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Selected environmental exposures and risk of neural tube defects

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SELECTED ENVIRONMENTAL EXPOSURES AND RISK OF NEURAL TUBE DEFECTS

by

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An Abstract

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ABSTRACT

With a birth prevalence of 1 in 1000, neural tube defects (NTD)s contribute considerably to morbidity and healthcare costs. Known genetic and environmental (non-inherited) risk factors for NTDs account for a small portion of risk, suggesting unidentified risk factors. In animal studies, maternal alcohol and pesticide exposures, independently, led to excess neural cell death, resulting in too few cells for neural tube closure. Human studies report no association between alcohol exposure and NTDs, but small to moderate positive associations for pesticide exposure. Such human etiologic studies of NTDs require a large base population, but frequently include only live births. Exclusion of cases by pregnancy outcomes may create ascertainment and response bias, complicating interpretation of findings.

Using data from the National Birth Defects Prevention Study (NBDPS) and the Iowa Registry for Congenital and Inherited Disorders (IRCID), the independent effects of maternal periconceptional (1 month prior through 2 months postconception) alcohol and occupational pesticide exposure on the development of NTDs were examined, and differences in Iowa NTD cases were characterized by pregnancy outcome.

Maternal reports of alcohol exposure were obtained for 1223 NTD case infants and 6807 control infants. Adjusted odds ratios, estimated using multivariate logistic regression, were near unity for NTDs by any maternal alcohol exposure, binge episode(s), and type(s) of alcohol consumed. Occupational pesticide exposure was assigned by industrial hygienists for mothers of 502 case and 2950 control infants. Adjusted odds ratios for any exposure and cumulative exposure to any pesticide,

insecticides only, and insecticides + herbicides + fungicides were near unity for NTDs. Insecticide + herbicide exposure was positively associated with spina bifida. Among the 279 Iowa NTD case infants ascertained by the IRCID, 167 live births and 112 were other pregnancy outcomes (fetal deaths and elective terminations), which increased in proportion over time. Selected infant and maternal characteristics of live births and other pregnancy outcomes were similar. NBDPS eligibility varied significantly by pregnancy outcome, but participation rates did not. NTD case mothers were similar to Iowa NBDPS control mothers.

Efforts were made to improve upon prior etiologic studies of these exposures and NTDs, including increased sample size and improved exposure specificity. Some exposure strata (e.g., herbicides only) and outcome strata (e.g., other rare subtypes) were limited by small numbers. All results may have been affected by response and ascertainment bias. Future studies should aim to use similarly detailed exposure classification methods, increase sample size in less prevalent NTD subtypes, and improve ascertainment of fetal deaths.

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PH.D THESIS

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LIST OF ABBREVIATIONS

5-MTHFA	5- methylenetetrahydrofolate reductase
aOR	adjusted odds ratio
BPA	British Pediatric Association
BRFSS	Behavioral Risk Factor Surveillance Study
CATI	computer assisted telephone interview
CDC	Centers for Disease Control and Prevention
CI	confidence interval
CNS	central nervous system
DFE	dietary folate equivalent
EDD	estimated date of delivery
ET	elective terminations
FAEE	fatty acid ethyl ethers
IRCID	Iowa Registry for Congenital and Inherited Disorders
JEM	job exposure matrix
LLR	log-likelihood ratio
MTHFR	methylenetetrahydrofolate reductase (
NBDPS	National Birth Defects Prevention Study
NC	not calculated
NIOSH	National Institute for Occupational Safety and Health
NTD	neural tube defects
OR	odds ratio
SAS	Statistical Analysis Software
USDA	United States Department of Agriculture

CHAPTER I

SELECTED ENVIRONMENTAL EXPOSURES AND RISK OF NEURAL TUBE DEFECTS

Public Health Significance

Neural tube defects (NTD)s, which result from failure of the neural tube to properly close, are one of the most common birth defects world-wide. In the United States, the birth prevalence of NTDs is estimated at 1 in 1000 births (reviewed by Detrait, George et al. 2005). In addition, an unknown number of NTD-affected pregnancies end in fetal death or elective termination. The most common types of NTDs are anencephaly and spina bifida. These defects are usually "open" NTDs, in which the neural tissue is exposed to the environment or covered only by a membrane. Less common are NTDs such as encephalocele, meningocele, and other rare subtypes, which are typically "closed" NTDs, in which the defect is covered by normal skin.

In all instances, anencephaly is fatal, either *in utero* or shortly after birth. Following extensive surgery, infants with spina bifida often survive. An estimated 50% of infants with spina bifida who received surgical repairs shortly after birth survived to the third decade of life (Hunt 1997). Yet, even with surgery, children with spina bifida frequently suffer from severe disability (Date, Yagyu et al. 1993), require continued medical treatment, and are at an increased risk of poor psychosocial adjustment (Zurmohle, Homann et al. 1998). Grosse et al. (2008) estimated the direct cost of spina bifida over a lifetime--medical, non-medical (developmental, education,

etc), and caregiver cost--to be $560,000 per case (2003 US dollars). As such, NTDs contribute considerably to national healthcare costs.

Embryology

The embryology of neural tube development has been studied extensively and is well understood. In contrast, the mechanisms which lead to NTDs, as well as the variation in anatomical location and severity of these defects, are not well understood (Botto, Moore et al. 1999). The neural tube is part of the central nervous system (CNS), which begins to form during the third week post-conception. Neurulation, the formation of the nervous system, starts with the formation of the neural plate, a thickening of ectoderm located along the middorsal region of the embryo. In humans, the neural plate develops into the neural tube via a two-step process. During primary neurulation, the lateral edges of the neural plate elevate as cells differentiate and migrate toward the neural plate edges forming the neural folds. As the neural folds continue to elevate, they meet at the midline and fuse to form the neural tube (Greene & Copp 2006). Primary neurulation and closure of the neural tube concludes by four weeks postconception. Secondary neurulation or canalization occurs at the caudal end of the primary neural tube when mesenchymal cells form a space that connects to the lumen of the primary neural tube (Hall, Friedman et al. 1988).

Open NTDs occur when the neural folds fail to meet and fuse during primary neurulation (Greene & Copp 2006), whereas incomplete secondary neurulation leads to closed NTDs (Copp, Greene et al. 2003). Failure of the neural tube to close at different regions results in the clinical variation seen in the anatomical location of

NTDs. For example, the effects of disruption of secondary neurulation are limited to the lower sacral and coccygeal regions of the neural tube (Hall, Friedman et al. 1988). Traditionally, closure of the neural tube was thought to begin at the cervical region and extend cranially and caudally (reviewed by Cabaret, Loget et al. 2007). Other evidence (Van Allen, Kalousek et al. 1993) suggests a multi-site neural tube closure mechanism in mammals, which implicates multiple, specific sites from which closure begins and proceeds cranially and caudally, rather than a single site. This latter mechanism would largely account for clinical variation in anatomical location of open NTDs, with each precise closure site being associated with a specific NTD subtype.

Risk Factors

Genetic

A small proportion of NTDs are associated with known genetic syndromes. Examples include Meckel syndrome, and aneuploid conditions, such as Trisomy 13 and Trisomy 18 (Rampersaud, Melvin et al. 2006). Non-syndromic NTDs are also thought to have some genetic basis as evidenced by a 2 to 5% risk of recurrence for mothers with a previous NTD-affected birth, a 50-fold increase over the general population risk (Risch 1990). In addition, a positive family history has been associated with an increased risk of an NTD-affected pregnancy (Partington & McLone 1995), although no major, single gene has been implicated in humans (De Marco, Merello et al. 2006).

Animal models, in particular murine models, also support a genetic basis for non-syndromic NTDs and provide insight into specific genetic risk factors. In total, more than 100 murine genes have be implicated in neural tube formation and more than 80 gene mutations have been linked to murine NTD development (reviewed by Detrait, George et al. 2005). These mutations are at loci which are highly heterogeneous by function with some showing complex inheritance patterns (reviewed by Juriloff & Harris 2000). Because early developmental processes such as neural tube development, are thought to be highly conserved across species, the same genes or similar genes in the same pathway in mice may be involved in human neural tube development, and in turn, NTD development (Rampersaud, Melvin et al. 2006). To date, none of the relevant murine genes with known human homologues have been associated with NTD development in humans (reviewed by Detrait, George et al. 2005).

Due to the known role of folate in NTD prevention (described below), considerable research has been focused on genes involved in the folate metabolic pathway. In humans, more than 25 proteins involved in this pathway have been identified; however, few of the corresponding genes have been associated with an increased risk of NTDs (reviewed by De Marco, Merello et al. 2006). One of the most extensively studied genes, the methylenetetrahydrofolate reductase (MTHFR) gene, produces the enzyme for which it is named. This enzyme converts 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate (5-MTHFA), a product used to convert homocysteine to methionine. A meta-analysis found that maternal and infant homozygosity for the C677C→T variant were independently associated with

increased risk of hyperhomocysteinemia and NTDs, pooled odds ratio (OR) of 1.8 (95% confidence interval (CI): 1.4, 2.2; 15 studies) and 2.0 (95% CI: 1.5, 2.8; 9 studies), respectively (Botto & Yang 2000). Homozygosity for the C677C→T is relatively common, occurring in about 1 to 15% (varies by race/ethnicity) of the population (Botto & Yang 2000). Studies suggest that the effects of homozygosity for the C677C→T on plasma homocysteine levels can been attenuated with folic acid supplementation (reviewed by Rozen 2009), explaining in part the decrease in NTD prevalence following folic acid supplementation.

Environmental

Environmental risk factors are thought to play a role in NTD development, but few have been consistently replicated in human studies. Among these, randomized controlled trials indicated that folic acid supplementation could prevent at least one-half of all NTDs (Medical Research Council 1991; Czeizel & Dudas 1992) and, as a result, folic acid fortification of enriched grains became mandatory in the United States in 1998; subsequently, NTD rates decreased by an estimated 26% (Centers for Disease Control and Prevention 2004). Also, increased risk of NTDs has been associated with maternal diabetes (Becerra, Khoury et al. 1990), obesity (Rasmussen, Chu et al. 2008), use of antiepileptic medications (Lammer, Sever et al. 1987), and race/ethnicity, a risk factor that may be genetic or environmental (e.g., diet), with Hispanics having the highest prevalence of NTDs (Canfield, Ramadhani et al. 2009). Even so, these risk factors are thought to account for only a small proportion of NTDs, suggesting the presence of other risk factors (Cavalli 2002).

Animal studies have shown increased occurrence of NTDs among offspring exposed *in utero* to either alcohol or pesticides (Yanaguita, Gutierrez et al. 2008; Slotkin 2004). In murine models, *in utero* exposure to 2 ethanol injections 4 hours apart, 8 hours post-conception (corresponding to the time period of neural tube development) produced excessive cell death of premigratory neural crest cells (Kotch & Sulik 1992), which arise from the neuroepithelium. Similarly, *in utero* exposure to chlorpyrifos, one of the most frequently used organophosphate insecticides, led to excessive cell death in neuroepithelium during neurulation in rat embryos (Roy, Andrews et al. 1998). In both examples, such excessive cell death may have resulted in insufficient cells to allow fusion of the neural folds, resulting in NTDs (Copp, Greene et al. 2003; Greene & Kopp 2006). Additionally, animal studies have also found that alcohol exposure can result in folic acid deficiency (Yanaguita, Gutierrez et al. 2008), which suggests that alcohol exposure might play an indirect role in the development of NTDs through folic acid depletion. Specifically, alcohol administration in rats resulted in an increase in folic acid excretion in the kidney followed by a decrease in plasma folate levels (McMartin 1984; Muldoon & McMartin 1994).

Unlike animal studies, human studies of maternal alcohol exposure and risk of NTDs have produced inconsistent results. Among five studies of maternal alcohol exposure and risk of NTDs, four reported no association (Shaw, Velie et al. 1996; Shaw, Nelson et al. 2002; Suarez, Cardarelli et al. 2003; McDonald, Armstrong et al. 1992). A single study reported a significantly increased risk of NTDs (adjusted odds ratio (aOR): 2.1; 95% CI: 1.1, 4.0) for women who reported exposure of alcohol at

least once a week (Grewal, Carmichael et al. 2008); an estimate only significant in adjusted analysis. By comparison, most studies of maternal pesticide exposure and NTDs reported positive associations, although such estimates were small to moderate (Zhang, Wen-wei et al. 1992; Blatter, Roeleveld et al. 1996; Blanco Munoz, Lacasana et al. 2005; Lacasana, Vazquez-Grameix et al. 2006).

Both maternal alcohol and pesticide exposure are relatively common exposures. In a recent national survey, 10% of women reported using alcohol during pregnancy, whereas more than 50% of women who could become pregnant, reported using alcohol (Centers for Disease Control and Prevention 2004). Residential exposure to pesticides is very common although the majority of exposure is expected to occur at low doses (Garcia 1998). A recent study (Castorina, Bradman et al. 2010) examined the presence of 26 pesticide metabolites (from pesticides such as chlorpyrifos, carbaryl, naphthalene, lindane, etc.) in urine samples of pregnant women (taken at 26 weeks) who lived near agricultural land and detected 11 pesticide metabolites, 8 which occurred in more than 50% of samples. Compared to a national sample of pregnant women (National Health and Nutrition Examination Study), 7 metabolites were significantly more common in the agriculture sample and 2 were significantly more common in the national sample (Castorina, Bradman et al. 2010). Occupational pesticide exposure is less common than residential exposure, but is expected to occur at increased dose levels (Garcia 1998).

Animal and human studies of alcohol and pesticide exposures and risk of NTDs have produced inconsistent results. This inconsistency may be attributable to small sample sizes and lack of specificity in exposure measurements. In human

studies, small sample sizes limited stratification by NTD subtype. Because risk may differ by NTD subtype (Mitchell 2005), this may have diluted risk estimates. Lack of specificity in exposure assessment limited stratification by exposure types (e.g., pesticide class, alcohol type) which may have biased results toward or away from the null depending on 1) how types differed between cases and controls and 2) the association between each type and NTDs.

In studies of alcohol exposure and NTDs, only two studies (Grewal, Carmichael et al. 2008; Mills & Graubard 1987) stratified by NTD subtype. Also, small sample sizes and/or limited data restricted previous studies of alcohol exposure from evaluating relevant covariates, including those known to be associated with NTDs (e.g., diabetes). Finally, insufficient specificity of alcohol exposure data did not allow prior studies to examine NTD risk by type of alcohol consumed. Larroque et al. (1992) found a positive correlation between red cell folate levels and alcohol exposure in pregnant women. These authors noted that much of the alcohol consumed was beer, which can contain folates. Stark et al. (2005) found similar results in pregnant African American women, noting a non-significant, but positive trend between beer and the most common folate metabolite, 5-MTHFA, and a significant negative association between wine cooler consumption and 5-MTHFA plasma levels.

Similarly, prior studies of occupational pesticide exposure and NTDs were limited, in part, by small sample sizes (Shaw, Wasserman et al. 1999; Nurminen, Rantala et al. 1995) and variability in the quality of exposure assessment (Daniels, Olshan et al. 2001). Prior studies frequently used only self-reported, dichotomous

pesticide exposure (yes/no) or job title. Industrial hygienist review of occupational exposures is thought to result in less exposure misclassification compared to self-reports of exposure or job title alone (Fritschi, Siemiatycki et al. 1996). Only two studies (Nurminen, Rantala et al. 1995; Shaw, Wasserman et al. 1999) of pesticide exposure and NTDs were identified in which industrial hygienist assigned pesticides exposure, but both studies had limited statistical power. Also, no studies were identified in which maternal pesticide exposure was stratified by specific pesticide type. It is unlikely that all pesticides cause NTDs and combining all pesticides may dilute findings. Unfortunately, because of the small number of women exposed to specific pesticide types in NTD studies, it is unlikely that a specific pesticide can be linked to NTDs; however analyses by pesticide class (e.g., insecticides, herbicides, and fungicides) would represent an improvement over previous studies.

Etiologic Studies of NTDs and Bias

Elucidating the risks associated with NTDs and maternal exposures such as alcohol and pesticides can be difficult, specifically because development of the neural tube concludes within four weeks postconception, often before a woman is aware of her pregnancy. The occurrence of an NTD may also impact the pregnancy outcome. Pregnancies affected by an NTD may be more likely to end in a fetal death. Similarly, due to advanced detection methods, such as ultrasound and alpha-fetoprotein tests, a large proportion of NTD cases are prenatally diagnosed and subsequently electively terminated. A study of six US states found that 9 to 42% of NTDs were electively terminated (years 1985 to1993, total number of years varied by

state) (Cragan, Roberts et al., 1995). Another study reported exceptionally high rates (83%) of elective termination in prenatally diagnosed anencephaly cases (Forrester, Merz et al. 1998).

Little is known about the differences between mothers of live born NTD-affected infants and other pregnancy outcomes. One study (Velie & Shaw 1996) found differences in maternal characteristics by pregnancy outcome with mothers of electively terminated NTD cases significantly more likely to be white, older, employed, and have attained higher education levels than mothers of live born or stillborn NTD cases.

Inclusion or exclusion of cases due to pregnancy outcome can create bias, more specifically, ascertainment and response bias. The risk of ascertainment bias occurs when inclusion or exclusion of cases is mitigated by pregnancy outcome and the risk factor under study is also associated with pregnancy outcome. Risk factors may be associated with pregnancy outcome either directly by increasing risk of fetal death, or indirectly by increasing the likelihood of an elective termination. As such, risk estimates in studies of the effect of these risk factors would be biased towards the null if fetal deaths or elective terminations were excluded. Similarly, if participation in studies varies by pregnancy outcome, studies of NTDs may suffer from response bias if true differences in risk factors under study exist among pregnancy outcomes. No study was identified that characterized participation rates of mothers of NTD cases by pregnancy outcome in a large, population-based case-control study. Understanding the presence and potential impact of ascertainment and response bias is crucial to interpreting studies of NTDs.

National Birth Defects Prevention Study

The relative rarity of any one birth defect requires a large base population to ascertain a sufficient number of cases for etiologic studies. The National Birth Defects Prevention Study (NBDPS) is one of the largest case-control studies of birth defects in the United States. Beginning in 1997, data have been collected by 10 centers (years of data collection vary by center): Arkansas, California, Iowa, Massachusetts, New Jersey, New York, North Carolina, Texas, Utah, and metropolitan Atlanta, covering an annual birth population of 482,000, or 10% of births nationally (Yoon, Rasmussen et al. 2001). The NBDPS collects data on over 30 defects, including NTDs. Clinical geneticists use standardized case definitions and medical record data to determine case eligibility (Rasmussen, Olney et al. 2003). Each center also randomly selects control infants using birth certificates or hospital records. Control infants are unaffected live births collected over the same time period and in the same geographic regions as case infants.

Mothers of eligible case and control infants are sent an introductory packet by mail no earlier than 6 weeks after the infant's estimated date of delivery (EDD). The EDD was used, as opposed to the actual date of delivery, to ensure a similar time frame between conception and contact of mothers of live births and those of fetal deaths or elective terminations. Two weeks following the introductory mailing, follow-up calls are conducted to answer questions and schedule or conduct a computer-assisted telephone interview (CATI). Using a standardized script, NBDPS interviewers collect data on maternal exposures including infectious, chemical, physical, nutritional, and behavioral factors. Interviews are conducted within 24

months following the EDD of an infant. After completing the interview, the mother, father, and child (if living) are asked by mail to provide buccal cell samples through use of a cytobrush collection kit.

The NBDPS is ideal for etiologic studies of birth defects, such as NTDs. Its population-based methodology, relatively large sample size, and geographically and racially diverse sample minimize selection bias. In addition, ascertainment bias is reduced by collection of live births (all centers), fetal deaths of 20 weeks or greater gestation (6 centers), and elective terminations (five centers) (Yoon, Rasmussen et al. 2001).

Goal and Significance

The long term goal of this project is to improve our understanding of selected environmental exposures and risk for NTDs. In addition, this report will describe data quality by examining exclusion of selected NTD-affected pregnancy outcomes and the risk of ascertainment and response bias. Ultimately, better understanding of risk factors for NTD will improve interventions aimed at reducing NTD prevalence.

Approach

The study aims were to: 1) examine the association between maternal periconceptional alcohol exposure and NTDs; 2) examine the association between maternal periconceptional occupational pesticide exposure and NTDs; and 3) characterize Iowa NTD cases by pregnancy outcome. To estimate the association between maternal exposure to alcohol and pesticides, data collected by the NBDPS

were used; assignment of maternal occupational pesticide exposure was conducted by industrial hygienists at Battelle Center for Public Health Research and Evaluation (Seattle, Washington) in collaboration with the National Institute for Occupational Safety and Health and the NBDPS Occupational working group. Data from the Iowa Registry for Congenital and Inherited Disorders (IRCID), the ascertainment source for the Iowa arm of the NBDPS, were used to characterize differences in proportions, selected maternal and infant characteristics, NBDPS participation rates, and risk estimates by pregnancy outcome

CHAPTER II

PERICONCEPTIONAL MATERNAL ALCOHOL EXPOSURE AND RISK OF NEURAL TUBE DEFECTS

Summary of Findings

Neural tube defects (NTD)s, which occur when the neural tube fails to close during early gestation, are one of the most common birth defects world-wide. Alcohol is a known teratogen and has been shown to induce NTDs in animal studies although most human studies have failed to replicate these results. Mechanisms by which alcohol may induce NTDs include, directly through excessive cell death of premigratory neural crest cells resulting in too few cells to close the neural tube, and indirectly through reduction of plasma folate levels. Using data from the National Birth Defects Prevention Study, the association between maternal reports of periconceptional (1 month prior through 2 months postconception) alcohol exposure and NTDs was examined. NTD cases and unaffected live born control infants, delivered from 1997 through 2005, were included. Interview reports of alcohol exposure (quantity, frequency, variability, and type) were obtained from 1223 case mothers and 6807 control mothers. Adjusted odds ratios (aOR)s and 95% confidence intervals (CI)s were estimated using multivariable logistic regression analysis. For all NTD cases combined, the aOR for mothers who reported any alcohol exposure was near unity compared to those who reported no periconceptional alcohol exposure. In addition, risk estimates for all NTD cases combined and reports of one or more binge episodes as well as type(s) of alcohol consumed were not significant. Results were similar among NTD subtypes. These findings suggest a lack of association between

maternal periconceptional alcohol exposure and NTDs; however, these results should be interpreted cautiously. Alcohol exposure and dose may be underreported given the negative social stigma associated with alcohol consumption during pregnancy; this is particularly relevant in this study in which data is collected postpartum, when the health of the infant in known. In addition, the lack of association seen here may be due to selective early pregnancy loss of fetuses with NTDs; such cases are difficult to ascertain and were not available to include in this study. Future studies should aim to increase sample sizes for less prevalent subtypes, reduce exposure misclassification, and ascertain maternal exposure information for NTD cases which are lost in early pregnancy.

Introduction

Neural tube defects (NTD)s are one of the most common birth defects world-wide. In the US, approximately 1 out of every 1000 infants are born with an NTD annually, with more aborted spontaneously and electively terminated (reviewed by Detrait, George et al. 2005). Anencephaly is fatal in all cases whereas children with spina bifida frequently suffer from severe disability (Date, Yagyu et al. 1993) and require continued medical treatment (Zurmohle, Homann et al. 1998). In 2003 dollars, Grosse et al. (2008) estimated the direct lifetime costs of spina bifida to be $560,000 per child. Randomized controlled trials indicated that folic acid supplementation could prevent at least one-half of all NTDs (Medical Research Council 1991; Czeizel & Dudas 1992), leading to mandatory folic acid fortification of enriched grains in the US in 1998. Although NTD rates decreased by an estimated

26% following folic acid fortification (Centers for Disease Control and Prevention 2004), persistence of NTDs suggests the presence of other risk factors (Cavalli 2002).

Alcohol is a known teratogen, with effects that may be more pronounced during organogenesis (Yanaguita, Gutierrez et al. 2008). A potential association between alcohol exposure and NTDs was first described in a case series more than 25 years ago (Freidman 1982), but the biological mechanisms by which this may occur are not well understood. One proposed mechanism is that prenatal alcohol exposure leads to excessive cell death (Bannigan & Burke 1982). Exposure to ethanol in pregnant mice beginning at 8 hours post-conception (equivalent to the third to fourth week of human development), led to excessive cell death of premigratory neural crest cells (Kotch & Sulik 1992). Excessive loss of these cells can result in an insufficient number of cells to allow fusion of the neural folds (Copp, Greene et al. 2003; Greene & Kopp 2006). Another proposed mechanism is that alcohol exposure can contribute to folic acid deficiency and thereby play an indirect role in NTD development (Yanaguita, Gutierrez et al. 2008). Alcohol administration in rats resulted in an increase in folic acid excretion in the kidney followed by a decrease in plasma folate levels (McMartin 1984; Muldoon & McMartin 1994).

Published human studies of maternal alcohol exposure and NTDs have produced results inconsistent with animal studies. Two studies (McDonald, Armstrong et al. 1992; Mills & Graubard 1987), which did not control for folic acid intake, found no association between alcohol exposure during the first trimester of pregnancy and NTDs. Among studies that controlled for folic acid supplementation, three (Shaw, Velie et al. 1996; Shaw, Nelson et al. 2002; Suarez, Cardarelli et al.

2003) reported no association with any level of alcohol exposure examined, and one (Grewal, Carmichael et al. 2008) reported a significantly increased risk of NTDs (OR: 2.1; 95% CI: 1.1,4.0) associated with maternal reports of alcohol exposure of at least once per week.

Each of these prior studies was limited by insufficient exposure assessment, as none examined NTD risk by type of alcohol consumed. Larroque et al. (1992) reported a positive correlation between red cell folate levels and alcohol exposure in pregnant women. These authors found that much of the alcohol consumed was beer, which can contain folates. Stark et al. (2005) found similar results in pregnant African American women, with a non-significant, but positive trend between beer exposure and plasma 5-MTHFA (the most common folate metabolite) and a significant negative association between wine cooler exposure and 5-MTHFA plasma levels. Also, due to limited data and/or small sample sizes, few previous studies were able to evaluate relevant covariates, including variables known to be associated with NTDs (e.g., diabetes). In addition, due to small sample sizes, only two studies (Grewal, Carmichael et al. 2008; Mills & Graubard 1987) stratified by NTD subtype, and no study examined encephalocele or other rare subtypes. Findings have shown that some risk factors (e.g., low folic acid levels) are associated with most NTD subtypes whereas others have only been associated with specific subtypes (e.g., valporic acid), supporting heterogeneity in NTD development and, in turn, risk factors (Mitchell 2005). This potential heterogeneity argues for stratification by NTD subtype; the inability to do so could possibly dilute reported risk estimates.

A recent report from the Behavioral Risk Factor Surveillance System (BRFSS) showed that 10% of women reported using alcohol during pregnancy, and over 50% of women of reproductive age reported using alcohol (BRFSS, 2004). Given that the closure of the neural tube occurs between 3 and 4 weeks post-conception, often before a pregnancy is recognized, comprehensive investigation of the relation of alcohol exposure and NTDs is needed. As such, data from the National Birth Defects Prevention Study (NBDPS), an ongoing, population-based, multi-center case-control study, were used to investigate the association between maternal reports of the quantity, frequency, variability, and type of alcohol exposure and NTD subtype.

Design and Methods

The NBDPS was designed to investigate genetic and non-inherited risk factors for over 30 major, structural birth defects including NTD subtypes (British Pediatric Association codes [BPA, 1979]) anencephaly and craniorachischisis (742.00-742.09), spina bifida (741.00-741.99), encephalocele, cranial meningocele, encephalomyelocele, and other rare subtypes (742.00-742.09). Case definitions for each eligible defect required confirmatory diagnostic procedures. The NBDPS excluded cases that are known to be secondary to a single gene disorder or a chromosome abnormality. Clinical geneticists at each NBDPS center reviewed reports from medical records to determine case eligibility. A brief description of NBDPS methods is provided below; additional detail is reported elsewhere (Yoon,

Rasmussen et al. 2001; Rasmussen, Moore et al. 2001; Rasmussen, Olney et al. 2003). Each center obtained institutional review board approval for the NBDPS.

Data included in this report were collected at 10 centers (Arkansas, California, Iowa, Massachusetts, New Jersey, New York, North Carolina, Texas, Utah, and metropolitan Atlanta), which cover an annual birth population of 482,000, or about 10% of births nationally (Yoon, Rasmussen et al. 2001). For this analysis, eligible case deliveries were live births, fetal deaths, and elective terminations with estimated dates of delivery (EDD)s from October 1, 1997 through December 31, 2005 and diagnosed with at least one NTD subtype (anencephaly, craniorachischisis, spina bifida, encephalocele, cranial meningocele, or encephalomyelocele). NTD cases were further classified as isolated (no additional major defects) or multiple (one or more additional defects in a separate organ system). Eligible control infants were live births without a structural birth defect delivered during the same time period and randomly selected using birth certificates or hospital records from the same regions as the NTD cases. Mothers of eligible NTD case and control infants were recruited to complete a computer-assisted telephone interview (CATI) no earlier than 6 weeks and no later than 24 months after the EDDs of the infants. The CATI asked about maternal exposures, including infectious, chemical, physical, nutritional, and behavioral factors. Case and control mothers who reported use of folate antagonist medication (aminopterin sodium, carbamazepine, cholestyramine resin, methotrexate, oxcarbazepine, pyrimethamine, sulfasalazine, triamterene, trimethoprim, phenytoin, primidone, phenobarbital, valproate sodium) during the periconceptional period (1 month prior [B1] through 2 months postconception [P1 and P2]) and/or were

diagnosed with type 1 or type 2 diabetes before or during the index pregnancy were excluded. These exclusions were applied because both maternal type 1 and type 2 diabetes and use of antiepileptic medications (e.g., valproic acid) have been consistently associated with an increased risk of an NTD delivery (Becerra, Khoury et al. 1990; Lammer, Sever et al. 1987).

The NBDPS interview collected quantity, frequency, and variability data for exposure and the type(s) of alcohol consumed. Maternal periconceptional alcohol exposure was assessed using a previously developed approach (Romitti, Sun et al. 2007). Mothers who reported exposure to alcohol during the periconceptional period were queried about the month(s) during which they drank (yes/no), the average number of drinking days per month (frequency), the average number of drinks per drinking day (quantity), the maximum number of drinks on one occasion per drinking month (variability), and type(s) of alcohol consumed (beer, wine, and/or distilled spirits).

Mothers were classified as exposed if they reported drinking alcohol during one or more periconceptional months. Including exposure reported in B1 allowed for analysis of mothers with unrecognized pregnancies who might have extended pre-pregnancy exposure patterns into P1 or later. Also, the date of conception, although based on a systematic calculation using available medical record data, could vary from the true date of conception; the calculated B1 may actually encompass the period of conception and therefore exposures may be relevant. The months, P1 and P2, encompass the relevant gestational period for development of NTDs. Maternal reports were excluded from analyses if, for any pregnancy month or trimester, alcohol

exposure (yes/no) was missing or unknown or if reports of average drinking for any month were greater than 150 drinks.

Alcohol exposure was categorized by quantity-frequency reported. The average number of drinks per each drinking month was calculated by multiplying the frequency of drinking (reported average number of drinking days per month) and the quantity of drinks (reported average number of drinks per drinking day) for that month. A periconceptional average (total average number of drinks/month divided by number of months drank) and maximum average number of drinks per month (highest reported number of drinks for a periconceptional month) were calculated for each mother. Using a 30-day month, four categories of exposure rates were used: monthly to weekly (1-4 drinks per month); weekly to every other day (5-15 drinks per month); every other day to daily (16-30 drinks per month); and daily with more than one drink per day (>30 drinks per month).

Exposure was further categorized by binge drinking, measured using both sex-neutral (Naimi, Brewer et al. 2003) and sex-specific (Wechsler, Dowdall et al. 1995) norms. Sex-neutral norms included five or more drinks per day on average, on one occasion, or both, whereas sex-specific norms for females included four or more drinks per day on average, on one occasion, or both. Lastly, exposure was categorized by type(s) of alcohol consumed including beer only, beer plus other alcohol type (wine and/or distilled spirits), or other alcohol type only.

Covariates evaluated included maternal age at delivery (<21, 21-25, 26-30, 31-35, >35), race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, Other), education (<12 years, 12 years, 13-15 years, 16 or more years), gravidity (0,

1, 2, 3 or more), pre-pregnancy body-mass index (<18.5, 18.5-24.9, 25-29.9, 30+), periconceptional smoking (yes/no), and NBDPS center. Additionally, food folate (<600 ug or ≥600 ug) and periconceptional use of folic acid – containing supplements (yes/no) intake were examined. Food folate intake was assessed using the responses to the food frequency module in the NBDPS interview that measured food intake 1 year pre-conception (Willet Food Frequency questionnaire) and reports of breakfast cereals consumed during the P1 and P2. Dietary folate equivalents (DFE)s were estimated using the reported food frequencies, the standardized serving size on which a question item was based, and the United States Department of Agriculture National Standard Reference 16-1 (United States Department of Agriculture 2004). Also, the NBDPS interview queried mothers regarding their intake of vitamins and supplements for a period of 3 months prior to conception through delivery. For each supplement reported, mothers were asked to provide start and stop dates (or if dates were unknown, duration of use) and frequency of intake. Each supplement was assessed to determine whether or not it contained folic acid. Eligible mothers were classified into two groups, those who took folic acid-containing supplements during the periconceptional period and those who did not.

Analyses were conducted using SAS software, version 9.2 (SAS Institute, Inc. 2007). Descriptive analyses of selected infant and maternal characteristics were conducted by NTD subtype and compared to controls using the chi-square statistic. Crude odds ratios (OR)s and 95% confidence intervals (CI)s were estimated to examine associations between any maternal periconceptional alcohol exposure, average and maximum average drinks per month, binge episodes, and alcohol type

and all NTD cases combined. Results of descriptive analyses were used to construct the most parsimonious multivariable logistic regression model for alcohol exposure and NTDs. The logistic regression model was built using backward selection. Covariates included in the preliminary model were those which, based on the descriptive analysis, were associated ($p< 0.10$) with any alcohol exposure (yes/no) and/or outcome. Backward selection was used to exclude covariates from the preliminary model beginning with the least statistically significant covariate (highest p-value) based on the Wald chi-square statistic. As each covariate was removed, the fit of the full model and reduced model were compared using the log-likelihood ratio (LLR) test. Covariates for which the LLR was significant ($p<0.05$), were re-entered into the model. In addition, regardless of LLR values, covariates for which exclusion from the model resulted in a change in parameter estimate of alcohol exposure variable by greater than 20% were re-entered in the model. Based on the final multivariable logistic model, adjusted odds ratios (aORs) were estimated to characterize the association between all NTD cases combined and any periconceptional alcohol exposure, quantity-frequency of exposure, binge episodes, and type of alcohol consumed. Crude and adjusted analyses were also conducted by NTD subtype.

Results

Interview data were collected from mothers of 1223 (68% of eligible) NTD cases and 6807 (66% of eligible) control infants. Of these, 56 case and 204 control interviews were excluded due to: incomplete interviews (case=17; control=104);

maternal diagnosis of type 1 or type 2 diabetes prior to or during the index pregnancy (case=18; control=42); and maternal periconceptional exposure to folic acid antagonists (case=21; control=58). To improve homogeneity of NTD subtype groups, maternal interviews for an additional 7 NTD cases were excluded due to diagnosis of multiple NTD subtypes for each case. Among the 1160 NTD cases included in the analyses, 328 were diagnosed with anencephaly or craniorachischisis, 703 with spina bifida, and 129 with encephalocele or other rare subtype. The median time between the estimated date of delivery and completed interview was 9.5 months for case and 8.9 months for control mothers.

As shown in Table II.1, anencephaly cases were more likely to be female than male and when, compared to control infants, each NTD case subtype was more likely to be preterm (< 37 weeks gestation). NTD cases with other rare subtypes were more likely than were anencephaly or spina bifida cases to be diagnosed with multiple defects. Compared to control mothers, case mothers (all NTD cases combined and each subtype) were significantly ($p < 0.05$) more likely to be Hispanic, less educated, and to differ in proportions by center. Mothers of spina bifida cases tended to be younger and have a pre-pregnancy body mass index of 30 or more, whereas mothers of anencephaly cases were less likely to have reported periconceptional smoking; mothers of spina bifida and anencephaly cases were each more likely to have had 4 or more pregnancies than control mothers. Case and control mothers did not differ in use of folic acid-containing supplements or food folate consumption.

Thirty percent of case mothers and approximately 36% of control mothers reported periconceptional alcohol exposure (Table II.2); duration of use was similar

between the two groups. Case mothers were more likely to report exposure to beer only, whereas control mothers were more likely to report exposure to other alcohol types. Duration and type of alcohol consumed were similar among mothers of infants of each NTD subtype. Reports of periconceptional alcohol exposure for case and control mothers stratified by 6-month intervals were similar as time to interview increased, although in control mothers, the frequency was somewhat lower for those with the shortest and longest length of time (18 to 24 months) between EDD and interview, 38% to 25%, respectively (data not shown).

Crude analysis suggested that any maternal periconceptional alcohol exposure was negatively associated with NTDs combined (data not shown). Similarly, any periconceptional exposure with no binge episodes was negatively associated with NTDs combined, whereas crude OR estimates for binge episodes were near unity. No dose-response relationship was observed for maximum average number of drinks per month. In addition, crude ORs were of a similar magnitude for the different type(s) of alcohol consumed. Little difference in ORs was found among NTD subtypes or phenotypes (isolated and multiple) within subtypes.

After adjustment for maternal race/ethnicity, education, pre-pregnancy body mass index, periconceptional smoking, and NBDPS center, any maternal periconceptional alcohol exposure was not associated with all NTD cases combined or any NTD subtype. Similarly, when compared to no periconceptional alcohol exposure, for all NTD cases combined, no significant associations were found for maximum average monthly drinks (Table II.3) or sex-specific binge episodes (Table II.4). Results for binge episodes did not appreciably change when using sex-neutral

norms (data not shown). For maximum monthly average and sex-specific binge episodes, few appreciable differences in OR estimates were found between all NTD cases combined, each NTD subtype, and each NTD phenotype. No patterns were seen with increasing number of maximum average drinks, although the largest estimated OR for all NTD cases combined and each NTD subtype was found for the highest category of drinking (30 or more drinks per month). Adjusted analyses for all NTD cases combined, each subtype, and each phenotype for reported type(s) of alcohol consumed were not significant (Table II.5).

The aORs calculated for periconceptional maximum average monthly drinks one or more binge episodes were stratified by type(s) of alcohol consumed (Table II.6). For maximum average monthly drinks, aORs were near unity. Likewise, aORs for one or more binge episodes by alcohol type(s) were non-significant, although aORs were elevated for all NTD cases combined and spina bifida cases for mothers reporting one or more binge episodes and exposure of beer only. Adjusted analyses were also calculated for periconceptional folic acid supplement consumption by reported type(s) of alcohol consumed and aORs were largely non-significant with the exception of mothers of spina bifida cases who reported no folic acid consumption and consumption of beer only (OR: 1.8, CI: 1.1, 2.9).

For all associations tested, sub-analyses stratified by pregnancy intendedness (yes/no) produced no appreciable differences in the aORs. Also, sub-analyses that excluded case and control infants with a family history (yes/no) of an NTD (controls=10; cases=9) did not materially change the aORs. Further, sub-analyses

restricted to the five centers which ascertained live births, fetal deaths, and elective terminations produced little change in aORs (data not shown).

Discussion

The current study did not identify significantly elevated associations between any maternal periconceptional alcohol exposure and all NTD cases combined or any NTD subtype. It also did not identify significantly elevated associations for reported maximum average monthly drinks for all NTD cases combined and most NTD subtypes. Additionally, little variation in risk was found by type(s) of alcohol consumed. Stratification of reported drinks per month, binge episodes, and folic acid use by type(s) of alcohol consumed largely produced ORs near unity. However, odds were significantly elevated in mothers of spina bifida cases who reported no folic acid consumption and beer exposure only. The few significant associations identified here could have been due to chance given the number of estimated ORs.

The analyses of maternal periconceptional alcohol exposure and NTDs utilized data from one of the largest US population-based, case-control studies of birth defects. Power calculations suggested that, with the available sample size and a 36% rate of periconceptional alcohol exposure in controls, our minimum detectable odds ratio for an association between any periconceptional alcohol exposure (yes/no) and NTDs combined was 1.15. Even so, the results were similar to the 6 previously published studies identified that examined the relation between maternal alcohol exposure and NTDs. Only one of these studies (Grewal, Carmichael et al. 2008) reported a significant, positive association (≥1 drink per week), but only in adjusted

analyses. A significant finding in adjusted analysis, but not mirrored in crude analysis, may indicate correlation of variables included in the adjusted model. As such, the finding should be interpreted cautiously.

The primary strength of this analysis was the large, population-based, geographically and ethnically diverse sample. The use of a population-based sample reduced the risk of selection bias. Comparison of selected maternal characteristics of controls versus all live births at each center has shown that NBDPS participants tend to be similar to all live births (Cogswell, Bitsko et al. 2009). Also, all cases were reviewed and verified by clinical geneticists, decreasing the risk of case misclassification. In addition, the large sample size allowed for stratification by NTD subtype, which is important due to the potential heterogeneity of risk factors among subtypes.

Exposure data collection was conducted through detailed maternal interview reports using a structured questionnaire. Detailed exposure data allowed assessment of alcohol exposure by quantity, frequency, and variability, as well as alcohol type, which had not been previously investigated. With the use of retrospective reports, the potential for differential recall existed between case and control mothers, although Verkerk et al. (1994) reported no significant differences in prospective and retrospective reports of alcohol between case and control mothers.

Several, previous animal studies showed an association between alcohol exposure and NTDs (Yanaguita, Gutierrez et al. 2008; Chen, Charness et al. 2005; Graham & Ferm 1985; Hunter, Tugman et al. 1994), although our analyses failed to corroborate these findings. Misclassification of timing and dose of alcohol could, in

part, explain discrepant findings between animal studies and this study. In the NBDPS, mothers were not queried regarding the precise volume of alcohol drinks consumed, rather general volumes (1 can of beer, 1 glass of wine, and 1 shot of liquor) were assumed. Varying alcohol concentrations between types of alcohol could have decreased differences in odds ratios by type(s) of alcohol consumed. Also, alcohol exposure and dose were self-reported, and may have been under-reported due to the negative social stigma associated with alcohol exposure during pregnancy, key in this study in which data is collected retrospectively, when the health of the infant is known. It is unclear if this would result in non-differential or differential exposure misclassification. An alternative explanation for the discrepant results with animal studies is that the lack of association seen here may be due to selective early pregnancy loss of fetuses with NTDs creating a ascertainment bias. A large proportion of pregnancies affected by NTDs result in fetal deaths (Center for Disease Control and Prevention). Such cases are difficult to include in retrospective case-control studies, as the pregnancy may not have been recognized or the defect may not be have been identified at the time of loss.

In summary, the association between periconceptional alcohol exposure and NTDs was investigated using a large, case-control study. Results were largely non-significant and no pattern of increasing risk per amount of alcohol consumed was found. Although previous studies corroborate these results and efforts were made to improve over previous studies, these results should be interpreted cautiously, as a number of limitations exist. Our study included more cases than previous studies; however, small numbers, particularly in subtype analyses, resulted in imprecise

estimates. Some risk factors affect the majority of NTD subtypes, for example low folic acid levels, others have only been associated with one or more specific subtypes (e.g., valproic acid) supporting heterogeneity in development (Mitchell 2005) and, in turn, risk factors. This argues for the need to stratify NTDs by subtype. As such, future studies should aim to increase sample sizes for less prevalent subtypes. In addition, future studies should aim to reduce exposure misclassification due to response and ascertainment bias by ascertaining exposure for NTD-affected pregnancies that result in induced or spontaneous loss.

Table II.1. Selected characteristics of infants and birth mothers by control and neural tube defect subtype, National Birth Defects Prevention Study, 1997-2005

	Controls Infants (n= 6603)		All NTD‡ Cases Combined (n=1160)		Anencephaly (n=328)		Spina Bifida (n=703)		Other Rare Subtype (n=129)	
Characteristic	n*	%†	n*	%†	n*	%†	n*	%†	n*	%†
Infant										
Sex										
Male	3334	50.5	536	48.2	126	43.2	352	50.8	58	46.0
Female	3264	49.5	575	51.8	166	56.8	341	49.2	68	54.0
Gestational Age (weeks)[a]										
< 37	620	9.4	510	44.0	246	75.0	200	28.4	64	49.6
≥ 37	5982	90.6	650	56.0	82	25.0	503	71.6	65	50.4
Phenotype										
Isolated	NA‡	-	1027	88.6	298	90.9	633	90.2	96	74.4
Multiple	NA	-	132	11.4	30	9.1	69	9.8	33	25.6
Mother										
Age at Delivery (years)[a]										
< 21	968	14.7	189	16.3	55	16.8	109	15.5	25	19.4
21-25	1581	23.9	266	22.9	67	20.4	166	23.6	33	25.6
26-30	1796	27.2	361	31.1	100	30.5	230	32.7	31	24.0
31-35	1571	23.8	221	19.1	70	21.3	126	17.9	25	19.4
> 35	687	10.4	123	10.6	36	11.0	72	10.2	15	11.6
Race/Ethnicity[a]										
Non-Hispanic White	3951	60.1	595	51.5	159	48.8	382	54.5	54	41.9
Non-Hispanic Black	750	11.4	105	9.1	26	8.0	58	8.3	21	16.3
Hispanic	1476	22.4	382	33.0	116	35.6	222	31.7	44	34.1
Other	399	6.1	74	6.4	25	7.7	39	5.6	10	7.8
Education (years)[a]										
< 12	1111	16.8	250	21.6	78	23.8	141	20.1	31	24.0
12	1625	24.6	329	28.4	90	27.4	196	27.9	43	33.3
13-15	1773	26.9	324	27.9	81	24.7	216	30.7	27	20.9

Table II.1 continued

16	2085	31.6	257	22.2	79	24.1	150	21.3	28	21.7
Gravidity[a]										
1	1929	29.2	298	25.7	76	23.2	183	26.0	39	30.2
2	1948	29.5	329	28.4	100	30.5	196	27.9	33	25.6
3	1351	20.5	226	19.5	57	17.4	146	20.8	23	17.8
> 4	1373	20.8	307	26.5	95	29.0	178	25.3	34	26.4
Pre-Pregnancy BMI‡ (kg/m^2)[a]										
Underweight (< 18.5)	353	5.6	47	4.3	19	6.2	24	3.6	4	3.3
Normal weight (18.5-24.9)	3548	56.0	549	50.4	161	52.6	313	47.3	75	62.0
Overweight (25-29.9)	1422	22.4	246	22.6	70	22.9	160	24.2	16	13.2
Obese (≥ 30)	1018	16.1	247	22.7	56	18.3	165	24.9	26	21.5
Periconceptional‡ Smoking[b]										
Yes	1243	18.8	187	16.1	39	11.9	127	18.1	21	16.3
No	5357	81.2	971	83.9	289	88.1	574	81.9	108	83.7
Periconceptional Folic Acid Intake										
Yes	4994	76.7	853	75.2	248	77.7	513	74.5	92	73.0
No	1518	23.3	281	24.8	71	22.3	176	25.5	34	27.0
Food Folate DFE										
< 600 ug daily	4152	62.9	749	64.6	225	68.6	439	62.4	85	65.9
≥ 600 ug daily	2449	37.1	411	35.4	103	31.4	264	37.6	44	34.1
NBDPS Center[a]										
Arkansas	825	12.5	146	12.6	41	12.5	88	12.5	17	13.2
California	836	12.7	228	19.7	75	22.9	132	18.8	21	16.3
Iowa	742	11.2	138	11.9	30	9.1	96	13.7	12	9.3
Massachusetts	844	12.8	71	6.1	16	4.9	46	6.5	9	7.0
New Jersey	569	8.6	69	5.9	10	3.0	51	7.3	8	6.2
New York	577	8.7	69	5.9	12	3.7	46	6.5	11	8.5
Texas	750	11.4	162	14.0	59	18.0	88	12.5	15	11.6
CDC‡/Atlanta, Georgia	712	10.8	136	11.7	37	11.3	80	11.4	19	14.7
North Carolina	386	5.8	64	5.5	29	8.8	27	3.8	8	6.2

Table II.1 Continued

Utah	362	5.5	77	6.6	19	5.8	49	7.0	9	7.0

***** Numbers may vary due to incomplete or missing data

† Due to rounding, percentages may not total 100

‡NTD, neural tube defect; NA, not applicable; BMI, body mass index; Periconceptional, 1 month before conception through 2 months post conception; CDC, Centers for Disease Control and Prevention

[a] $p < 0.01$ for all NTDs combined

[b] $p < 0.05$ for all NTDs combined

Table II.2. Reported patterns of maternal periconceptional alcohol exposure and type of alcohol consumed by control and neural tube defect subtype, National Birth Defects Prevention Study, 1997-2005

	Control Infants (n= 6603)		NTDs Combined (n=1160)		Anencephaly (n=328)		Spina Bifida (n=703)		Other Rare Subtype (n=128)	
Periconceptional Exposure*,†	n	%‡	n	%‡	n	%‡	n	%‡	n	%‡
Pattern of exposure[a]										
Any exposure	2357	35.7	348	30.0	86	26.2	223	31.7	39	30.2
1 month	1268	19.2	194	16.7	53	16.2	117	16.6	24	18.8
2 months	721	10.9	101	8.7	22	6.7	71	10.1	8	6.0
3 months	368	5.6	53	4.6	11	3.4	35	5.0	7	5.3
Type of alcohol[b]										
Beer only	488	7.4	90	7.8	20	6.1	61	8.7	9	7.0
Beer + other alcohol	630	9.5	94	8.1	20	6.1	60	8.5	14	10.9
Other alcohol only	1236	18.7	162	14.0	44	13.4	102	14.5	16	12.4

∝NTD, neural tube defect

*Excluded mothers with incomplete or missing alcohol exposure data for any month and mothers who reported >150 drinks for any month

† 1 month before conception through 2 months post conception

‡ Due to rounding, percentages may not total 100

[a]Missing, incomplete or questionable data on alcohol exposure were distributed as follows: controls (n=54), NTDs combined (n=19), anencephaly (n=7), spina bifida (n=11), other rare subtypes (n=1)

[b]Missing, incomplete or questionable data on alcohol type were distributed as follows: controls (n=57), NTDs combined (n=21), anencephaly (n=9), spina bifida (n=11), other rare subtypes (n=1)

Table II. 3. Adjusted odds ratio estimates for neural tube defects for the association with maternal reports of maximum average alcoholic drinks consumed per month, National Birth Defects Prevention Study, 1997-2005*†

	Drinks per month											
	0	1-4			5-15			16-30			> 30	
	n	n	Odds Ratio	95% Confidence Interval	n	Odds Ratio	95% Confidence Interval	n	Odds Ratio	95% Confidence Interval	n	Odds Ratio	95% Confidence Interval
Controls	4192	1081	Ref‡		745	Ref		333	Ref		181	Ref	
NTDs‡ Combined∞	793	171	1	0.8, 1.2	108	0.9	0.8, 1.2	36	0.7	0.5, 1.0	30	1.1	0.7, 1.6
Isolated	699	152	1	0.8, 1.2	100	1	0.8, 1.2	33	0.8	0.5, 1.1	24	1	0.6, 1.5
Multiple	94	18	1	0.6, 1.7	8	0.6	0.3, 1.3	3	NC‡	NC	6	1.8	0.7, 4.4
Anencephaly	235	42	0.9	0.6, 1.2	26	0.8	0.5, 1.3	10	0.8	0.4, 1.5	7	1	0.4, 2.1
Isolated	213	38	0.9	0.6, 1.3	24	0.9	0.6, 1.4	10	0.9	0.4, 1.7	7	1.1	0.5, 2.4
Multiple	22	4	NC	NC	2	NC	NC	0	NC	NC	0	NC	NC
Spina Bifida	469	108	1	0.8, 1.3	73	1	0.8, 1.3	23	0.7	0.5, 1.2	17	1	0.6, 1.7
Isolated	420	98	1	0.8, 1.3	68	1.1	0.8, 1.4	21	0.7	0.5, 1.2	14	0.9	0.5, 1.6
Multiple	49	9	1	0.5, 2.1	5	0.6	0.2, 1.8	2	NC	NC	3	NC	NC
Other Rare Subtype	89	21	1.2	0.7, 2.0	9	0.7	0.3, 1.5	3	NC	NC	6	2.2	0.9, 5.3
Isolated	66	16	1.2	0.6, 2.1	8	0.8	0.4, 1.8	2	NC	NC	3	NC	NC
Multiple	23	5	1.3	0.5, 3.5	1	NC	NC	1	NC	NC	3	NC	NC

*Missing, incomplete, or questionable data for exposure were distributed as follows: controls (n=71), all NTDs combined (n=22), anencephaly (n=8), spina bifida (n=13), and other rare subtypes (n=1)

† Adjusted for maternal race/ethnicity, education, pre-pregnancy body mass index, cigarette smoking, and center

∞1 spina bifida case was excluded due to insufficient information to classify the case as isolated or multiple

‡ NTDs, neural tube defects; Ref, Reference; NC, not calculated

Table II.4. Adjusted odds ratio estimates for neural tube defect subtype for the association with maternal reports of alcohol binge episodes, National Birth Defects Prevention Study, 1997-2005*†±

		No binge episodes			One or more binge episodes		
	0 drinks per month n	n	Odds Ratio	95% Confidence Interval	n	Odds Ratio	95% Confidence Interval
Controls	4192	1563	Ref‡		72	Ref	
NTDs‡ Combined	793	216	0.9	0.8, 1.1	28	1.0	0.8, 1.2
Isolated	699	194	0.9	0.8, 1.1	14	1.0	0.8, 1.3
Multiple∞	94	21	0.9	0.5, 1.4	4	1.0	0.5, 1.7
Anencephaly	235	55	0.8	0.6, 1.1	9	0.9	0.6, 1.3
Isolated	213	50	0.8	0.6, 1.2	8	1.0	0.6, 1.5
Multiple	22	5	0.7	0.3, 2.0		NC‡	NC
Spina Bifida	469	136	1.0	0.8, 1.2	5	1.0	0.8, 1.4
Isolated	420	125	1.0	0.8, 1.2	6	1.0	0.8, 1.4
Multiple	49	10	0.8	0.4, 1.6		1.1	0.5, 2.4
Other Rare Subtypes	89	25	1.0	0.7, 1.7	4	1.1	0.6, 2.0
Isolated	66	19	1.0	0.6, 1.7	0	1.0	0.5, 2.2
Multiple	23	6	1.2	0.5, 3.0		NC	NC

*Missing or incomplete data for exposure were distributed as follows: controls (n=76), NTDs combined (n=23), anencephaly (n=9), spina bifida (n=13), and other rare subtypes (n=1)

† Adjusted for maternal race/ethnicity, education, pre-pregnancy body mass index, cigarette smoking, and center

±Binge episode defined by sex-specific standards, ≥ 4 drinks in one sitting

‡ NTDs, neural tube defects; Ref, Reference; NC, not calculated

∞1 spina bifida case was excluded due to insufficient information to classify the case as isolated or multiple

Table II.5. Adjusted odds ratio estimates by neural tube defect subtype for the association with maternal reports of alcohol type, National Birth Defects Prevention Study, 1997-2005

		Beer Only			Beer + Other types∏			Other types		
	0 drinks per month n	No.	Odds Ratio	95% Confidence Interval	No.	Odds Ratio	95% Confidence Interval	No.	Odds Ratio	95% Confidence Interval
Controls	4192	488	Ref		629	Ref		1237	Ref	
NTDs‡ Combined	793	90	1.1	0.8, 1.4	94	1.0	0.8, 1.3	162	0.9	0.7, 1.0
Isolated	699	82	1.1	0.9, 1.4	81	1.0	0.7, 1.3	146	0.9	0.7, 1.1
Multiple∞	94	7	0.8	0.3, 1.7	13	1.2	0.7, 2.3	16	0.8	0.5, 1.4
Anencephaly	235	20	0.9	0.5, 1.4	20	0.8	0.5, 1.3	44	0.9	0.6, 1.2
Isolated	213	18	0.9	0.5, 1.5	18	0.8	0.5, 1.3	41	0.9	0.6, 1.3
Multiple	22	2	NC‡	NC	2	NC	NC	3	NC	NC
Spina Bifida	469	61	1.2	0.9, 1.6	60	1.0	0.8, 1.4	102	0.9	0.7, 1.1
Isolated	420	56	1.2	0.9, 1.7	53	1.0	0.7, 1.4	94	0.9	0.7, 1.2
Multiple	49	4	NC	NC	7	1.2	0.5, 2.7	8	0.7	0.3, 1.6
Other Rare Subtypes	89	9	1.0	0.5, 2.1	14	1.4	0.8, 2.7	16	0.9	0.5, 1.5
Isolated	66	8	1.2	0.5, 2.7	10	1.3	0.6, 2.7	11	0.8	0.4, 1.5
Multiple	23	1	NC	NC	4	NC	NC	5	1.3	0.5, 3.6

*Missing or incomplete data for exposure were distributed as follows: controls (n=57), NTDs combined (n=21), anencephaly (n=9), spina bifida (n=11), and other rare subtypes (n=1)

† Adjusted for maternal race, education, pre-pregnancy body mass index (continuous), cigarette smoking, and center

∏ Other types include wine and distilled spirits

∞1 spina bifida case was excluded due to insufficient information to classify the case as isolated or multiple

‡NTDs, neural tube defects; NC, Not calculated

Table II.6. Adjusted odds ratio estimates for maternal reports of maximum average drinks consumed per month, binge episodes per month, and folic acid intake by report of type(s) of alcohol consumed, by neural tube defect subtype, National Birth Defects Prevention Study, 1997-2005†

	Controls No.	All NTDs‡ Combined			Anencephaly			Spina Bifida			Other Rare Subtypes		
		No.	Odds ratio	95% Confidence Interval	No.	Odds ratio	95% Confidence Interval	No.	Odds ratio	95% Confidence Interval	No.	Odds ratio	95% Confidence Interval
Maximum drinks/month[a]													
No drinks	4192	793	Ref		235	Ref		469	Ref		89		
1-4 drinks	1079	169			40			108			21		
Beer only	205	35	0.9	0.6, 1.4	9	0.8	0.4, 1.7	22	1.0	0.6, 1.6	4	NC ‡	NC
Beer and other	179	31	1.2	0.8, 1.8	5	0.7	0.3, 1.8	22	1.4	0.9, 2.2	4	NC	NC
Only other	695	103	0.9	0.8, 1.2	26	0.9	0.6, 1.3	64	1.0	0.7, 1.3	13	1.2	0.6, 2.2
5-15 drinks	745	108			26			73			9		
Beer only	157	25	0.9	0.6, 1.4	5	0.7	0.3, 1.8	17	1.0	0.6, 1.7	3	NC	NC
Beer and other	233	37	1.1	0.7, 1.5	8	0.8	0.4, 1.8	25	1.2	0.7, 1.8	4	NC	NC
Only other	355	46	0.9	0.6, 1.2	13	0.9	0.5, 1.6	31	0.9	0.6, 1.4	2	NC	NC
16-30 drinks	333	36			10			23			3		
Beer only	78	17	1.4	0.8, 2.3	3	NC	NC	13	1.6	0.9, 2.9	1	NC	NC
Beer and other	122	13	0.7	0.4, 1.2	4	NC	NC	7	0.6	0.3, 1.3	2	NC	NC
Only other	133	6	0.3	0.1, 0.8	3	NC	NC	3	0.3	0.1, 0.9	0	NC	NC
> 30 drinks	180	30			7			17			6		
Beer only	45	12	1.7	0.9, 3.2	2	NC	NC	9	2.0	0.9, 4.1	1	NC	NC
Beer and other	92	13	0.9	0.5, 1.6	3	NC	NC	6	0.7	0.3, 1.6	4	NC	NC
Only other	43	5	0.9	0.3, 2.3	2	NC	NC	2	0.6	0.1, 2.4	1	NC	NC
Binge Episodes[b]													

Table II.6 Continued

No drinks	4192	793	Ref		235			469			89		
No episodes	1560	214			53			136			25		
Beer only	275	42	0.9	0.6, 1.3	10	0.7	0.4, 1.4	26	0.9	0.6, 1.4	6	1.3	0.6, 3.0
Beer and other	334	51	1.1	0.8, 1.5	9	0.7	0.3, 1.4	36	1.3	0.9, 1.9	6	1.1	0.4, 2.8
Only other	951	121	0.9	0.7, 1.1	34	0.9	0.6, 1.3	74	0.9	0.7, 1.1	13	0.9	0.5, 1.7
1 or more binge episodes	772	128			29			85			14		
Beer only	208	47	1.3	0.9, 1.9	9	1.0	0.5, 2.1	35	1.6	1.0, 2.3	3	NC	NC
Beer and other	288	43	0.9	0.6, 1.3	11	0.9	0.5, 1.7	24	0.8	0.5, 1.3	8	1.8	0.8, 3.9
Only other	276	38	0.9	0.6, 1.3	9	0.8	0.4, 1.7	26	0.9	0.6, 1.4	3	NC	NC
Folic acid intake[c]													
No drinks	4192	793			235	Ref		469	Ref		89	Ref	
No folic acid	394	75			15			51			9		
Beer only	113	30	1.5	0.9, 2.3	6	1.1	0.5, 2.6	22	1.8	1.1, 2.9	2	NC	NC
Beer and other	100	15	0.9	0.5, 1.5	2	NC	NC	10	0.9	0.5, 1.8	3	NC	NC
Only other	181	30	1.0	0.6, 1.4	7	0.8	0.4, 1.8	19	1.0	0.6, 1.6	4	NC	NC
Folic acid	1929	267			68			169			30		
Beer only	371	58	0.9	0.7, 1.2	13	0.7	0.4, 1.4	38	1.0	0.7, 1.4	7	1.2	0.5, 2.7
Beer and other	521	78	1	0.8, 1.3	18	0.9	0.5, 1.4	49	1.0	0.8, 1.4	11	1.4	0.7, 2.8
Only other	1037	131	0.9	0.7, 1.1	37	0.9	0.6, 1.3	82	0.9	0.7, 1.1	12	0.8	0.4, 1.5

† Adjusted for maternal race, education, pre-pregnancy body mass index (continuous), cigarette smoking, and center

[a]Missing or incomplete data for exposure were distributed as follows: controls (n=74), all NTDs combined (n=24), anencephaly (n=10), spina bifida (n=13), and other rare subtypes (n=1)

[b]Missing or incomplete data for exposure were distributed as follows: controls (n=79), all NTDs combined (n=25), anencephaly (n=11), spina bifida (n=13), and other rare subtypes (n=1)

[c]Missing or incomplete data for exposure were distributed as follows: controls (n=88), all NTDs combined (n=25), anencephaly (n=10), spina bifida (n=14), and other rare subtypes (n=1)

‡ NTD, neural tube defects; NC, not calculated

CHAPTER III

PERICONCEPTIONAL MATERNAL OCCUPATIONAL PESTICIDE EXPOSURE AND RISK OF NEURAL TUBE DEFECTS

Summary of Findings

Chemicals used as pesticides can cross the placenta and impact embryonic development. In animal studies, these chemicals have been shown to alter neuroepithelial cell proliferation and differentiation during neurulation and to lead to excessive neuroepithelial cell death that impacts closure of the neural tube. As such, these chemicals have been suggested as risk factors for neural tube defects (NTD)s. To date, reported adverse associations between NTDs and maternal occupational pesticide exposures have been small to moderate and have not been consistently observed. Using data from the National Birth Defects Prevention Study, a large, ongoing, multicenter case-control study, the association between maternal periconceptional (1 month prior through 2 months postconception), occupational pesticide exposure and NTDs was examined. Telephone interviews of mothers of 502 NTD case infants and 2950 unaffected live born control infants delivered from 1997 through 2003 were included. Using maternal interview reports of occupational exposures, industrial hygienists assigned exposure (yes/no) and cumulative exposure (mg) to each pesticide class (insecticides, herbicides, and fungicides) for each reported job. Multivariable logistic regression analysis was used to estimate adjusted odds ratios (aOR)s and 95% confidence intervals (CI)s for the association between maternal occupational pesticide exposure and all NTD cases combined and individual

NTD subtypes. AORs for any exposure and cumulative exposure to any pesticide, insecticides only, and insecticides + herbicides + fungicides were near unity for all NTD cases combined and each NTD subtype. Exposure to insecticides + herbicides was positively associated with spina bifida (aOR: 2.2; 95% CI: 1.1, 4.4). Additionally, elevated, but non-significant, aORs were found for cumulative exposure (0 mg, >0 to <9.48 mg, ≥9.48 mg) to insecticides + herbicides for all NTD cases combined and for spina bifida. Future studies should aim to increase sample size, particularly in less prevalent subtypes and those exposed to only herbicides, potentially utilizing highly exposed populations. In addition, future studies should aim to use similarly detailed exposure classification methods as well as collect data on exposure to pesticides outside of the work place.

Introduction

Neural tube defects (NTD)s affect 1 in every 1000 births in the United States (reviewed by Detrait, George et al. 2005). NTDs occur during neurulation, 21 to 28 days post-conception, when the neural tube fails to close. Few risk factors have been consistently associated with NTDs, although both genetic and environmental factors are thought to play a role in development (Detrait, George et al. 2005).

In humans, no single, major gene has been implicated as causal in the development of NTDs (De Marco, Merello et al. 2006), although a small proportion of NTDs are associated with known genetic syndromes and aneuploid conditions (Rampersaud, Melvin et al. 2006) and a positive family history is associated with an increased risk of an NTD-affected birth (Partington & McLone 1995). Low folic acid

levels remain the most widely recognized environmental (e.g., non-inherited) risk factor for NTDs, with randomized controlled trials showing that folic acid supplementation might prevent at least one-half of all cases (Medical Research Council 1991; Czeizel & Dudas 1992). In addition, NTDs have consistently been associated with maternal diagnosis of type 1 and type 2 diabetes (Becerra, Khoury et al. 1990) and use of antiepileptic medications during neurulation (Lammer, Sever et al. 1987).

Chemicals, such as those in pesticides, are known to cross the placenta and impact embryonic development. Mechanisms by which such chemicals may cause NTDs are poorly understood; however, animal studies provide some insight on possible mechanisms. Methyl carbamate, chlorpyrifos, and other organophosphate insecticides are cholinesterase inhibitors, and in animal studies, cholinesterase inhibition has been shown to alter cell proliferation and differentiation during neurulation (Slotkin 2004). In particular, chlorpyrifos has also been shown to lead to excessive neuroepithelial cell death during neurulation in rat embryos (Roy, Andrews et al. 1998), possibly resulting in too few cells for neural tube closure.

Pesticide exposure can occur in both the home and the workplace. Residential pesticide exposure can occur through air, water, or food contamination as well as home and garden use; occupational pesticide exposure can occur more directly through mixing of chemicals, equipment loading, application, equipment clean-up/repair, or disposal of empty containers. Residential pesticide exposure is expected to be common and occur at low doses, whereas occupational pesticide exposure is less common, but may occur at higher doses (Garcia 1998). Positive associations

have been reported between NTDs and maternal residential exposure to pesticides (White, Cohen et al. 1988; Garry, Schreinemachers et al. 1996; Shaw, Wasserman et al. 1999; Rull, Ritz, & Shaw 2006) and between conception during the growing season when pesticides would be applied (Garry, Schreinemachers et al. 1996; Kristensen, Irgens et al. 1997). The strength of reported associations between NTDs and maternal occupational pesticide exposure has been small to moderate and associations have not been consistently observed (Zhang, Wen-wei et al. 1992; Blatter, Roeleveld et al. 1996; Blanco Munoz, Lacasana et al. 2005; Lacasana, Vazquez-Grameix et al. 2006).

Previous studies of NTDs and occupational pesticide exposure have been limited by sample size and exposure assessment. Due to sample size limitations, few analyses were stratified by NTD subtype. Because NTD subtypes may have different etiologies (Mitchell 2005), combining NTDs subtypes may dilute the reported odds ratios. Blanco-Munoz et al. (2005) reported an odds ratio of 6.5 (95% CI: 1.4, 29.6) for pesticide exposure and anencephaly, an estimate considerably higher than those reported from other studies that combined NTD subtypes (Nurminen, Rantala et al. 1995; Shaw, Wasserman et al. 1999). Different exposure assessment methods may have also contributed to inconsistent findings. Many studies used only self-reports of any exposure (yes/no) to occupational pesticides (Zhang, Wen-wei et al. 1992; Blatter, Roeleveld et al. 1996; Blanco Munoz, Lacasana et al. 2005; Lacasana, Vazquez-Grameix et al. 2006). Industrial hygienist review of occupational exposures is thought to result in less exposure misclassification compared to self-report or job title alone (Fritschi, Siemiatycki et al. 1996). Only two studies of NTDs and pesticide

exposure were identified that used industrial hygienist review to assess exposure (Nurminen, Rantala et al. 1995; Shaw, Wasserman et al. 1999); however, neither sufficient power to examine risks of maternal occupational exposure to pesticides. Also, in previous studies, exposure assessment frequently included jobs over the entire pregnancy period or jobs held at the time of delivery, rather than those held during the period in which NTDs develop (day 21 to 28 post-conception). Lastly, no studies of maternal occupational pesticide exposure were found that stratified exposure by specific pesticide type. It is unlikely that all pesticides cause NTDs and, therefore, testing for associations of all pesticides combined may bias findings towards the null. Because of the large number of pesticide formulations and overlapping exposure to pesticide types, it will likely be difficult to link a specific pesticide to NTDs; however, exposure assessment may be improved by stratifying by pesticide class (e.g., insecticides, fungicides, or herbicides).

In this study, data from the National Birth Defects Prevention Study (NBDPS), an ongoing population-based, multi-center case-control study, were used to examine the relation between NTDs and maternal occupational pesticide exposure. Specifically, using retrospective maternal interview reports of occupations, industrial hygienists assigned probability of any exposure (yes/no) and cumulative exposure (mg) to each pesticide class for each occupation, and these exposure estimates were used to examine associations with all NTD cases combined as well as individual NTD subtypes.

Design and Methods

The NBDPS is a multicenter study to investigate genetic and environmental risk factors for over 30 major structural birth defects. At each center, clinical geneticists reviewed data abstracted from medical records to determine case eligibility based on pre-defined case definitions, required confirmatory diagnostic procedures, and exclusion criteria (e.g., known chromosomal or single gene disorders). NBDPS methods are described briefly below; additional detail is published elsewhere (Yoon, Rasmussen et al. 2001; Rasmussen, Olney et al. 2003). Each center obtained institutional review board approval for the NBDPS.

For this analysis, eligible case and control deliveries were those with estimated dates of delivery (EDD) from October 1, 1997 through December 31, 2003 collected at 8 centers (Arkansas, California, Iowa, Massachusetts, New Jersey, New York, Texas, and CDC/Metropolitan Atlanta). Eligible case deliveries included live births, fetal deaths, and elective terminations diagnosed with an NTD subtype (British Pediatric Association codes [BPA 1979]) anencephaly and craniorachischisis (742.00-742.09), spina bifida (741.00-741.99), and encephalocele, cranial meningocele, encephalomyelocele, and other rare subtypes (742.00-742.09). NTD case infants were classified by subtype and by phenotype, either isolated (no additional major defects) or multiple (one or more additional defects in a separate organ system). Eligible control deliveries were live births without a structural birth defect and randomly selected by each center using birth certificates or hospital records.

Mothers of eligible NTD cases and eligible control infants were recruited to complete a telephone interview no earlier than 6 weeks and, in an effort to minimize recall bias, no later than 24 months after the EDDs of the infants. The telephone interview collected information about maternal infectious, chemical, physical, nutritional, and behavioral exposures. The interview also collected information on maternal occupation(s) from 3 months prior to conception through delivery. Occupational data included company name and description, job title and description, dates worked, and average hours worked per day and number of days worked per week.

Case and control infants were restricted to those whose mothers reported employment during all or part of the relevant periconceptional period (1 month pre-conception [B1] through 2 months post-conception [P1 and P2]). Excluding non-working mothers, rather than classifying them as unexposed, eliminated confounding by employment status and factors associated with employment status (e.g., the healthy worker effect whereby those employed tend to be healthier than those not employed [McMichael 1976]). In addition, case and control mothers who reported use of folate antagonist medication (aminopterin sodium, carbamazepine, cholestyramine resin, methotrexate, oxcarbazepine, pyrimethamine, sulfasalazine, triamterene, trimethoprim, phenytoin, primidone, phenobarbital, valproate sodium) during the periconceptional period and/or were diagnosed with type 1 or type 2 diabetes before or during the index pregnancy were excluded.

Based on an extensive literature review and reported dermal measurements of pesticide exposure, the National Cancer Institute previously developed job exposure

matrices (JEM)s to assign pesticide exposure by job title and description (Samanic, De Roos et al. 2008). Using these JEMs and NBDPS maternal interview reports, industrial hygienists from Battelle Center for Public Health Research and Evaluation (Seattle, Washington), in collaboration with the National Institute for Occupational Safety and Health (NIOSH) and the NBDPS occupational working group, assigned a probability (0, < 1%, 1-33%, 34-66%, 67-89%, and 90% or greater) of maternal occupational exposure to each of three classes of pesticides (insecticides, herbicides, and fungicides) for each maternal job reported. Further, the exposure assessment team used the JEMs to estimate the average number of hours (<2, 2-10, 11-19, or >19) exposed to each pesticide class based on a 40-hour work week. Lastly, the intensity, or dose, of exposure to each class of pesticides was estimated (<1, 1-9, 10-99, and 100 mg/hr or greater).

For each maternal job reported, the hours worked per week were calculated based on reported typical hours worked per day multiplied by the typical number of days per week worked. For reported jobs with missing hours per day and/or days per week (< 1% of all jobs), an 8-hour day and/or a 5-day work week were assumed. The exposure assessment team verified individual maternal reports that exceeded 12 hours per day and 7 days per week for accuracy, and imposed a 16-hour limit per day to 28 jobs.

For this analysis, mothers who had a probability of 0 for occupational pesticide exposure for each reported job during the periconceptional period were classified as unexposed, whereas mothers who had a probability > 0 for at least one reported job during the periconceptional period were classified as exposed. For each

job with a probability > 0, the assigned exposure intensity, typical hours exposed per week, and maternal reports of typical hours worked per week were used to estimate cumulative occupational exposure to each pesticide class as follows:

$$\text{exposure intensity, mg} \times \frac{\text{typical hours exposed per week}}{\text{40 hours per week}} \times \frac{\text{typical hours worked per week}}{\text{7 days per week}} \times \text{number of days worked, B1 - P2}$$

Cumulative exposure to each pesticide class was classified as less than 50% or 50% or more relative to cut-points based on median cumulative exposure in control mothers.

Covariates evaluated included maternal age at delivery (<21, 21-25, 26-30, 31-35, >35), race/ethnicity (non-Hispanic white, non-Hispanic black, Hispanic, Other), education (<12 years, 12 years, 13-15 years, >15), gravidity (0, 1, 2, ≥3), pre-pregnancy body-mass index (<18.5, 18.5-24.9, 25-29.9, ≥ 30), periconceptional smoking (yes/no), and NBDPS center. Additionally, food folate (<600 ug or ≥600 ug) and periconceptional use of folic acid-containing supplements (yes/no) intake were examined. Food folate intake was assigned using the responses to the food frequency module in the NBDPS interview that measured food intake 1 year pre-conception (Willet Food Frequency questionnaire) as well as reports of breakfast cereals consumed during the P1 and P2. Dietary folate equivalents (DFE)s were estimated using the reported food frequencies, the standardized serving size on which a question item was based, and the United States Department of Agriculture National Standard Reference 16-1 (United States Department of Agriculture 2004). Also, each vitamin and supplement reportedly used during the periconceptional period was

assessed to determine whether or not it contained folic acid. Mothers were classified into those who took folic acid-containing vitamins and/or supplements during the periconceptional period and those who did not.

Analyses were conducted using SAS software, version 9.2 (SAS Institute 2007). Using the chi-square test, descriptive analyses of selected infant and maternal characteristics were conducted comparing each NTD subtype relative to control infants. Crude odds ratios (ORs) and 95% confidence intervals (CIs) were estimated to examine associations between maternal periconceptional occupational exposure (yes/no) to any pesticides and all NTD cases combined as well as each NTD subtype. Similarly, crude ORs and 95% CIs were estimated to examine the associations between cumulative exposure in mg (0, 0 to 50%, >50%) to any pesticides and all NTD cases combined as well as each NTD subtype. Where the number of exposed case mothers was at least 5, analyses were also conducted by individual (insecticides only, herbicides only, and fungicides only) and combined (insecticides + herbicides, insecticides + fungicides, herbicides + fungicides, and insecticides + herbicides + fungicides) pesticide classes.

Results of descriptive analyses were used to construct the most parsimonious multivariable logistic regression model for NTDs and maternal periconceptional occupational pesticide exposure. Covariates included in the preliminary model were those which were associated ($p < 0.10$) with any pesticide exposure and/or outcome. Beginning with the least significant covariate (highest p-value), backward variable selection was used to exclude covariates from the preliminary model based on the Wald chi-square statistic. Covariates for which exclusion from the model resulted in

a change of greater than 20% in the parameter estimate(s) of pesticide exposure were re-entered in the model. If the pesticide exposure parameter estimate(s) changed by less than 20%, the fit of the full model and reduced model were compared using the log-likelihood ratio (LLR) test. Covariates for which the LLR test was significant ($p<0.05$) were re-entered into the model and those that were not significant remained excluded. Based on the final multivariable logistic regression model, adjusted odds ratios (aORs) and 95% CIs were estimated to characterize the association between NTDs (all NTD cases combined, NTD subtypes, and NTD phenotypes) and maternal periconceptional occupational exposure to any pesticide or pesticide class and cumulative exposure to any pesticide or pesticide class.

Results

Interview data were collected from mothers of 958 (68% of eligible) NTD cases and 5008 (66% of eligible) control infants; 521 case and 2997 control mothers met the criterion of employment during all or part of the relevant periconceptional period. Of these, 18 case and 47 control interviews were excluded due to incomplete maternal interviews (cases n=1; controls n=4), maternal diagnosis of type 1 or type 2 diabetes prior to or during the index pregnancy (cases n=7; controls n=15), or maternal periconceptional exposure to folic acid antagonists (cases n=10; controls n=28). To improve homogeneity of NTD subtype groups, the maternal interview for 1 NTD case diagnosed with multiple NTD subtypes was excluded. Among the 502 NTD cases included in the analyses, 131 were diagnosed with anencephaly or

craniorachischisis, 310 with spina bifida, and 61 with encephalocele or other rare subtype.

All NTD cases combined were more likely to be preterm (< 37 weeks gestation) than control infants (Table III.1). Case infants with a rare NTD subtype were more likely than those with anencephaly or spina bifida to be diagnosed with multiple defects. Compared to control mothers, case mothers (all NTD cases combined) were significantly ($p < 0.05$) more likely to be younger, Hispanic, less educated, have 4 or more pregnancies, and to be obese; the number of NTD cases also differed among centers. When stratified by subtype, differences between control mothers and spina bifida mothers tended to parallel those for all NTD cases combined, although mothers of cases with anencephaly and rare NTD subtypes only differed from control mothers by number of infants per center.

As shown in Table III.2, 30% of control mothers and 32% of case mothers were occupationally exposed to pesticides. The majority of exposure was to insecticides only. Compared to control mothers, median cumulative exposure to insecticides and fungicides was higher for mothers of anencephaly and spina bifida cases, whereas, for all 3 pesticide classes, median cumulative exposure was lower for mothers of rare subtypes compared to controls.

Crude analyses found no associations between all NTD cases combined or each NTD subtype and maternal periconceptional occupational exposure to any pesticides. Maternal exposure to insecticides + herbicides was significantly associated with all NTD cases combined (OR: 2.2, 95% CI: 1.3, 3.8) and with spina bifida (OR: 2.6, 95% CI: 1.4, 4.8), whereas estimates were elevated, but non-

significant, for anencephaly. In addition, exposure to insecticides + herbicides + fungicides was significantly associated with all NTD cases combined (OR: 1.5, 95% CI: 1.1, 2.1) and with anencephaly (OR: 2.1, 95% CI: 1.2, 3.6); estimates were elevated for other rare NTD subtypes combined.

After adjustment for body mass index and center, aORs for maternal periconceptional occupational exposure to any pesticides or insecticides only were non-significant for all NTD cases combined or individual NTD subtypes (Table III.3). Maternal exposure to insecticides + herbicides produced elevated, but non-significant estimates, for all NTD cases combined, but significantly elevated estimates for spina bifida (OR: 2.2, 95% CI: 1.1, 4.4). No significant associations were found for mothers exposed to any insecticides + herbicides + fungicides for all NTD cases combined or individual NTD subtypes, although estimates were elevated for anencephaly and other rare NTD subtypes. Sample size precluded analyses of exposure to herbicides only; however, numbers allowed estimation of odds of exposure to each pesticide class if exposure to the other remaining classes was considered irrelevant (e.g., exposure to herbicides regardless of exposure to insecticides and/or fungicides). Odds ratios were near unity for mothers exposed to insecticides and mothers exposed to fungicides but elevated risk for mothers exposed to herbicides (aOR: 1.3; 95% CI: 1.0, 1.9).

Examination of maternal cumulative exposure (0, >0 to 50%, >50%) to any pesticides and also to insecticides only produced aORs near unity for all NTD cases combined and each NTD subtype. The aORs for maternal cumulative exposure to insecticides + herbicides were also elevated, but non-significant, for all NTD cases

combined and for spina bifida cases. In addition, aORs for maternal cumulative exposure above the median (≥ 245.09 mg) insecticides + herbicides + fungicides were elevated for spina bifida cases. The majority of mothers exposed to insecticides + herbicides held at least one job in janitorial services (cases=95%, controls=85%). Jobs held by mothers exposed to insecticides + herbicides + fungicides included agricultural (cases=33%, controls=21%), food preparation (cases=21%, controls=28%), and supermarkets (cases=19%, controls=20%).

Additional analyses included estimation of odds ratios 1) by phenotype (isolated or multiple), 2) excluding those with a family history of NTDs, and 3) restricting to centers that include live births, fetal deaths, and elective termination. .For each pesticide class examined, aORs for NTDs with isolated defects were similar to those of all phenotypes combined (data not shown). For all NTD cases combined with multiple defects (n=26), when compared to no maternal exposure, cumulative exposure (>84.375 mg) to any pesticides was significant (aOR: 2.1, 95% CI: 1.2, 3.9); small numbers precluded additional analyses by phenotype. In addition, sub-analyses by family history of an NTD (yes/no) produced no appreciable difference in the aORs. Restriction of analyses to centers which collected live births, stillbirths, and elective terminations also produced little change in the results.

Discussion

Maternal periconceptional occupational pesticide exposure (yes/no) was not associated with all NTD cases combined or individual NTD subtypes. Similarly, exposure to insecticides only or insecticides + herbicides + fungicides, was not

significantly associated with NTDs. Exposure to insecticides + herbicides was significantly associated with spina bifida whereas aORs for all NTD cases combined were elevated. Results for maternal cumulative exposure to a pesticide were similar to those for any exposure to that class.

Comparing the results of those exposed to insecticides only and those exposed to insecticides + herbicides, would suggest the risk of NTDs lies with herbicide exposure; however, exposure to insecticides + herbicides + fungicides did not produce significant odds ratios. It seems unlikely that exposure to fungicides would attenuate risk found with exposure to insecticides + herbicides. The variation in risk was not due to higher cumulative exposure in those exposed to insecticides + herbicides compared to those exposed to insecticides + herbicides +fungicides; as exposure was more than twice as high in the later group (Table III.3). When these mothers were examined by job title, the majority of mothers exposed to insecticides + herbicides were janitorial services (e.g., maids, housekeepers, etc.) whereas mothers exposed to insecticides + herbicides + fungicides were most likely employed in agriculture, supermarkets (e.g., cashiers), and food preparation (e.g. cooks). It is possible that these jobs are exposed to different types of pesticides, pesticides that carry different risk, or that mothers' residential exposure, for which this study could not account, varies by occupational exposure.

In animal studies, pesticides have been shown to negatively impact neurulation via cholinesterase inhibition, affecting neuroepithelial cell proliferation and differentiation (Rull, Ritz et al. 2006) or causing cell death (Roy, Andrews et al. 1998). Three prior studies in humans reported significant associations between NTDs

and pesticide exposures (Blatter, Roeleveld et al. 1996; Blanco Munoz, Lacasana et al. 2005; Lacasana, Vazquez-Grameix et al. 2006). Two of these studies (Blanco Munoz, Lacasana et al. 2005; Lacasana, Vazquez-Grameix et al. 2006) were limited to cases diagnosed with anencephaly and assignment of pesticide exposure from job title, comparing those employed in agriculture to those not employed in agriculture. The odds ratios reported in each study were markedly higher than those reported in the current study although imprecise (OR: 6.5, 95% CI: 1.4, 29.6; OR: 4.57, 95% CI: 1.1, 20.0). The third study (Blatter, Roeleveld et al. 1996), also restricted to mothers employed in agriculture and reported an association for spina bifida only (OR: 3.4, 95% CI: 3.0, 9.0).

The current case-control study of maternal occupational exposure to pesticides and NTDs used data from one of the largest U.S. population-based studies of birth defects. Use of these data is a strength of this study. Each case was reviewed by geneticists using accepted clinical criteria and diagnostic tests for classification. In addition, comparison of selected maternal characteristics of control mothers who participated in NBDPS and all live births in the same geographic regions showed that control participants tend to be similar to live births (Cogswell, Bitsko et al. 2009). These strengths reduced the risk of case misclassification and selection bias, respectively.

Assignment of maternal occupational exposure to pesticides was conducted by industrial hygienist review, the 'gold standard,' unlike many previous studies which used maternal self-reports only. Classification through industrial hygienist review is expected to decrease the risk of exposure misclassification compared to use of self-

report or job title alone to classify exposure (Fritschi, Siemiatycki et al. 1996). Two prior studies of NTDs and pesticide exposure that used industrial hygienist review to assess exposure (Nurminen, Rantala et al. 1995; Shaw, Wasserman et al. 1999) were limited by sample size. Also, in previous studies the exposure assessment frequently included jobs over the entire pregnancy or jobs held at the time of delivery, rather than only jobs held during the relevant periconceptional period, as was evaluated in this study. Finally, no prior studies reported stratifying exposure by pesticide class.

Although efforts were made to improve exposure and outcome classification compared to previous studies, limitations remained. Our sample of NTD case and control mothers was larger than previous studies, yet small sample sizes limited some analyses for NTD subtypes, phenotypes, and pesticide classes, resulting in either imprecise odds ratios or the inability to calculate odds ratios for specific strata. Also, although the extensive interview of NBDPS allowed us to account for a variety of possible covariates, exposure to pesticides outside the workplace, such as pesticide use at home, living on a farm, or residing near land in crop production, was not determined and could not be adjusted for in the analyses. Although residential exposure to pesticides at low levels is expected to be common (Garcia 1998) for both case and control mothers, it is unknown how such exposure may vary between case and control mothers. Residential exposure to pesticides could confound the relation being tested if residential exposure to pesticides is associated with NTDs and occupational exposure. Lastly, overlap among pesticide class exposure was high, limiting analysis to individuals exposed to insecticides only, insecticides + herbicides, and insecticides + herbicides + fungicides.

In summary, the association between NTDs and maternal periconceptional occupational exposure to pesticides was investigated using a large, case-control study. Results suggested that joint exposure to insecticides + herbicides was associated with spina bifida in addition to elevated risks among those with multiple defects exposed to pesticides. Although previous studies corroborate these results and efforts were made to improve over previous studies, these results should be interpreted cautiously, due to the limitations described previously. Future studies should aim to increase sample size, particularly in less prevalent subtypes and those exposed to only herbicides, and may benefit from characterizing associations in highly exposed populations. In addition, future studies should aim to use similar detailed exposure assessment while also collecting data on exposure to pesticides outside of the workplace.

Table III.1. Selected characteristics of infants and birth mothers for control infants and neural tube defect subtypes, National Birth Defects Prevention Study, 1997-2003

	Control Infants (n= 2950)		All NTDs‡ (n=502)		Anencephaly (n=131)		Spina Bifida (n=310)		Other Rare Type (n=61)	
Characteristic	n*	%†	n	%	n	%	n	%	n	%
Infant										
Sex										
Male	1468	49.8	230	48.4	46	41.8	158	52.0	26	42.6
Female	1480	50.2	245	51.6	64	58.2	146	48.0	35	57.4
Gestational Age (weeks)[a]										
< 37	251	47.3	222	44.2	103	78.6	91	29.4	28	45.9
≥ 37	2699	52.7	280	55.8	28	21.4	219	70.7	33	54.1
Phenotype										
Isolated	NA		441	87.9	117	89.3	280	90.3	44	72.1
Multiple	NA		61	12.2	14	10.7	30	9.7	17	27.9
Mother										
Age at Delivery (years)[b]										
<21	361	12.2	70	13.9	19	14.5	43	13.9	8	13.1
21-25	664	22.5	112	22.3	28	21.4	70	22.6	14	23.0
26-30	845	28.6	160	31.9	41	31.3	103	33.2	16	26.2
31-35	746	25.3	96	19.1	31	23.7	50	16.1	15	24.6
>35	334	11.3	64	12.7	12	9.2	44	14.2	8	13.1
Race/Ethnicity										
Non-Hispanic White	1910	64.9	295	58.8	75	57.3	188	60.6	32	52.5
Non-Hispanic Black	373	12.7	57	11.4	16	12.2	31	10.0	10	16.4
Hispanic	521	17.7	124	24.7	33	25.2	74	23.9	17	27.9
Other	139	4.7	26	5.2	7	5.3	17	5.5	2	3.3
Education (years)[a]										
< 12	295	10.0	72	14.3	20	15.3	44	14.2	8	13.1
12	729	24.7	152	30.3	39	29.8	94	30.3	19	31.1
13-15	885	30.0	145	28.9	32	24.4	97	31.3	16	26.2
> 15	1039	35.2	133	26.5	40	30.5	75	24.2	18	29.5

Table III.1 Continued

Gravidity[b]										
1	899	30.5	137	27.3	38	29.0	82	26.5	17	27.9
2	922	31.3	148	29.5	41	31.3	90	29.0	17	27.9
3	606	20.5	101	20.1	23	17.6	66	21.3	12	19.7
> 3	522	17.7	116	23.1	29	22.1	72	23.2	15	24.6
Pre-Pregnancy BMI (kg/m^2)‡[a]										
Underweight (<18.5)	150	5.2	17	3.5	6	4.7	9	3.0	2	3.5
Normal weight (18.5-24.9)	1662	57.5	249	51.4	75	59.1	140	46.7	34	59.6
Overweight (25-29.9)	652	22.6	104	21.5	28	22.0	67	22.3	9	15.8
Obesity (≥ 30)	425	14.7	114	23.6	18	14.2	84	28.0	12	21.1
Periconceptional‡ Smoking[b]										
Yes	600	20.3	91	18.1	112	85.5	62	20.0	10	16.4
No	2350	79.7	411	81.9	19	14.5	248	80.0	51	83.6
Periconceptional Folic Acid Intake										
Yes	2295	79.0	382	77.2	105	80.8	230	75.7	47	77.0
No	611	21.0	113	22.8	25	19.2	74	24.3	14	23.0
Pre-pregnancy Food Folate										
<600 ug daily	1892	64.1	324	64.5	89	67.9	195	62.9	40	65.6
≥ 600 ug daily	1058	35.9	178	35.5	42	32.1	115	37.1	21	34.4
NBDPS Center[a]										
Arkansas	369	12.5	83	16.5	24	18.3	49	15.8	10	16.4
California	343	11.6	83	16.5	29	22.1	51	16.5	3	4.9
Iowa	409	13.9	80	15.9	16	12.2	58	18.7	6	9.8
Massachusetts	424	14.4	35	7.0	7	5.3	20	6.5	8	13.1
New Jersey	414	14.0	47	9.4	9	6.9	34	11.0	4	6.6
New York	334	11.3	43	8.6	7	5.3	27	8.7	9	14.8
Texas	311	10.5	71	14.1	21	16.0	40	12.9	10	16.4
CDC‡/Atlanta, Georgia	346	11.7	60	12.0	18	13.7	31	10.0	11	18.0

* Numbers may vary due to incomplete or missing data

Table III.1 Continued
† Due to rounding, percentages may not total 100

‡ NTDs, neural tube defects; NA, not applicable; BMI, body mass index; B1-P2; CDC, Centers for Disease Control and Prevention

a $p < 0.01$ for all NTDs

b $p < 0.05$ for all NTDs

Table III.2. Maternal periconceptional occupational pesticide exposure by control and neural tube defect subtype, National Birth Defects Prevention Study, 1997-2003*

	Control Infants (n= 6603)		All NTDs		Anencephaly		Spina Bifida		Other Rare Subtype	
Periconceptional Exposure	n=2950		n=502		n=131		n=310		n=61	
Pesticide Exposure	n	%†	n	%	n	%	n	%	n	%
Any	888	30.1	162	32.3	45	34.4	97	31.3	20	32.8
None	2042	69.2	334	66.5	83	63.4	210	67.7	41	67.2
Pesticide Exposure by Class∞										
Insecticides Only	616	20.9	91	18.1	23	17.6	58	18.7	10	16.4
Insecticides + Herbicides	52	1.8	19	3.8	4	3.1	14	4.5	1	1.6
Insecticides + Herbicides + Fungicides	211	7.2	52	10.4	18	13.7	25	8.1	9	14.8
Mean Pesticides Exposure Among Exposed (mg)	n	Median	n	Median	n	Median	n	Median	n	Median
Insecticides	885	62.6	162	96.4	45	96.4	97	84.4	20	59.5
Herbicides	266	49.8	71	40.2	22	57.9	30	35.5	10	33.4
Fungicides	217	64.3	52	63.9	18	86.4	25.0	96.4	9	34.7

*Missing, incomplete, or questionable data on pesticides exposure was distributed as follows: controls (n=20), all cases (n=6), anencephaly (n=3), spina bifida (n=3), other rare subtypes (n=0)

∞ No case and 3 control mothers were exposed to herbicides only, no case or control mothers were exposed to fungicides only or jointly exposed to herbicides and fungicides, and no case and 6 control mothers were jointly exposed to insecticides and fungicides

† Due to rounding, percentages may not total 100

Table III.3. Adjusted odds ratio estimates for neural tube defect subtypes associated with maternal occupational exposure to pesticides, National Birth Defects Prevention Study, 1997-2003*†

	Controls	Combined NTDs			Anencephaly			Spina Bifida			Other rare subtypes		
Pesticide Exposure[a]	No.	No.	Odds Ratio	95% Confidence Interval	No.	Odds Ratio	95% Confidence Interval	No.	Odds Ratio	95% Confidence Interval	No.	Odds Ratio	95% Confidence Interval
None	2042	334	Ref‡		83	Ref		210	Ref		41	Ref	
Any Exposure													
Any Pesticides	888	162	0.9	0.8, 1.2	45	1	0.6, 1.5	97	0.9	0.7, 1.2	20	1	0.5, 1.7
Insecticides Only	616	91	0.8	0.6, 1.0	23	0.8	0.5, 1.3	58	0.8	0.6, 1.1	10	0.8	0.4, 1.6
Insecticides + Herbicides	52	19	1.8	1.0, 3.3	4	NC	NC	14	2.2	1.1, 4.4	1	NC	NC
Insecticides + Herbicides + Fungicides	211	52	1.2	0.8, 1.7	18	1.6	0.9, 2.8	25	0.9	0.6, 1.5	9	1.7	0.7, 3.9
Cumulative Exposure (mg)[∞]													
Any Pesticides													
>0 and <84.375	450	63	0.8	0.6, 1.1	14	0.7	0.4, 1.3	42	0.8	0.6, 1.2	7	0.8	0.4, 1.9
≥84.375	438	99	1.1	0.8, 1.4	31	1.3	0.8, 2.0	55	1	0.7, 1.4	13	1.1	0.5, 2.2
Insecticides Only													
>0 and < 62.625	329	34	0.7	0.4, 1.0	4	NC‡	NC	23	0.7	0.4, 1.1	7	1.2	0.5, 2.8
≥62.625	287	57	0.9	0.7, 1.3	19	1.2	0.7, 2.0	35	1	0.6, 1.4	3	NC	NC
Insecticides + Herbicides													
>0 and < 9.48	26	10	1.8	0.8, 4.1	2	NC	NC	8	2.3	0.9, 5.7	0	NC	NC
≥9.48	26	9	1.8	0.8, 4.3	2	NC	NC	6	2.2	0.8, 5.9	1	NC	NC
Insecticides + Herbicides +Fungicides													
>0 and <245.09	105	22	1.1	0.7, 1.8	7	1.1	0.7, 1.8	9	1.3	0.6, 2.9	6	0.8	0.4, 1.6
≥245.09	106	30	1.3	0.8, 2.1	11	1.3	0.8, 2.1	16	1.8	0.9, 3.8	3	MC	NC

Table III.3 Continued

*Missing or incomplete data for pesticides exposure were distributed as follows: controls (n=71), all cases (n=22), anencephaly (n=8), spina bifida (n=13), and other rare subtypes (n=1)

‡ NTD, neural tube defect; Ref, Reference; NC, not calculated

[a] Analyses adjusted for maternal body mass index and center

[α] Cut-points were based on exposure in control mothers and calculated as exposure less than the median (50%) and more than the median

CHAPTER IV

NEURAL TUBE DEFECTS AND PREGNANCY OUTCOME: BIAS?

Summary of Findings

Few risk factors have been consistently associated with NTD development. Etiologic study of NTDs requires a large base population; however many studies include only live born NTD cases. Little is known about the differences among mothers of NTD-affected live births, fetal deaths, and elective terminations. Inclusion or exclusion of NTD cases due to pregnancy outcome may create an ascertainment or response bias, making it difficult to interpret risk estimates. This study used data collected by the Iowa Registry for Congenital and Inherited Disorders (IRCID) for the National Birth Defects Prevention Study (NBDPS) to estimate differences in NBDPS eligibility, participation rates, and selected maternal characteristics by NTD pregnancy outcome. Subjects included Iowa mothers with an NTD-affected pregnancy with an estimated date of delivery from January 1, 1998 through December 31, 2005 and Iowa mothers of unaffected live born infants over the same time period with completed NBDPS maternal interviews. The proportion of NTDs by pregnancy outcome (live births versus fetal deaths and elective terminations) over time as well as differences in NBDPS eligibility, participation rates, and selected maternal and infant characteristics were examined. In addition, Iowa NBDPS interview data were used to describe differences in selected characteristics between NTD cases and control infants. Of the 279 NTD cases ascertained by the IRCID, 167 were live births and 112 were other pregnancy outcomes (fetal deaths or elective terminations). From 1998 through 2005, the proportion of these

other pregnancy outcomes increased by over 10%. When compared by selected infant and maternal characteristics, mothers of live births and other pregnancy outcomes were similar. Overall, 81% of NTD cases (n=226) were eligible for the NBDPS, although eligibility varied significantly by pregnancy outcome (live born infants=89% versus other pregnancy outcomes=69%, $p < 0.01$). Participation rates did not vary significantly between eligible, mothers of live born NTD cases and mothers of other pregnancy outcomes (live born=66% versus other pregnancy outcomes=55%, respectively, $p=0.08$). When case mothers were compared to control mothers by selected characteristics, there were no significant differences, although mothers of live born case deliveries were more likely to be obese. The IRCID is an ideal birth defect surveillance system in that it uses active surveillance and well-established case ascertainment methods; however, only one NTD-affected early fetal death (<20 weeks gestation) was ascertained. To better understand the impact of ascertainment and response bias on risk estimates, future studies should aim to include all fetal deaths and assess differences in known risk factors for NTDs by pregnancy outcome.

Introduction

An estimated 1 in 1000 infants are born with a neural tube defect (NTD) annually in the United States (Detrait, George et al. 2005), and more are spontaneously aborted or electively terminated. The most common types of NTDs are anencephaly and spina bifida, which typically present as open NTDs; such NTDs occur when neural tissue is exposed to the environment or only covered by a membrane. Less common are encephalocele and meningocele, which typically present as closed NTDs, in which the

defect is covered by normal skin. Anencephaly is fatal in all cases; infants with spina bifida frequently survive following surgery. Although no single major gene has been implicated as causal in the development of NTDs (De Marco, Merello et al. 2006), these defects are thought to result in part from genetic risk factors. Environmental (non-inherited) factors are also thought to play a role in NTD development; however established risk factors, such as folate levels (Medical Research Council 1991; Czeizel & Dudas 1992), maternal diabetes (Becerra, Khoury et al. 1990), obesity (Shaw, Velie et al. 1996), and use of antiepileptic medications (Lammer, Sever et al. 1987), account for only a small proportion of prevalent NTDs, indicating that unidentified risk factors for NTDs remain.

Development of the neural tube occurs within 28 days of conception, frequently before a woman is aware of her pregnancy, making detection of maternal risk factors particularly difficult. In addition, NTDs are associated with pregnancy outcome (e.g., live birth, fetal death, or elective termination). NTD-affected pregnancies are more likely to result in fetal death compared to unaffected pregnancies (Centers for Disease Control and Prevention 2004). Also, because NTDs occur during early pregnancy and can be detected by ultrasound or other prenatal tests (e.g., alpha feto-protein), a large proportion of NTDs are prenatally diagnosed and subsequently electively terminated (Forrester, Merz, et al. 1998). Forrester et al. (1998) reported an increasing trend in prenatal diagnosis of spina bifida cases from 1987/1988 to 1995/1996 in Hawaii, with rates increasing from 46 to 67%. Over the same time period, among all anencephaly cases prenatally diagnosed, 83% were electively terminated (Forrester, Merz, et al. 1998).

Further, a study of six states found that 9 to 42% of NTDs were electively terminated (years 1985-1993, total years varied by state) (Cragan, Roberts et al. 1995).

Little is known about the differences among mothers of NTD-affected live births, fetal deaths, and elective terminations. One study by Velie and Shaw (1996) found that, when compared to mothers of live born and stillborn (fetal deaths at 20 weeks or greater gestation) NTD cases, mothers of electively terminated NTD-affected cases were significantly more likely to be white, older, employed, and have attained higher education levels.

The relative rarity of NTDs requires a large base population in order to ascertain sufficient numbers of NTD cases for etiologic studies. As such, studies of NTDs frequently use cases identified by birth defect surveillance systems; however, many of these systems monitor live births only, or monitor live births and selected fetal deaths (e.g., 20 weeks or greater gestation) and elective terminations (e.g., prenatally diagnosed birth defects). According to the National Birth Defects Prevention Network (2009), only 21 of the 46 state-based birth defect surveillance programs collected data on at least some electively terminated deliveries with birth defects, with only 11 of those programs collecting data on elective terminations at all gestational ages (Table IV.1).

Inclusion or exclusion of cases by pregnancy outcome may create biases, more specifically, ascertainment bias and response bias. Ascertainment bias can impact risk estimates, particularly when the exposure variable being tested is associated with ascertainment of the case (pregnancy outcome) and the outcome of interest, in this instance NTD development. Consider an exposure with a dose-response effect, where an increasing dose leads to increasing severity of an NTD as an example. If NTD severity is

associated with an increased risk of fetal death or elective termination, exclusion of fetal deaths and aborted cases would bias a risk estimate towards the null, or in extreme cases, generate a seemingly protective effect. Similarly, response bias due to differences in participation rates by pregnancy outcome may affect risk estimates. No study was found that examined participation rates of mothers of NTD cases by pregnancy outcome in a large, population-based study. Without improved understanding of the impact of the survival and response biases, interpretation of risk estimates in studies of NTDs is difficult.

This study used data collected by the Iowa Registry for Congenital and Inherited Disorders (IRCID) for the National Birth Defects Prevention Study (NBDPS) to examine the proportion of NTDs by pregnancy outcome over time as well as differences in NBDPS eligibility, participation rates, and selected maternal and infant characteristics. Also, Iowa NBDPS interview data were used to estimate differences in selected characteristics between NTD case and control infants by pregnancy outcome.

Design and Methods

Established in 1983, the IRCID uses active surveillance to collect population-based information about live births, fetal deaths, and elective terminations diagnosed with birth defects, including NTDs, occurring to Iowa residents. Beginning with pregnancies with an estimated date of delivery of October 1, 1997, mothers of infants ascertained by the IRCID and diagnosed with one of over 30 eligible defects were recruited for the NBDPS. The NBDPS, one of the largest United States studies of birth defects, collects data on a variety of maternal exposures including infectious, chemical, physical,

nutritional, and behavioral factors. Clinical geneticists review hospital and medical records to determine case eligibility based on pre-defined definitions of each defect, required confirmatory diagnostic procedures, and exclusion criteria. Control infants for the NBDPS include unaffected live births during the same time period and in the same geographic regions randomly selected from birth certificates or hospital discharge data. Additional details about the NBDPS are described elsewhere (Yoon, Rasmussen et al. 2001; Rasmussen, Moore et al. 2001; Rasmussen, Olney et al. 2003).

For this analysis, mothers of live births, fetal deaths, and electively terminated fetuses with estimated dates of delivery (EDD)s from January 1, 1998 through December 31, 2005 diagnosed with an NTD and ascertained by the IRCID were included. Controls included mothers of live born infants who completed the NBDPS interview over the same time frame. In Iowa, control infants were randomly selected from birth certificates for the NBDPS. Pregnancy outcome--live birth, fetal death, or elective termination--was previously assessed by IRCID abstractors using vital and medical records. For this analysis, live births were compared to the other pregnancy outcomes (fetal death and elective terminations) combined.

To estimate the change in pregnancy outcomes over time, the proportion of each outcome was calculated in two-year increments from 1998 through 2005; the Mantel-Haenszel chi-square statistic was used to test for an increasing or decreasing trend in proportions. NTD cases were also compared for differences in selected infant (sex and gestational age) and maternal (age, race/ethnicity, education, gravity, pre-pregnancy body mass index, periconceptional folic acid use, smoking, and alcohol exposure); chi-square analyses and Student's t-tests were used to test for significant differences between groups

for categorical and continuous characteristics, respectively. Mothers of NTD cases were compared by eligibility of the index infant to participate in the NBDPS and by pregnancy outcome. Mothers of eligible case infants were stratified by pregnancy outcome and compared by participation (yes/no) in the NBDPS, defined as completion of the maternal NBDPS interview. The chi-square statistic was calculated to evaluate differences in eligibility and participation rates by pregnancy outcome. Case mothers who completed the NBDPS interview were stratified by pregnancy outcome and compared to those who did not complete the interview to examine differences in selected characteristics. Lastly, Iowa case and control mothers who completed the NBDPS interview, were compared for differences in selected characteristics using data collected for the NBDPS maternal interview.

Results

IRCID Cases by Pregnancy Outcome

Of the 279 NTD cases with EDDs from January 1, 1998 through December 31, 2005 ascertained by IRCID, 167 (60%) were live births and 112 (40%) were other pregnancy outcomes (fetal deaths=32; elective terminations=80). From 1998/1999 to 2004/2005, the proportion of these other pregnancy outcomes rose from 33 to 44% (Table IV.2), a non-significant trend (Mantel-Haenszel $p = 0.15$). Mothers with live births and those with other pregnancy outcomes were similar for age, race/ethnicity, education, and gravidity (Table IV.3). Stratification of NTD cases by subtype showed that those with other pregnancy outcomes were significantly more likely ($p < 0.01$) to be diagnosed with anencephaly than live born NTD cases (59% versus 13%). Conversely, live born NTD

cases were more likely to be diagnosed with spina bifida than those with other pregnancy outcomes (74% versus 32%).

NBDPS Eligibility and Participation

Of the 279 NTD cases ascertained by the IRCID, 81% (n=226) were eligible for the NBDPS. Eligibility varied significantly by pregnancy outcome, with 89% of live births compared to 69% of other pregnancy outcomes eligible for NBDPS. Participation rates did not differ significantly between eligible live born NTD outcomes and other pregnancy outcomes (66% versus 55%, p=0.08). Overall, mothers who had at least 12 years of education were more likely to participate in the NBDPS than those with less than 12 years of education (71% versus 49%, $p < 0.01$). In addition, non-Hispanic white mothers were more likely to participate than mothers in other race/ethnicity groups (65% versus 41%, $p = 0.01$). Overall participation rates also differed significantly by NTD subtype; mothers of infants diagnosed with spina bifida were more likely to participate (70%) than mothers of infants with anencephaly (47%) or other rare NTD subtypes (64%). When stratified by pregnancy outcome, NBDPS participation by mothers of live born infants only differed by education and NTD subtype with 72% of mothers of spina bifida cases participating compared to only 35% of mothers of anencephaly cases. Participation among mothers of other pregnancy outcomes differed by race/ethnicity, but did not differ by NTD subtype; 52% and 60% of mothers of infants diagnosed with anencephaly and spina bifida participated, respectively.

Based on maternal reports from the NBDPS interview, case mothers of live births and those of other pregnancy outcomes were similar in terms of age, race/ethnicity, education, gravity, pre-pregnancy body mass index, periconceptional folic acid use,

smoking, and alcohol exposure. NTD infants by pregnancy outcome were similar by sex and phenotype but varied significantly by gestational age at delivery as the majority (95%) of other deliveries occurred prior to 37 weeks compared to only 30% of live births. In addition, NTD infants varied significantly ($p < 0.01$) by NTD subtype as other pregnancy outcomes were more likely to be diagnosed with anencephaly compared to live born infants (60% versus 7%). Live born infants were more likely to be diagnosed with spina bifida compared to other pregnancy outcomes (81% versus 36%).

NBDPS Cases and Controls

There were 751 Iowa control mothers with completed interviews over the 1998 to 2005 time frame. Control infants and NTD casaes were similar by sex, but NTD cases were significantly more likely ($p < 0.01$) to have been born at less than 37 weeks gestation. Compared to Iowa control mothers, mothers of NTD case infants were similar in age, race, education, gravidity, periconceptional folic acid use, and food folate consumption. Case mothers were significantly more likely ($p = 0.01$) to have a pre-pregnancy body mass index higher than 30, which is classified as obese. Results from comparisons between mothers of live born case infants and mothers of control infants mirrored those of comparisons between all cases and controls. Mothers of other pregnancy outcomes did not significantly differ from controls by any selected characteristic, including pre-pregnancy body mass index.

Sub-analyses comparing live births and elective terminations found that, from 1998/1999 to 2004/2005, the proportion of electively terminated NTD cases nearly doubled, rising from 20 to 39%, a significantly increasing trend (Mantel-Haenszel $p =$

0.03). Results of subsequent analyses mirrored those of all pregnancy outcomes combined. Small numbers precluded sub analyses comparing live births and fetal deaths.

Discussion

Overall, other pregnancy outcomes, fetal deaths and elective terminations, accounted for 40% of the NTD cases ascertained by the IRCID from January 1, 1998 through December 31, 2005. The proportion of other pregnancy outcomes increased by more than 10% during this time frame. NBDPS eligibility varied by pregnancy outcome, although NBDPS participation rates were similar among pregnancy outcomes. Little difference in selected maternal characteristics was found among pregnancy outcomes. In general, mothers of NTD case infants were similar to mothers of Iowa NBDPS controls, although mothers of live born cases were more likely to be obese.

No significant differences were identified in participation rates of case mothers by pregnancy outcome or in selected characteristics between those who participated and those who did not, suggesting that studies using this population would be unlikely to be affected by response bias. However; this study only explored selected infant and maternal characteristics and did not examine differences in all characteristics known to be associated with NTDs. Differences in pregnancy outcome by NTD subtype were not surprising due to the variable viability of each subtype. However, lack of differences between mothers by pregnancy outcome was inconsistent with a previous report (Velie & Shaw 1996) which compared mothers of live born and stillborn (fetal deaths at 20 weeks or later) NTD cases to electively terminated NTD cases which found numerous differences between mothers. These results suggest that exclusion of the other pregnancy

outcomes included in the study (elective terminations and late fetal deaths) may not induce an ascertainment bias when examining the selected characteristics included in our study. Rates of elective termination were within the range (9 to 42%) of those reported previously by Cragan et al. (1995) and, like previous reports, showed an increasing trend (Forrester, Merz et al. 1998). This increase in elective terminations may be due, at least in part, to an increase in prenatal diagnosis over this time period.

The IRCID is an ideal birth defect surveillance system in that it uses active surveillance and well-established case ascertainment methods, and is expected to have near complete ascertainment of NTD-affected live births, late fetal deaths (20 weeks or later gestation), and elective terminations. NTD cases were reviewed and verified by clinical geneticists, decreasing the risk of case misclassification. Pregnancy outcome was assessed by trained abstractors reducing the risk of misclassification of pregnancy outcome. In addition, unlike many other birth defect surveillance systems, the IRCID collects live births, fetal deaths, and elective terminations, regardless of gestational age. However, ascertainment of early fetal deaths remains limited. Of the 32 fetal deaths ascertained by IRCID, only one occurred prior to 20 weeks gestation; yet early fetal deaths are expected to be more common than late fetal deaths.

Future studies should aim to assess known or suspected risk factors such as diabetes and consumption of folic acid antagonists by pregnancy outcome; sample size precluded such analyses in this study. In addition, future studies would ideally make efforts to ascertain all fetal deaths, as these early fetal deaths may differ from late fetal deaths and elective terminations and contribute to survival and response biases. With improved ascertainment and, in turn, improved understanding of the impact of

ascertainment and response biases, results could be used to better interpret study risk estimates.

Table IV.1. Surveillance methods of 46 state-based birth defect surveillance systems stratified by collection of elective terminations

	Ascertainment of ETs‡ (all gestational ages) (n=11)	Partial Ascertainment of ETs* (n=10)	No Ascertainment of ETs (n=25)
Surveillance Methods			
Active	7	4	2
Passive	2	3	14
Active and passive	2	3	9

*Includes ascertainment in 7 states of ETs over 20 weeks gestational age, 2 states of ETs prenatally diagnosed with a birth defect, and 1 state of only ETs with diagnosed neural tube defects

‡ ETs, elective terminations

Table IV.2. Neural tube defect cases by delivery type; Iowa Registry for Congenital and Inherited Disorders, 1998-2005

Birth Years	Live Births		Other Pregnancy Outcomes*	
	n	%	n	%
All Years	167		112	
1998-1999	47	66.2	24	33.8
2000-2001	49	62.0	30	38.0
2002-2003	36	53.7	31	45.3
2004-2005	35	56.5	27	43.6

*Other pregnancy outcomes includes fetal deaths and elective terminations

Table IV.3. Selected characteristics of neural tube defect cases by pregnancy outcome; Iowa Registry for Congenital and Inherited Disorders, 1998-2005*

	Live Births (n=167)		Other Pregnancy Outcomes (n=112)		p
Mother					
Age at Delivery (years)					
<21	36	21.6	25	22.3	
21-25	28	16.8	18	16.1	
26-30	55	32.9	35	31.3	0.99
31-35	29	17.4	19	17.0	
>35	19	11.4	15	13.4	
Race/Ethnicity					
Non-Hispanic White	142	87.1	86	84.3	0.52
Other	21	12.9	16	15.7	
Education (years)					
< 12	76	46.3	27	42.2	0.57
≥ 12	88	53.7	37	57.8	
Gravidity					
1	8	6.5	11	12.4	
2	50	40.3	30	33.7	0.45
3	30	24.2	22	24.7	
≥ 4	36	29.0	26	29.2	
Infant					
NTD‡ Subtype					
Anencephaly	22	13.2	66	58.9	
Spina Bifida	123	73.7	36	32.1	<0.01
Other rare subtypes	22	13.2	10	8.9	

*Totals may vary due to missing data

‡NTD, neural tube defect

CHAPTER V

INTERPRETATION OF FINDINGS AND FUTURE RESEARCH DIRECTIONS IN THE STUDY OF NEURAL TUBE DEFECTS

Despite a dramatic 25 to 30% decrease in birth prevalence over the past decade, neural tube defects (NTD)s remain the second most common birth defect in the United States (Detrait, George et al. 2005). Survival varies by NTD subtype; anencephaly is fatal in all cases, whereas infants with spina bifida often survive following extensive surgery. Few risk factors have been consistently associated with NTDs. Prior etiologic studies have not often ascertained sufficient sample sizes and may have been limited by response and ascertainment bias. In addition, exposure measurements used in prior studies have been subject to systematic misclassification and frequently lacked specificity.

Using data from the National Birth Defects Prevention Study (NBDPS), the associations between selected maternal exposures, periconceptional (1 month prior through 2 months postconception) maternal alcohol and occupational pesticide exposure, and NTDs were examined. Using data collected by the Iowa Registry for Congenital and Inherited Disorders (IRCID), differences in eligibility, participation, and selected maternal and infant characteristics by NTD pregnancy outcome (live births versus fetal deaths and elective terminations) were evaluated. Following is an interpretation of the findings in the context of potential biases and study strengths, and suggestions for future research directions in the etiologic study of NTDs.

Interpretation of Findings and Information Bias

The NBDPS, is a large, ongoing, population-based case-control study of birth defects, covering 10% of births nationally (Yoon, Rasmussen et al. 2001; Rasmussen, Moore et al. 2001; Rasmussen, Olney et al. 2003). Since 1997, the NBDPS has collected information on cases for over 30 defects, including NTDs, with case eligibility assigned by clinical geneticists. NBDPS controls are unaffected live births, delivered during the same time period and in the same geographic regions as case infants. Mothers of eligible cases and controls are asked to complete a telephone interview no earlier than 6 weeks and no later than 24 months following the estimated date of delivery of an infant. The NBDPS interview collects data on a number of maternal exposures including periconceptional alcohol exposure and maternal occupation(s). Using these latter data, maternal occupational exposure to three classes of pesticides (insecticides, herbicides, and fungicides) was assigned by industrial hygienists.

Maternal Periconceptional Alcohol Exposure

Similar to previous studies (McDonald, Armstrong et al. 1992; Mills & Graubard 1987; Shaw, Velie et al. 1996; Shaw, Nelson et al. 2002; Suarez, Cardarelli et al. 2003), current analyses showed that nearly all odds ratio estimates for the association between maternal periconceptional alcohol exposure and NTDs were near unity. Efforts to improve upon prior studies included use of a large, population-based sample and increased specificity of exposure through inclusion of maternal reports of types of alcohol consumed. Despite these improvements, the lack of a positive association may be explained in a number of ways. First, there may be in fact no positive association between maternal periconceptional alcohol exposure and NTDs, although this conclusion

is inconsistent with animal studies (Kotch & Sulik 1992; McMartin 1984; Muldoon & McMartin 1994), Notably, genes that regulate neurulation are expected to be highly conserved among species, and therefore risk factors for NTDs are expected to be similar among species (Rampersaud, Melvin et al. 2006). Although it is possible that alcohol does not uniformly impact neural tube development in humans and animals.

The study methodology used might also explain the differences found between animal and human studies. In particular, use of retrospective, self-reported alcohol exposure may have resulted in both non-differential misclassification due to underreporting and differential exposure misclassification due to recall bias. With regard to underreporting, as the time increases between exposure and reporting, maternal reports of exposures are expected to decrease similarly among cases and controls (Kelsey, Whittemore et al. 1996). To reduce misclassification bias, NBDPS interviews are completed within 24 months subsequent to the estimated date of delivery (EDD) of an infant. In the current study, the median times between the EDD and completed interview were similar between case mothers (9.5 months) and control mothers (8.9 months). Stratification by six-month intervals between EDD and completed interview showed, case mother reports of alcohol exposure were similar as time to interview increased. Likewise, control mother reports of alcohol exposure were similar as time to interview increased, although the frequency was somewhat lower for those with the longest length of time (18 to 24 months) between EDD and interview, 38% to 25%, respectively.

Underreporting of alcohol exposure may have also occurred due to the negative social stigma associated with alcohol consumption during pregnancy. This underreporting is particularly relevant in the current study, as data are collected after the

NTD has been diagnosed. Because non-differential exposure misclassification tends to bias estimates towards the null, such bias could explain, in part, the lack of association found between maternal periconceptional alcohol exposure and NTDs.

Recall bias may have also impacted the results found. It has been speculated that mothers of case infants are more likely to recall exposures when compared to control mothers (Kelsey, Whittemore et al. 1996), which would bias an estimate away from the null. Verkerk et al. (1994) compared absolute differences in the mean maternal alcohol exposure between prospective and retrospective reports of mothers of infants with and without birth defects. In addition, these researchers calculated risk estimates using retrospective and prospective reports for a number of outcomes (e.g., stillbirth, restricted growth, etc.). They found that variation between prospective and retrospective reports of case and control mothers was similar; thus, differential misclassification was minimal. These results provide some evidence that recall bias may not greatly influence risk estimates in studies using retrospective maternal reports of alcohol exposure. With estimates presented here near unity, recall bias did not appear to influence the results.

In addition to the potential for non-differential and differential misclassification of alcohol exposure, information bias, due to misclassification error, may have influenced the results. The NBDPS interview did not ask mothers about the volume of alcoholic drinks consumed, rather standard volumes (1 can of beer, 1 glass of wine, and 1 shot of liquor) were assumed, potentially resulting in misclassification of dose. As such, actual exposure could have been over- or underestimated due to the lack of precision in the measurement of alcohol drink volume. The effect on the odds ratio estimate, towards or away from the null, would be dependent on whether the alcohol quantity was over- or

underestimated and if the misclassification affected control mothers, case mothers, or both. Similarly, variation in true drink volume (relative to assumed standard drink volume), could have influenced odds ratios for alcohol type. It is well-established that the risk of NTDs increases as plasma folate levels decrease (Medical Research Council 1991; Czeizel & Dudas 1992). Also, in animal studies, alcohol exposure has been shown to decrease plasma folate through increased folate secretion, potentially indirectly resulting in NTDs (Yanaguita, Gutierrez et al. 2008). Beer contains folates and plasma folate levels have been shown to increase with beer exposure, potentially negating a decrease in plasma folate due to alcohol exposure (Stark, Pawlosky et al. 2005; Larroque, Kaminski et al. 1992). No prior studies were identified that stratified alcohol exposure by type of alcohol. Such analyses were expected to produce associations that differed by alcohol type, although odds ratios in the current study were near unity for each alcohol type. Use of standard rather than actual volumes may have attenuated he results. In our analyses, stratification by alcohol type produced estimates near unity.

Maternal Occupational Pesticide Exposure

Similar to relevant, prior studies (Blatter, Roeleveld et al. 1996; Blanco Munoz, Lacasana et al. 2005; Lacasana, Vazquez-Grameix et al. 2006) a small to moderate increased risk of NTD-affected pregnancies was identified for mothers occupationally exposed to insecticides + herbicides and also elevated risks were identified among those mother exposed to insecticides + herbicides + fungicides. Efforts to improve upon prior studies included use of a relatively large, population-based sample, industrial-hygienist assessment of pesticide exposure, and improved specificity of pesticide exposure

assessment, allowing some sub-analyses by pesticide class(es) and cumulative exposure (mg).

Unlike reports of maternal alcohol exposure, maternal reports of jobs worked may be less prone to bias as their occurrence would be less variable than alcohol exposure and less likely to be affected by social desirability bias. Previous work supports this assumption (Eskenazki & Pearson 1988). Compared to detailed clinical interview by occupational health professionals, mothers were found to be able to reliably provide retrospective reports of job title and typical number of days and hours worked during pregnancy. Given this, minimal non-differential and differential misclassification of maternal job reports was expected in the current study.

Exposure assessment by industrial hygienists has been suggested as the 'gold standard' for assigning pesticide exposure (Fritschi, Siemiatycki et al. 1996). Two prior studies pesticide exposure and NTDs used industrial hygienist reports, but each had limited sample size (Nurminen, Rantala et al. 1995; Shaw, Wasserman et al. 1999). Other prior studies assigned occupational pesticide exposure based on self-report or job-title (Blanco Munoz, Lacasana et al. 2005; Lacasana, Vazquez-Grameix et al. 2006). Self-report of occupational pesticide exposure is expected to result in non-differential exposure misclassification, with cases and controls equally likely to under-report exposure, biasing estimates towards the null (Fritschi, Siemiatycki et al. 1996); thus the true risk of NTDs may differ than those reported in studies using self-reported pesticide exposure. Occupational pesticide exposure assignment by industrial hygienist review, as used the current study in is expected to decrease the risk of exposure misclassification compared to self-report (Fritschi, Siemiatycki et al. 1996).

In an effort to overcome the limitations of self-report, some investigators have utilized job title to assign pesticide exposure. Three studies (Blatter, Roeleveld et al. 1996; Blanco Munoz, Lacasana et al. 2005; Lacasana, Vazquez-Grameix et al. 2006) were identified which used job title to assign occupational pesticide exposure; those in agricultural work were assigned as exposed, whereas others in non- agricultural occupations were assigned as not exposed. Each of these studies reported an increased risk of NTDs for any pesticide exposure and these risks, although imprecise, were considerably higher than those reported in the current study. Importantly, one might expect pesticide exposure in agricultural populations to be extreme and likely not reflective of that in the broader working population used in our study. If a dose-response relationship between pesticide exposure and NTDs exists, extreme exposure levels could explain why studies using agricultural populations noted an increased risk of NTDs for any pesticide exposure whereas the current study did not. Rather, significant, but relatively small elevations in risk were found for mothers with occupational exposure to insecticides + herbicides. For other pesticide combinations, the occupational pesticide exposure levels in NBDPS population may not have reached levels seen in agricultural populations previously studied. Even so, reports have suggested that use of job title to assign exposure for agricultural occupations can produce exposure misclassification, as exposure within these occupations varies widely, biasing estimates towards the null (MacFarlane, Glass et al. 2009; Harris, Sass-Kortsak et al. 2005). Again, use of industrial hygienist review was expected to reduce this misclassification bias and may, in part, account for the smaller effect seen in the current study relative to prior studies.

The potential for misclassification bias must also be considered in the approach used to calculate cumulative pesticide exposure. In estimating risk of cumulative exposure to insecticides + herbicides and insecticides + herbicides + fungicides, 1 mg of exposure to each pesticides class was assumed to carry an equivalent risk. Classification of a mother as having a cumulative exposure above or below the median was based on addition of cumulative exposure in each class. It is difficult to predict how this assumption would bias the odds ratios generated without knowledge of the true variation of risk between 1 mg of exposure in each pesticide classes; however cumulative exposure to different pesticide classes was frequently equivalent for each job reported.

With the limitations in exposure assessment described above, results taken at face value suggest that exposure to insecticides + herbicides results in an increased risk of NTDs, whereas exposure to insecticides only or to insecticides + herbicides + fungicides does not. If true, this would imply that the elevated association is due to herbicide exposure. Sample size precluded analyses of exposure to herbicides only, yet, numbers were sufficient to estimate odds of exposure to each pesticide class if exposure to the other remaining classes was irrelevant (e.g., exposure to herbicides regardless of exposure to insecticides and/or fungicides). In this analysis, odds ratios were near unity for mothers exposed to insecticides and mothers exposed to fungicides but elevated risk for mothers exposed to herbicides (aOR: 1.3; 95% CI: 1.0, 1.9). Also, it seems unlikely that addition of fungicides would have attenuated risks associated with insecticides + herbicides. Such variation could not be explained by higher rates of cumulative exposure in those exposed to insecticides + herbicides; for case mothers, both median and mean exposure to insecticides +herbicides (median: 8.0 mg; mean: 34.8 mg) was considerably

lower than that to insecticides + herbicides + fungicides (median: 326.9 mg; mean: 5628.3 mg).

Alternatively, mothers in our study exposed to insecticides + herbicides may have been exposed to different pesticides, perhaps those associated with a greater risk of NTD development, than mothers exposed jointly to insecticides + herbicides + fungicides, but data by pesticide type were not available. Also, although this study improved upon prior studies in terms of exposure specificity, due to small numbers in certain subgroups analyses could only be conducted for three pesticide class combinations (insecticides only, insecticides + herbicides, and insecticides + herbicides + fungicides

Interpretation of Study Findings and Bias

The large, population-based sample used in the current study, was expected to reduce selection bias; however, certain biases may have remained, particularly response and ascertainment bias. Understanding the impact of bias is important to interpretation of study findings. Response bias would occur if those who chose to participate in the NBDPS were different than those who chose not to participate. Comparison of selected maternal characteristics of NBDPS control mothers to all live births at each center has shown that NBDPS control mothers tend to be similar to all live births (Cogswell, Bitsko et al. 2009); however, the question remains if NBDPS case mothers who participated were similar to those who did not. In addition to response bias, ascertainment bias may have impacted results as only five of the ten centers included fetal deaths 20 weeks or greater gestational age and elective terminations.

To assess the potential for response and ascertainment bias in the studies of maternal periconceptional alcohol and occupational pesticide exposure, IRCID data were analyzed. Specifically, participation of Iowa case mothers with NTD-affected pregnancies identified for NBDPS and differences in selected maternal and infant characteristics by pregnancy outcome (live born versus fetal deaths and elective terminations). Overall, other pregnancy outcomes (fetal deaths and elective terminations) accounted for 40% of the NTD cases ascertained by the IRCID from January 1, 1998 through December 31, 2005. Although NBDPS eligibility varied by pregnancy outcome, NBDPS participation rates were similar between case mothers with live borns and those with other pregnancy outcomes. Overall, characteristics of case mothers were similar between participants and non-participants. Also, little difference in selected infant and maternal characteristics was found among NTD pregnancy outcomes. Additionaly, mothers of Iowa NTD cases were similar to those of Iowa control infants, although mothers of live born cases were more likely to be obese.

Because participation rates did not vary by NTD pregnancy outcome, this suggests that there was little if any response no bias based on pregnancy outcome for Iowa case mothers. In addition, results showed that for most infant and maternal characteristics, differences between pregnancy outcomes were non-significant. Still, analyses of the effects of periconceptional alcohol and occupational pesticide exposure on NTDs were likely prone to ascertainment bias. Due to ascertainment difficulties, no early fetal deaths (less than 20 weeks) were included in the NBDPS. Particularly relevant is that prior studies (Henriksen, Hjollund, et al. 2004; Sokol 1980) have found a positive association between maternal alcohol exposure at the time of conception and early fetal

death. This evidence, with that of animal studies showing a positive association between alcohol exposure and NTDs, leaves open the question of whether early alcohol exposure, particularly binge exposure, increases the risk of NTDs and leads to early fetal death. This question cannot be answered by the current analyses due to the inability to obtain this pregnancy outcome.

Future Directions

Study Design

Due to the relative rarity of NTDs, this population-based study used retrospective data collection, which introduced numerous sources of information biases as described above. In observational studies, the 'gold standard' is prospective data collection; however, improving assessment of periconceptional alcohol or pesticide exposure via prospective data collection would require extreme resources to ascertain a sufficient number of cases. For example, the Danish National Birth Cohort, which enrolled 100,000 pregnancies, would be expected to identify only about100 NTD cases (Olsen, Melbye et al. 2001).

Subject Selection

Feasibility of ascertaining fetal deaths of all gestational ages in retrospective study designs is limited. Although mothers can self-report early fetal deaths if recognized, such losses are rarely clinically examined to determine if the delivery was affected by an NTD. Improved ascertainment of fetal deaths will require improvements in surveillance methodologies. In the absence of feasible methodology for ascertaining

all fetal deaths, future studies can proceed by improving ascertainment of late fetal deaths and elective terminations to reduce the risk of ascertainment bias.

Data Collection

Beyond using prospective studies, future studies can aim to reduce information bias, namely exposure misclassification, in other ways. In the current study, improvements in exposure assessment were in part due to an increase in specificity (i.e., alcohol type and pesticide class). These should be replicated in future studies but can also be expanded upon. For example, future studies may implement improvements by not only increasing the number of subjects per class, but examining pesticides by specific types. To do so, researchers may consider utilizing populations with above average exposure levels, for example agricultural workers.

In addition to the above described methodological efforts to limit information bias, exposure measurement via biomarkers holds promise for improvement in exposure assessment in future studies. Biomarkers, cellular and molecular indicators of exposure, disease, or pre-disposition to disease, can measure exposure more reliably than self-report (reviewed by Littner & Bearer 2007). However, biomarkers of periconceptional exposure pose numerous challenges. To limit the need of a cohort study due to the above described limitations, a biomarker for exposures relevant to NTDs must be able to measure exposure months after actual exposure with high sensitivity and specificity. In addition, ideal biomarkers are minimally invasive and can be collected over a relatively long window of time. The biological samples collected to measure biomarkers might include maternal (e.g., urine hair, blood, etc.), fetal (e.g., amniotic fluid, blood, etc.), or newborn (e.g., cord blood, placenta, etc.) (reviewed by Littner & Bearer 2007).

Although numerous biomarkers for alcohol exposure have been identified; no single biomarker is yet conducive to measuring periconceptional alcohol exposure with high sensitively and specificity (reviewed by Littner & Bearer 2007). Many identified biomarkers of alcohol use are successful only in measuring recent alcohol exposure. One of the more promising protractedly measured biomarkers is fatty acid ethyl esters (FAEE)s, metabolites produced by the interaction between alcohol and fatty acids (reviewed by Littner & Bearer 2007). Studies have successfully measured one or more FAEEs in infant meconium, the first fecal matter produced by a newborn (sensitivity rates of 72 to 88%; specificity rates of 51 to 83%) (Bearer, Santiago et al. 2005; Bearer, Jacobson et al. 2003; Bearer, Lee et al. 1999). Yet, studies of NTDs, meconium, which begins to form during the thirteen week of gestation, represents only alcohol exposure occurring after the relevant gestation age of neural tube development. FAEEs have been detected in other biological samples, such as cord blood and hair, but studies of the sensitivity and specificity for these samples in measuring FAEEs to estimate prenatal alcohol exposure are limited. Additional studies are needed to identify appropriate biomarkers to measure periconceptional alcohol exposure.

A number of biomarkers of prenatal pesticide exposure have also been discovered and validated; however, such biomarkers are often specific to a particular pesticide and would not be feasible to incorporate into a study with heterogeneous pesticide exposures. Like alcohol, biomarkers of organophosphates (metabolites) have been validated in meconium (Wyatt & Barr 2001. Amniotic fluid has also been tested as a source for biomarkers of pesticides with promising results. One study was able to detect a number of pesticide metabolites in amniotic fluid (Bradman, Barr et al. 2003). Although amniotic

fluid may be one of the few methods ascertaining early exposure, obtaining amniotic fluid is highly invasive posing risk to the fetus and, thus, not an ideal biological sample. In addition, samples would need to be collected prospectively. Like biomarkers for alcohol, additional studies are necessary to identify biomarkers useful in estimating maternal pesticide exposure, retrospectively.

In addition to improvements in exposure specificity, data collection efforts in future studies should aim to include possible covariates such as those included in our study (e.g., diabetes, etc.) and others. Namely, exposure to pesticides outside of the workplace may be relevant.

In conclusion, although odds ratios for maternal alcohol exposure and NTDs were largely near unity the current risks study improved upon previous works by expanding exposure specificity to include alcohol type. Additionally, some elevated odds ratios were found for maternal occupational pesticide exposure and NTDs using improved specificity of exposure assignment. Finally, although preliminary, our study of NTDs by pregnancy outcome provides valuable insight into the variation and lack of variation in selected characteristics among pregnancy outcomes.

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