10	0.850	0.685
Mmp7	0.850	0.685
Myc	0.707	0.246
Nab2	0.870	0.712
Nfkbia	1.023	0.972
Nos2	0.977	0.617
Nrip1	0.691	0.792
Odc1	0.954	0.829
Pparg	0.435	0.147
Ptch1	0.775	0.296
Ptgs2	0.477	0.116
Rbp1	0.977	0.830
Sele	0.740	0.331
Selp	1.289	0.467
Tank	0.707	0.546
Tcf7	0.812	0.889
Tert	0.850	0.480
Tfrc	0.659	0.143
Pmepa1	1.259	0.693
Tnf	0.707	0.282
Trp53	0.675	0.101
Vcam1	1.122	0.828
Vegfa	0.775	0.621
Wisp1	0.691	0.790
Wnt1	0.740	0.124
Wnt2	0.086	0.331

APPENDIX M. Fold change of genes related to signal transduction pathways in GD9 embryos with open neural tubes from VA-treated dams compared to control group

Gene	Fold Change	*p*-value
Atf2	1.633	0.396
Bax	1.078	0.792
Bcl2	0.917	0.989
Naip1	0.836	0.485
Birc2	0.347	0.263
Birc3	0.591	0.292
Birc5	1.053	0.627
Bmp2	0.363	0.011
Bmp4	0.663	0.016
Brca1	0.960	0.850
Ccl2	0.855	0.839
Ccl20	0.836	0.485
Ccnd1	1.633	0.366
Cd5	0.526	0.403
Cdh1	0.427	0.273
Cdk2	0.817	0.580
Cdkn1a	0.960	0.706
Cdkn1b	1.965	0.344
Cdkn2a	0.240	0.103
Cdkn2b	0.982	0.997
Cebpb	0.817	0.384
Csf2	0.836	0.485
Cxcl1	0.798	0.336
Cxcl9	1.21	0.518
Cyp19a1	0.836	0.485
Egr1	0.744	0.698
Ei24	1.078	0.878
En1	0.679	0.114
Fas	0.896	0.785
Fasl	0.39	0.352
Fasn	0.982	0.970
Fgf4	0.141	0.002
Fn1	0.480	0.007
Fos	0.679	0.964
Foxa2	0.762	0.155
Gadd45a	0.817	0.410
Greb1	0.982	0.898
Gys1	1.005	0.898
Hhip	0.982	0.856
Hk2	0.982	0.810
Hoxa1	0.054	0.005
Hsf1	1.078	0.666
Hspb1	0.169	0.027
Icam1	0.663	0.108
Igfbp3	0.514	0.024
Igfbp4	0.917	0.551
Ikbkb	1.267	0.337
Il1a	0.836	0.876
Il2	0.836	0.485
Il2ra	0.836	0.485
Il4ra	0.875	0.555
Irf1	1.029	0.874
Jun	0.798	0.102
Lef1	1.238	0.569
Lep	1.029	0.786
Lta	0.875	0.774
Mdm2	0.109	0.328
Mmp10	0.836	0.485
Mmp7	0.836	0.485
Myc	0.619	0.099
Nab2	1.053	0.919
Nfkbia	1.078	0.899
Nos2	3.584	0.377
Nrip1	0.711	0.457
Odc1	0.938	0.365
Pparg	0.514	0.203
Ptch1	0.762	0.573
Ptgs2	0.372	0.067
Rbp1	0.064	0.276
Sele	0.798	0.517
Selp	0.836	0.485
Tank	4.412	0.942
Tcf7	0.875	0.842
Tert	0.982	0.959
Tfrc	0.780	0.298
Pmepa1	1.455	0.481
Tnf	0.695	0.169
Trp53	1.078	0.481
Vcam1	1.267	0.731
Vegfa	0.982	0.988
Wisp1	0.399	0.328
Wnt1	0.591	0.273
Wnt2	0.022	0.000

APPENDIX N. Fold change of genes related to signal transduction pathways in GD9 embryos with closed neural tubes from IFNγ+VA-treated dams compared to IFNγ only control group

Gene	Fold Change	*p*-value
Atf2	1.252	0.311
Bax	1.252	0.179
Bcl2	0.885	0.577
Naip1	0.927	0.932
Birc2	1.090	0.451
Birc3	0.865	0.910
Birc5	0.906	0.684
Bmp2	0.463	0.000
Bmp4	0.826	0.248
Brca1	1.017	0.789
Ccl2	1.090	0.988
Ccl20	0.807	0.589
Ccnd1	2.082	0.211
Cd5	1.252	0.589
Cdh1	0.508	0.075
Cdk2	0.807	0.802
Cdkn1a	1.116	0.205
Cdkn1b	0.508	0.215
Cdkn2a	0.071	0.078
Cdkn2b	0.571	0.307
Cebpb	1.116	0.675
Csf2	0.927	0.932
Cxcl1	0.927	0.932
Cxcl9	0.175	0.201
Cyp19a1	0.927	0.932
Egr1	0.719	0.794
Ei24	1.507	0.169
En1	0.927	0.608
Fas	1.065	0.858
Fasl	1.116	0.599
Fasn	3.383	0.329
Fgf4	0.403	0.069
Fn1	0.545	0.011
Fos	0.558	0.093
Foxa2	1.224	0.064
Gadd45a	0.571	0.048
Greb1	1.252	0.144
Gys1	0.971	0.877
Hhip	1.374	0.157
Hk2	0.641	0.145
Hoxa1	0.080	0.016
Hsf1	0.906	0.438
Hspb1	0.164	0.126

Gene	Fold Change	*p*-value
Icam1	1.342	0.070
Igfbp3	0.971	0.737
Igfbp4	1.691	0.044
Ikbkb	1.090	0.243
Il1a	0.703	0.414
Il2	0.927	0.932
Il2ra	0.927	0.932
Il4ra	3.014	0.339
Irf1	1.065	0.882
Jun	0.949	0.702
Lef1	0.865	0.454
Lep	0.927	0.977
Lta	0.927	0.932
Mdm2	0.497	0.724
Mmp10	0.927	0.932
Mmp7	0.927	0.932
Myc	0.807	0.337
Nab2	1.652	0.159
Nfkbia	1.542	0.155
Nos2	0.034	0.253
Nrip1	0.719	0.736
Odc1	1.142	0.373
Pparg	0.753	0.594
Ptch1	1.196	0.441
Ptgs2	0.753	0.634
Rbp1	167.924	0.088
Sele	0.598	0.353
Selp	0.753	0.420
Tank	0.153	0.487
Tcf7	0.687	0.303
Tert	1.041	0.710
Tfrc	1.017	0.840
Pmepa1	3.977	0.333
Tnf	0.927	0.932
Trp53	0.906	0.507
Vcam1	1.311	0.350
Vegfa	1.342	0.382
Wisp1	0.826	0.644
Wnt1	2.035	0.067
Wnt2	0.021	0.031

APPENDIX O. Fold change of genes related to signal transduction pathways in GD9 embryos with open neural tubes from IFNγ+VA-treated dams compared to IFNγ only control group

Gene	Fold Change	*p*-value
Atf2	0.865	0.452
Bax	1.342	0.267
Bcl2	0.266	0.169
Naip1	0.927	0.913
Birc2	0.042	0.262
Birc3	0.083	0.028
Birc5	0.626	0.789
Bmp2	0.584	0.007
Bmp4	0.656	0.011
Brca1	0.826	0.563
Ccl2	1.196	0.781
Ccl20	1.116	0.876
Ccnd1	1.041	0.842
Cd5	1.281	0.647
Cdh1	0.497	0.009
Cdk2	0.598	0.263
Cdkn1a	1.281	0.216
Cdkn1b	1.142	0.926
Cdkn2a	0.164	0.105
Cdkn2b	0.545	0.165
Cebpb	0.545	0.088
Csf2	0.927	0.913
Cxcl1	0.927	0.913
Cxcl9	0.598	0.387
Cyp19a1	0.927	0.913
Egr1	1.065	0.687
Ei24	0.949	0.699
En1	0.584	0.066
Fas	0.485	0.275
Fasl	0.927	0.913
Fasn	1.065	0.706
Fgf4	0.771	0.343
Fn1	0.545	0.099
Fos	0.571	0.039
Foxa2	0.949	0.867
Gadd45a	0.558	0.134
Greb1	0.865	0.604
Gys1	0.826	0.101
Hhip	1.196	0.405
Hk2	0.612	0.021
Hoxa1	0.211	0.031
Hsf1	1.116	0.543
Hspb1	0.313	0.170

Gene	Fold Change	*p*-value
Icam1	0.885	0.675
Igfbp3	0.641	0.064
Igfbp4	1.224	0.331
Ikbkb	1.065	0.307
Il1a	2.082	0.347
Il2	0.927	0.913
Il2ra	0.927	0.913
Il4ra	2.284	0.022
Irf1	1.017	0.978
Jun	0.703	0.014
Lef1	0.949	0.967
Lep	0.826	0.772
Lta	0.994	0.826
Mdm2	0.010	0.019
Mmp10	0.927	0.913
Mmp7	0.927	0.913
Myc	0.520	0.050
Nab2	1.406	0.268
Nfkbia	0.971	0.898
Nos2	0.328	0.420
Nrip1	0.520	0.018
Odc1	0.807	0.064
Pparg	0.789	0.082
Ptch1	0.753	0.767
Ptgs2	0.703	0.434
Rbp1	0.949	0.737
Sele	0.994	0.749
Selp	1.168	0.947
Tank	1.116	0.968
Tcf7	0.885	0.875
Tert	0.927	0.729
Tfrc	0.845	0.155
Pmepa1	1.065	0.674
Tnf	0.927	0.913
Trp53	0.826	0.458
Vcam1	1.142	0.612
Vegfa	1.116	0.677
Wisp1	0.906	0.630
Wnt1	1.041	0.851
Wnt2	0.045	0.034

APPENDIX P. Fold change of genes related to signal transduction pathways in GD9 embryos with open neural tubes from tap water exposed dams compared to control group

Gene	Fold Change	*p*-value
Atf2	1.374	0.451
Bax	0.865	0.568
Bcl2	1.168	0.684
Naip1	1.196	0.605
Birc2	0.753	0.660
Birc3	1.311	0.428
Birc5	0.906	0.853
Bmp2	0.789	0.725
Bmp4	1.090	0.706
Brca1	0.845	0.342
Ccl2	1.578	0.180
Ccl20	1.196	0.605
Ccnd1	0.845	0.454
Cd5	1.652	0.084
Cdh1	1.142	0.592
Cdk2	1.017	0.837
Cdkn1a	0.865	0.345
Cdkn1b	1.691	0.449
Cdkn2a	2.082	0.292
Cdkn2b	1.615	0.109
Cebpb	1.406	0.251
Csf2	1.196	0.605
Cxcl1	1.196	0.605
Cxcl9	1.542	0.419
Cyp19a1	1.196	0.605
Egr1	0.906	0.741
Ei24	0.994	0.799
En1	1.196	0.334
Fas	1.065	0.647
Fasl	1.168	0.651
Fasn	1.196	0.979
Fgf4	0.753	0.442
Fn1	0.927	0.867
Fos	0.612	0.672
Foxa2	0.927	0.871
Gadd45a	1.142	0.550
Greb1	1.041	0.735
Gys1	0.736	0.361
Hhip	1.224	0.423
Hk2	0.807	0.967
Hoxa1	1.615	0.253
Hsf1	1.142	0.610
Hspb1	1.771	0.450

Gene	Fold Change	*p*-value
Icam1	0.771	0.743
Igfbp3	1.311	0.422
Igfbp4	0.789	0.314
Ikbkb	1.065	0.719
Il1a	1.017	0.853
Il2	1.196	0.605
Il2ra	1.196	0.605
Il4ra	0.687	0.614
Irf1	0.719	0.058
Jun	1.224	0.338
Lef1	1.196	0.551
Lep	1.196	0.605
Lta	1.196	0.605
Mdm2	1.855	0.552
Mmp10	1.196	0.605
Mmp7	1.196	0.605
Myc	0.771	0.848
Nab2	0.272	0.174
Nfkbia	0.753	0.742
Nos2	1.943	0.404
Nrip1	1.116	0.537
Odc1	0.845	0.496
Pparg	1.507	0.409
Ptch1	1.116	0.854
Ptgs2	1.615	0.467
Rbp1	0.080	0.594
Sele	1.196	0.605
Selp	1.196	0.605
Tank	5.127	0.521
Tcf7	0.671	0.935
Tert	0.474	0.286
Tfrc	0.885	0.834
Pmepa1	1.142	0.775
Tnf	1.196	0.605
Trp53	0.885	0.679
Vcam1	1.090	0.549
Vegfa	0.753	0.310
Wisp1	1.731	0.541
Wnt1	0.285	0.109
Wnt2	1.406	0.462