

TCE Meeting Presentation Supporting Material for J. DeSesso

Paul Dugard

to:

Marc Rigas

05/03/2010 10:04 PM

Please respond to Paul Dugard

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Dear Dr Rigas:

Please find attached supporting material for Dr DeSesso's presentation on May 10. This was originally submitted as part of the Aerospace Industries Alliance comments during the public review of the IRIS draft and has been abstracted for the convenience of the panel members.

Thank you.

Paul Dugard

**Comments on the Public Review Draft of EPA's IRIS
Toxicological Review for TCE: Developmental Effects**

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INTRODUCTION

EPA's assessment of TCE uses data on heart defects as a major endpoint for setting the RfD and RfC. The data selected to support this decision are from studies that are poorly designed and flawed. Furthermore, EPA neither incorporates nor accounts for more robust data from guideline- and GLP-compliant studies that show no increase in congenital heart defects.

- The human data are based on studies with inadequate exposure information, making it impossible to determine whether or not exposure occurred and, if it did, to what levels of TCE.
 - There are also deficiencies in the human data in terms of the background rates of cardiac malformations (Bove et al., 1995), and differences in the outcome of different studies (Goldberg et al., 1990, versus the Baltimore Washington Infant Study – Wilson et al.; 1998).
- The animal data reporting a link between TCE and heart defects all come from the same laboratory and were an accumulation of data over ten years (Johnson et al. 2003, Dawson et al. 1993).
 - In the Johnson and Dawson studies, there were a number of deficiencies in study design and reporting of data that make the interpretation of data tentative at best.
 - The major effect reported in the Johnson and Dawson studies was an increase in the incidence of atrial septal defects (or the foramen ovale, which closes around the time of birth) which may be related to the procedure for examining fetuses or the timing of the dissection relative to the development of the fetus, rather than actual heart defects.
- Two additional GLP- and guideline-compliant studies showing no effect on heart development were conducted by Fisher et al. (2001) and Carney et al. (2006).
- Thus, EPA uses weak human data; incomplete and flawed animal data; and in vitro/in ovo data (which are of questionable relevance to environmental exposures) to make a mechanistic argument that TCE causes heart defects. Although EPA notes some of the database deficiencies, EPA uses a “strength of evidence” approach, rather than a “weight of evidence” analysis, by basing the RfD only on the studies reporting a positive effect and ignoring the data from subsequent well-conducted GLP studies that show no increase in heart defects associated with TCE (Fisher et al., 2001; Carney et al., 2006).

EPA Evaluation of Animal Data on Heart Defects and Comments

The EPA review of TCE (US EPA, 2009) uses the Johnson et al. (2003) and Dawson et al. (1993) data to establish reference levels for exposure - an RfC of 0.001 ppm and an RfD of 0.0004 mg/kg/day. The fetal heart malformation data reported in Johnson et al. (2003) are used to support both of these values (US EPA, 2009; see Tables 5.1.23 and 5.1.24 and the associated text). There are several limitations with this approach:

- The Johnson et al. (2003) publication includes the Dawson et al. (1993) data and appears to be an accumulation of data over an approximate 10-year period.
 - This was not made clear in the Johnson paper, and it required a letter to the editor (Hardin et al., 2004) for the authors to respond and explain this situation (Johnson et al., 2004). There is no indication in the paper reporting the combined data (Johnson et al., 2003) about which data came from Dawson et al. (1993) and which data came from subsequent studies. Over the course of a decade, there could have changes in the lot of TCE used in the studies, differences in the animal supplier or animal health, changes in

the experience of investigators and technicians, and changes in the procedure used for heart examination. All of these could affect the results.

- Dawson et al. (1993) do not mention the number of pregnant dams that were assigned to each treatment group and Dawson et al. (1993) used the fetus as the unit for statistical analysis. In developmental toxicity studies, the unit for statistical analysis is based on the dam or litter. This method helps to account for the litter effect (based on the concept that offspring of a given female tend to react more similarly to challenges than offspring from different females) and prevents inappropriate inflation of statistical significance.
 - These mistakes give the appearance that the authors were unaware of how to design studies, or how to analyze and present developmental toxicity data.
- **For the purposes of risk assessment and setting of regulatory standards, studies like Johnson et al. (2003) and Dawson et al. (1993), with deficiencies such as those mentioned above, should only be used in a support role when a database of other, more well-designed studies is available. Johnson et al. (2003) should not be used as the critical study for establishing regulatory exposure levels.**
 - The Johnson et al. (2003) and Dawson et al. (1993) studies have significant limitations regarding the reporting of standard maternal and fetal parameters.
 - Johnson et al. (2003) do not provide data on maternal and fetal parameters other than cardiac malformations, only mentioning that “maternal and fetal variables, including noncardiac congenital abnormalities, showed no significant differences between treated and control groups.”
 - Dawson et al. (1993) did not provide any control data for maternal and fetal parameters, other than cardiac abnormalities. Consequently, there is no way to assess the impact of exposure on any parameter other than cardiac abnormalities, including such parameters as maternal body weight and body weight gain, fetal weight, and fetal viability.
 - Johnson et al. (2004) note that “Control values were consistent throughout our studies.” However, there is no way for the reader to determine this.
 - Without evaluating all of the maternal and fetal parameters, it is not possible to get a clear idea of how the animals are responding to treatment and whether the endpoint values (e.g. cardiac defects) are within historical ranges.
 - **Studies where major components of the results are not reported or the missing data have not been evaluated by the risk assessors may be useful in supporting other, more complete, data sets, but are of questionable value as primary studies in establishing an exposure standard.**
 - Johnson et al. (2003) indicate that their goal was to determine whether there was a threshold level of TCE in drinking water above which the incidence of congenital cardiac defects in the rodent increased significantly. The doses reported were 0, 2.5, 250, 1,500, and 1,100,000 ppb. Does their study design and statistical analysis permit the testing of a hypothesis derived from this goal?
 - Their study pools discrete data from at least two separate studies and an accumulation of data over several years and is an unbalanced design (55 dams in the control vs. 9-13 in the treatment groups).
 - They report that their data could indicate that a threshold effect exists at a level between 1.5 and 1,100 ppm.

- **It would be prudent to have a qualified statistician look at this database and the statistical evaluations used to determine if the analysis was appropriate. The reported “threshold effect” has a range of three orders of magnitude. This is not very useful in establishing reference levels.**
- In discussing the dose-response pattern in Johnson et al. (2003), the authors specifically mention the response observed at the highest exposure level (1,100,000 ppb) relative to control. With regard to the results seen in the other three dose levels, they only mention that “Intermediate exposure levels produced intermediate response rates.” While the latter statement may be true, the intermediate levels did not produce a clear dose-response relationship.
 - The incidence of heart defects in fetuses was 2.1, 0, 4.5, 5.0 and 10.5% in controls, 2.5, 250, 1500 and 1,100,000 ppb exposure groups, respectively. The extreme range of exposure levels (440,000-fold difference between low and high exposure levels, and >700-fold between the 1500 and 1,100,000 ppb exposure levels) is not mirrored by a remarkable difference in the incidence of heart defects (2.1% in controls and only 10.5% incidence at the highest exposure level).
- To make the analysis more difficult to interpret independently, **the fetus and not the dam (litter) was used as the experimental unit.** EPA has noted that Johnson “has provided individual litter incidence data to the USEPA for independent statistical analysis (P. Johnson, personal communication, 2008) (see Section 6, dose-response)” (US EPA, 2009, p 857). It is unclear why EPA refers to “Section 6, dose-response” regarding this additional data, since it does not appear that anything in this section/sub-section details these data or how they were used. It is unclear if EPA has examined these data. At a minimum, EPA should make the data available and explain how it has been incorporated into EPA’s risk assessment.
- **The dose-response pattern is another area where the input of a qualified statistician/modeler would be prudent.**
- Johnson et al. (2003) comment that TCE exposure using an in vitro chick model has been shown to have effects on several elements of epithelial–mesenchymal cell transformation in endocardial cushions (tissue that becomes part of the atrioventricular valves and septum) at concentration ranges that correlate with their findings.
 - They note a concentration range of 50-250 ppm (although it isn’t clear if this is the only concentration range used in the referenced studies), which is bounded by the Johnson et al. (2003) concentration range, but then, almost any range would be, given the extreme range that Johnson et al. used.
 - More importantly, an application of X ppm in an in vitro chick embryo study is in no way comparable to an application of X ppm in drinking water in an in vivo rat study.
- **Use of in vitro/in ovo data with questionable relevance to environmental exposures as mechanistic support for heart defects reported in poorly conducted whole animal studies and weak human studies does not build a strong case for using heart defects as the basis for risk assessment, and compounds the problem of overstating the importance of the data.**

- Generally, the draft assessment focuses too much on one set of studies that show a putative positive response to low-exposure levels of TCE, instead of considering the overall data base and the limitations of the focus studies.
 - The draft assessment is not a “weight of evidence” evaluation but a “strength of evidence” evaluation (NRC, 1994). All the focus is on those studies that found a compound-related effect and no attention was given to the strengths and weaknesses of those studies that found no compound-related effects. Data from GLP-compliant animal studies that were carefully designed to probe the existence of potential links between TCE or its metabolites and heart or eye defects have shown no associations at exposure levels that are several orders of magnitude higher than those expected in environmental or occupational settings.
 - Fisher et al. (2001) specifically investigated the cardiac teratogenic potential of TCE, TCA, and DCA in groups of 19 – 20 pregnant Sprague-Dawley rats. The rats received oral bolus doses of TCE (500 mg/kg/day, in soybean oil), TCA (300 mg/kg/day, in water) or DCA (300 mg/kg/day, in water) on gestational days 6 – 15. On gestational day 21, fetuses were removed by laparohysterectomy and hearts were examined and microdissected under a stereomicroscope by an investigator experienced in the procedure (Dr. Paula Johnson, author of Johnson et al. (2003)). The rates of cardiac malformations among treated animals did not differ from control rates. Also, TCE caused no change in the weight of fetuses and did not inhibit maternal weight gain at the high dose level¹ used in this study.
 - An inhalation study of TCE in pregnant Charles River CD IGS rats (Carney et al., 2001; 2006) exposed groups of 27 animals to filtered air or to atmospheric concentrations of TCE up to and including the limit dose (600 ppm) for 6 hours/day on each of gestational days 6 – 20. Although maternal toxicity (decreased body weight gain) was elicited at the highest dose, TCE exposure caused no increase in gross, skeletal, or visceral (including heart and eye) malformations at any of the concentrations tested.
 - Some early studies of TCA and DCA in pregnant Long-Evans rats (Smith et al., 1989, 1992) reported ocular malformations. In a follow-up to the Fisher et al. (2001) study, Warren et al. (2006) reported that examination of the heads showed that none of the chemicals used in the Fisher et al. (2001) study elicited gross ocular malformations. Morphometric analysis of the lens area, globe area and interocular distances revealed reductions of these parameters only in the TCA- and DCA-treated fetuses, but the overall smaller sizes of the fetuses in those groups were sufficient to explain the reductions.
 - **Weight of evidence clearly must consider all of the data, both positive and no effect data. When the majority of the positive data are derived from clearly flawed studies using methods that give results that are not replicable in other laboratories, it is difficult to understand how the Agency can justify using only these data as the basis for a regulatory assessment.**
- While there were similar methods used for examining hearts in fetuses in the Dawson and Johnson laboratories and Dr. Johnson collaborated on the Fisher et al. (2001) study, there were

¹ For purposes of estimating the comparability of the dosages in the Fisher and Johnson studies, the following rough estimates can be made. In the Johnson drinking water study, the high dose was 1100 ppm TCE in the water. If the rats drank 20 mL/day, they received ~22 mg TCE/day. In the Fisher gavage study, the rats were administered 500 mg/kg/day. If the rats weighed 350 g, they received ~175 mg TCE/day.

several differences among the 3 studies as noted in the EPA review, as well as possibly significant differences in heart preparation not noted by EPA (see Table 1 below).

Table 1. Comparison of Methods Used in the Dawson et al. (1993), Johnson et al. (2003), and Fisher et al. (2001)

Study	Stock of animals	Source of animals	Route of exposure	Dose	Vehicle	Treatment days GD	Day of sperm GD	Day of sacrifice GD	Heart preparation
Dawson et al. 1993	Sprague Dawley	Harian, Indianapolis?	Drinking water	1.5 and 1100 ppm	Tap water	1-22	1?	22?	flushed with 2% glutaraldehyde after heart removal, fixed for 24 hrs in the same solution, transferred to 0.1 mol/L phosphate buffer
Johnson et al. 2003	Sprague Dawley	Harian?	Drinking water	2.5 & 250 ppb, 1.5 & 1100 ppm	Distilled water	1-22	1?	22?	flushed with 10% formalin, transferred to 10% formalin
Fisher et al. 2001	Sprague Dawley	Charles River, Raleigh	Gavage	500 mg/kg	Soybean oil (TCE & RA); IERO* water (TCA, DCA)	6-15	0	21	flushed in situ via the left ventricle with staining solution for better visualization (1:3 hematoxylin-saline solution), then removed and immersion fixed in 10% buffered formalin

* IERO = ion exchange/reverse osmosis

- Table 1 details differences in preparation of the heart for dissection. Dawson et al. (1993) and Johnson et al. (2003) both removed the heart first, then flushed with a fixative. Fisher et al. (2001) flushed the heart in situ via the left ventricle with a staining solution for better visualization (1:3 hematoxylin-saline solution), perhaps a more physiologically normal situation, then removed the heart and immersion fixed it in 10% buffered formalin.
- One major difference in the data from the Dawson/Johnson laboratory versus the Fisher laboratory appears to be the incidence of atrial septal defects (Table 2). The types of atrial septal defects reported by Dawson/Johnson et al. are not detailed in any of the papers except for the statement that they are “secundum in type” (Dawson et al., 1993).
 - Since the septum primum and septum secundum both grow rapidly around the time of birth to close the foramen ovale (Momma et al., 1992), this may represent normal in developmental timing such as occurs with other structures that are maturing around the time of birth in the rat, (e.g., skeletal ossification of sternbrae, vertebral centra, etc., or development of the renal papilla).
 - Whether the different methods of flushing the hearts may have disturbed the position of the septum which would not be closed on the day of sacrifice is unclear.
 - Even more troubling, however, is that neither Dawson et al. (1993) nor Johnson et al. (2003) provide maternal or fetal weight data, so it is impossible to know whether there were differences in fetal weight that would suggest a delay in development. Also, data on other aspects of fetal development (e.g., skeletal ossification) were not presented to give any clues about developmental stage.
 - Fisher et al. (2001) report no significant difference from water-treated control animals in maternal weight, uterine weight, number of implantations or fetal weight for TCE at 500 mg/kg. In that study, the percent of fetuses with atrial septal defects was approximately

the same in the two groups. Thus, there are a lot of questions about the incompleteness of the data presented in the Dawson et al. (1993) and Johnson et al. (2003) papers, in addition to the obvious design flaws and protracted length of time over which the studies were conducted. Without concurrent control data, it is very difficult to evaluate small changes in heart development that may or may not be related to TCE exposure.

Table 2. Comparison of Atrial Septal Defects in the Three Papers*

Study/Data	Treatment Groups						
	Control Tap water	TCE – Prepreg only 1.5 ppm	TCE – Prepreg only 1100 ppm	TCE – Preg only 1.5 ppm	TCE – Preg only 1100 ppm	TCE – Prepreg & Preg 1.5 ppm	TCE – Prepreg & Preg 1100 ppm
Dawson et al. 1993							
No. of atrial septal defects/no hearts examined (%)	1/232 (0.4)	3/130 (2.3)	7/147 (4.8)	4/181 (2.2)	7/105 (6.7)	5/256 (2.0)	19/435 (4.4)
Johnson et al. 2003	Control Distilled water	TCE – 2.5 ppb	TCE – 250 ppb	TCE – 1.5 ppm	TCE – 1100 ppm		
No. of atrial septal defects/no hearts examined (%)	7/606 (1.2)	0/144 (0)	1/110 (1.0)	4/181 (2.2)	7/105 (6.7)		
Fisher et al. 2001	Control IERO** Water	TCA 300 mg/kg in IERO water	DCA 300 mg/kg in IERO water	Control Soybean oil	TCE 500 mg/kg in soybean oil	Retinoic acid – 15 mg/kg in soybean oil	
No. of atrial septal defects/no hearts examined (%)	2/273 (1.0)	2/269 (1.0)	3/298 (1.0)	6/367 (1.6)	4/290 (1.4)	3/155 (1.9)	

*Data in the shaded boxes were reported in both the Dawson et al. 1993 and the Johnson et al. 2003 papers.

**IERO = ion exchange/reverse osmosis

- Another difference is in the incidence of ventricular septal defects (VSDs).
 - Johnson et al. (2003) reported membranous VSD occurrences as 0.33% in controls; 1.7% at 1.5 ppm; and 2.9% at 1,100 ppm. For muscular VSDs, they reported 0.33% in controls; 0.55% at 1.5 ppm; and 0.95% at 1,100 ppm.
- In the Fisher et al. (2003) study, there are no cases of VSD in TCE-treated fetuses, even though there were 2 cases of membranous VSD and one case of muscular VSD in soybean-treated controls (incidence of 0.54% and 0.27%, respectively).
- There are significant questions about examination of the hearts in the Dawson/Johnson studies, as well as questions about whether effects on the atrial septum (the primary defect reported) are actually a reflection of developmental delays, because the atrial septum is developing around the time of birth. In addition, there was no increase in VSDs in a carefully-controlled study (Fisher et al. 2001), while Johnson et al. (2003) reported a low increase in incidence with TCE exposure. Unfortunately, data on maternal and fetal body weight or other indicators of development (e.g., skeletal ossification) are missing from the reports by Dawson/Johnson. Consequently, it is not possible to assess the developmental importance of their findings.
- The NRC (2006) report states that ventricular septal defects (VSDs) were the most commonly observed cardiac problems in both animal studies and the epidemiological studies. This observation is provided as support to the idea that TCE can induce heart defects. However, as indicated earlier, the Johnson et al. (2003) study reported a much higher incidence of atrial septal defects than VSDs.

- There are serious questions about whether or not atrial septal defects are actual defects or simply due to delays in development (an adaptive response that is usually reversible). In addition, VSDs are the most common heart defect in the human population, making up anywhere from ~14-25% of CHD cases (American Heart Association, 2005b; Hoffman and Kaplan, 2002), regardless of whether or not TCE exposure is involved.
- TCE reportedly alters endocardial cushion proliferation at low doses when administered *in ovo*, but whether or not this in turn increases the incidence of CHD is unclear. An increase in cellular proliferation in the cardiac cushion and outflow tract has been noted in the *in ovo* study by Drake et al. (2006a). In this study, 0.2, 4, and 200 nm/egg concentrations of TCE were injected into the yolks of eggs during cardiac cushion formation at Hamburger Hamilton (HH) stages 13, 15, 17, and 20. At the 4 nm/egg concentration and higher, an increase in cardiac cushion proliferation was observed in parallel with alterations in cardiac blood flow patterns. However, the same authors also noted in a later paper that this same increase in cellular proliferation was observed when TCE was administered at HH 18, 21, and 23, but this latter experiment the increased proliferation was not linked to any kind of functional cardiac alterations, illustrating that the two are not necessarily linked (Drake et al., 2006b).
- **Thus, it is unclear whether the effects on cellular proliferation of endocardial cushions seen in chick studies are related to septal defects, and it is unlikely that the changes reported from direct egg injection studies with high levels of TCE are relevant to whole animal or human exposure levels.**

EPA Evaluation of Human Data on Heart Defects and Comments

The existing human data are deficient for risk assessment, but even so they do not support an association between TCE exposure and cardiac defects in human infants.

- A shortcoming that is common to all of the epidemiology studies is the lack of accurate exposure information and poor control of confounding factors. In the instance of the Arizona aquifer, the authors were clear to point out that their data showed “a significant association but not a cause and effect relation between parental exposure to the contaminated water area” and cardiac defects. By this, they meant that the parents of affected children were present in the land area overlying the aquifer during early gestation – but not that they had necessarily drunk or used contaminated water. Thus, it is not clear whether exposure occurred or to how much. With respect to the Baltimore-Washington Infant Study, interviews with parents identified activities and occupations that were likely to have involved organic solvents and degreasing substances. TCE is among the substances that could have been used, but it was not singled out as a causative agent and there is no information on levels of exposure. These data sets fail to clearly identify a specific causative agent and do not quantify exposure levels, making these data sets insufficient for an assessment of risk for a particular chemical (i.e., TCE).
- NRC (2006) cited the findings in Bove et al. (2002), a study that re-analyzed the data presented in the widely disputed Goldberg et al. (1990) study. Goldberg et al. (1990) reported an increased incidence of congenital heart defects (CHD) in Tucson, AZ, but this report was criticized for its data analysis and sampling techniques. Bove et al. (2002) reported that 10-11% of households in Tucson had at least one member that had worked or resided in the TCE contaminated area. In contrast, it was stated that 39.2% of babies born with CHD had at least one parent who had resided or worked in a contaminated area. This was based on interviews of 143 of the 365 CHD

cases. Bove et al. (2002) claimed that if it was assumed that the remaining 172 cases had a similar proportion of exposed parents, then the prevalence of CHD in the exposed areas during the first trimester of pregnancy would be about 2.3 times that in the uncontaminated areas. No confidence interval for this was provided. One major problem with this evaluation is that whether the mother and/or father was exposed to the TCE was not considered, and the pathway by which paternal exposure would contribute to an increase in CHD is unclear. Additionally, because socioeconomic status and demographics were not integrated with the geographical distribution of the population, it is possible that a higher proportion of births occurred in the part of town with TCE-contaminated water. In many parts of the county, certain areas of a region are more heavily populated with households with children. The control group here is for the overall Tucson population and not childbearing families. The absence of an appropriate control group is a potential confounding factor that was not considered. Another issue is that the control incidence of CHDs was stated to be 2.6/1,000 births, which is well below the expected U.S. background CHD rate of 8/1,000 births as reported by the American Heart Association (2005a). Therefore, it appears that the Bove et al. (2002) study suffers from many of the same problems as the original Goldberg et al. (1990) study.

- The NRC (2009) report updated the conclusions of the IOM (2003) report and concluded that “there continues to be inadequate/insufficient evidence” for a link between TCE and congenital malformations in humans.
- **As discussed above, the human data cited by the assessment are inadequate for risk assessment and do not support a link between TCE and heart defects.**

CONCLUSIONS

- EPA used a strength of evidence rather than a weight of evidence in their assessment of the data on cardiac defects. That is, only the positive data showing effects were considered in selecting data as the basis for the RfD and RfC rather than considering the whole body of data. EPA’s guidelines clearly indicate the importance of using a weight of evidence approach.
- All of the data showing cardiac defects in whole animal studies come from a single lab and have significant study design flaws and inadequate data reporting.
- More carefully controlled GLP-studies did not show an increase in cardiac defects, including the study by Fisher et al. (2001) in which Dr. Johnson (of Johnson et al. 2003) participated.
- The human data used by EPA as support for a link between TCE and heart defects are inadequate for risk assessment.

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