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Cardiac Teratogenicity of Trichloroethylene Metabolites

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Objectives. **The hypothesis of this study was that metabolites of trichloroethylene (TCE), dichloroethylene (DCE) and related compounds were responsible for fetal cardiac teratogenic effects seen when TCE or DCE is consumed by pregnant rats during organogenesis. Identification of teratogenic metabolites would allow more accurate assessment of environmental contaminants and public health risks from contaminated water or possibly municipal water supplies which, when chlorinated, may produce these potentially dangerous chemicals.**

Background. **Human epidemiologic studies and previous teratogenic studies using chick embryos and fetal rats have shown an increased incidence of congenital cardiac lesions in animals exposed to TCE and DCE.**

Methods. **Metabolites and compounds studied in drinking water exposure included: trichloroacetic acid (TCAA), monochloroacetic acid (MCAA), trichloroethanol (TCEth), carboxy methylcystine (CMC), trichloroacetaldehyde (TCAld), dichloroacetaldehyde (DCAld), and dichlorovinyl cystine (DCVC). Compounds were administered to pregnant rats during fetal heart development.**

Results. **Fetuses of rats receiving 2,730 ppm TCAA in drinking water were the only group that demonstrated a significant increase in cardiac defects (10.53%) compared with controls (2.15%) on a per fetus basis ($p = 0.0001$, Fischer's exact test), and a per litter basis ($p = 0.0004$, Wilcoxon and $p = 0.0015$, exact permutation tests). Trichloroacetic acid also demonstrated an increased number of implantation and resorption sites ($p < 0.05$) over controls. Other maternal and fetal variables showed no statistically significant differences between treated and untreated groups.**

Conclusions. **Of the metabolites tested, only TCAA appeared to be a specific cardiac teratogen in the fetus when imbibed by the maternal rat.**

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Halogenated hydrocarbons, such as trichloroethylene (TCE), and its metabolic products, are among the most common water supply contaminants in the United States and around the world (1). Also, in the presence of natural organic material, chlorination of municipal water to eliminate coliform bacteria has been shown to produce a number of quantitatively important chlorinated organic compounds, including the TCE metabolites chloral hydrate (TCAld), trichloroacetic acid (TCAA) and dichloroacetic acid (DCAA) (2–4). The United States population consumes chlorinated drinking water, which is a major source of ingestion of these chlorinated organic compounds (5). Thus, both contaminated water and municipal water are both potential sources of exposure to TCE and its metabolites.

An epidemiologic study in the Southwestern United States found an association between the presence of TCE in contaminated water and an increased incidence of major cardiac malformations in children born to mothers residing in contaminated areas (6). Although this case-controlled study demonstrated an association between the halogenated hydrocarbon contaminants and congenital heart defects, it was not designed to establish a cause and effect relation. As a result, in vivo studies were undertaken to determine the possible teratogenicity of TCE and dichloroethylene (DCE) in both avian and rat models.

Avian studies (7,8) demonstrated that TCE and DCE had both general and cardiac teratogenic effects on the developing chick. Subsequent controlled studies in the pregnant rat model (9,10) determined an increased frequency of cardiac teratogenicity in fetuses after direct intrauterine exposure to TCE and DCE. Compounds were then administered to the dam in drinking water during the critical days of fetal organogenesis to more closely simulate human exposure conditions. Similar increases in cardiac teratogenesis were observed. Other researchers demonstrated an increased frequency of specific congenital cardiac malformations following treatment with chlorinated hydrocarbons in other rodent models (11–19). A recent study of whole embryo exposure to TCE and other related compounds (including metabolites), demonstrated embryotoxicity and various developmental abnormalities (20).

Our purpose was to determine whether the parent compounds (TCE or DCE), or one or more of their metabolites was linked with the presence of cardiac defects. Compounds tested in this study included TCAA, monochloroacetic acid (MCAA), trichloroethanol (TCEth), carboxy methylcystine

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Abbreviations and Acronyms

CMC = carboxy methylcystine
DCAA = dichloroacetic acid
DCAld = dichloroacetaldehyde
DCE = dichloroethylene
DCVC = dichlorovinyl cystine
MCAA = monochloroacetic acid
TCAA = trichloroacetic acid
TCAld = trichloroacetaldehyde
TCE = trichloroethylene
TCEth = trichloroethanol

(CMC), TCAld, dichloroacetaldehyde (DCAld) and dichlorovinyl cystine (DCVC). Based on work by others, the most suspect metabolites were weak acids such as TCAA or MCAA or an alcohol such as TCEth (4,11–20). A nonchlorinated hydrocarbon was included to determine if the chlorine atom played a role in teratogenicity.

Methods

This study was conducted in Association for Assessment and Accreditation of Laboratory Animal Care accredited and Institutional Animal Care and Use Committee governed facilities at the University of Arizona Animal Care Center. Animals were quarantined for 7 days before study. Study groups consisted of virus free, young, sexually mature Hsd:Sprague Dawley SD rats (Harlan Sprague Dawley, Inc., Indianapolis, Indiana). Females, 225 ± 30 g, were housed in pens of four, and the males, 300 ± 50 g, were housed individually. All rats had access to water and Teklad 4% Mouse Rat diet (Teklad, Madison, Wisconsin) ad libitum. Each animal was identified by an ear notch code. The number of animals in each group was determined by a power calculation to detect a threefold increase in the malformations over controls.

Daily vaginal smears or impedance measurements (Estrous Impedance Monitor, Fine Scientific Instruments, Inc., Phoenix, Arizona) were obtained from all females to determine stages of the estrous cycle. When in proestrus, female rats were placed in a cage with one male overnight. The presence of a vaginal plug and/or spermatozoa in the vaginal smear the following morning was considered indicative of day 1 of pregnancy. Each rat was carefully observed throughout pregnancy and weight gain was monitored and recorded daily.

Administration of agents. Control animals for this study received distilled water throughout pregnancy. On day 1 of pregnancy, and continuing throughout pregnancy, their regular drinking water was replaced with treated water. Compounds tested included TCAA, MCAA, TCEth, CMC, TCAld, DCAld and DCVC. Solutions of the various compounds were prepared by dilution with distilled water and, if necessary, titrated with NaOH to a pH of approximately 7.0, a pH similar to that of the control water. Each water bottle was placed in a specially made metal casing to reduce light exposure and subsequent chemical breakdown. The amount of water consumed by each pen of animals (4 maximum) was monitored and recorded every 24 h. Bottles were cleaned and fresh solutions prepared daily.

Levels of metabolites were based on the dosage equivalent to that expected if all of the high dose of TCE (1,100 ppm, the limit of solubility, and the maximal TCE dose tested) was to breakdown completely into that given metabolite. The level of TCAA was similar to the dose tested by other investigators for comparison purposes (11). To achieve uniformity between the tri- and monochloroacetic acids, MCAA was also given at an equivalent dose. Dichloroacetic acid was not tested because this had already been performed by other investigators (12).

Examination of fetuses. For all groups, on day 22 of gestation, approximately 1 day before parturition, each pregnant rat was weighed before euthanasia in a carbon dioxide chamber. An examination was then conducted for any abnormalities (external and internal) and the gravid uterus and ovaries removed. The uterus was opened, exposing all fetuses, implantation sites (sites where the embryo implanted in the uterus, but did not mature beyond implantation, leaving only a metrial gland) and resorption sites (sites where fetal development began, but stopped at some point during gestation with only decaying fetal tissue remaining). The position of each site was recorded. Fetuses and placentas were examined in situ, then removed and individually examined externally for any morphologic abnormalities. All fetal placements, weights, placental weights, crown rump (C/R) lengths and any gross fetal abnormalities were evaluated by an experienced veterinarian. Using an Optivisor (Donegan Optical Co., Inc., St. Lenexa, Kansas) for magnification, the thoracic and abdominal cavities were opened. All abdominal organs were inspected for any congenital abnormalities. Exposing the thoracic cavity allowed observation of the great arterial and venous connections to the heart in situ. Pulmonary and vena caval attachments were then incised, as distal to the heart as possible, and the heart removed. A 27-gauge needle was placed apically in the left ventricle and the heart gently flushed with 2% gluteraldehyde solution. Each heart was then placed in an individual vial that was labeled with a seven digit code (for future "blind" assessment) and placed in the same solution for 24-h fixation. The heart was then transferred to a 0.1 mol/L phosphate buffer solution for storage.

Examination of hearts. Individual hearts were dissected and evaluated using a Nikon SMZ-2T light microscope with an attached TV camera and monitor (Nikon, Chandler, Arizona). This allowed excellent visualization and manipulation. Initially, the heart was examined for any gross morphologic abnormalities from both dorsal and ventral aspects. The heart was then examined in a step by step protocol which is detailed by Dawson et al. (10). This method allows visualization of the atrial septum, aortic and pulmonary vessels, semi-lunar and atrioventricular valves and the ventricular septum. All confirmed abnormalities were agreed upon by the three investigators: a veterinarian, a pathologist and a pediatric cardiologist. All abnormal specimens were then photographed using a

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Table 1. Average Amount of Drinking Water Consumed per Maternal Rat

Water Treatment	Normal	TCAA	MCAA	TCEth	TCAld	DCAld	CMC	DCVC
Concentration in ppm	Water	2,730	1,570	1,249	1,232	174	473	50
Equivalent dosage to ppm (mg/ml)	N/A	2.73	1.57	1.249	1.232	0.174	0.473	0.05
Dosage (mg/kg/d)	N/A	291	193	153	151	21	58	6
Average amount of "drinking water" consumed on a daily basis per maternal rat (ml)	46	38	21	53	42	55	55	40
Number of days consuming "drinking water"	21	20*	20*	21	21	21	21	21
Average dose per rat per day (mg/rat/d)	N/A	103	33	67	51	10	26	2
Average total consumed during pregnancy by the rat (mg/rat)	N/A	2,092	612	1,396	1,070	200	546	41

*Exposure was consistent, but due to occasional mechanical failure, days were missed in some rats, thus bringing the average days of consuming treated water in some groups to <21 days.

Nikon N2020 camera mounted on the light microscope. Decoding of the hearts, with respect to treatment, occurred only after final examination of all hearts and fetuses.

Statistical analysis. For an effect size of a threefold increase over background heart defects, a power analysis of 90%, with an alpha error of 0.05 and a beta error of 0.1, determined that a sample size of 100 was needed for statistical significance. Statistics for the individual fetal data were analyzed by using Fisher's exact test. The Wilcoxon and exact permutation tests were used to determine the significance of the litter outcome (21).

Results

Data compiled for treatment groups of all compounds included: 1) maternal parameters; 2) in situ uterine abnormalities observed at necropsy on day 22; 3) fetal parameters; 4) types of cardiac anomalies, and 5) percent congenital fetal cardiac anomalies calculated both on a per fetus basis and a per litter basis. Table 1 contains concentration and dosing information.

Maternal observations. All maternal rats (138 total) were healthy throughout the study and without evidence of toxicity. Steady weight gain occurred throughout pregnancy in all groups. Ovaries had normal morphologic features. All 138 pregnancies except for two, in which total resorption occurred, progressed uneventfully. Observations, shown in Table 2, included weight gain, pregnancy complications and uterine examination. Weight gain during pregnancy was not significantly different for any group of dams. There were no pregnancy complications, and all uterine morphologic examinations were normal.

Implantation sites, resorption sites and fetuses. The uterus was examined for implantation sites, resorption sites, and live and dead fetuses. External inspection of live fetuses (n = 932) and dead fetuses (n = 9), excluding one fetus that was too autolyzed to examine, demonstrated no gross morphologic congenital abnormalities in any group. No differences were found between treated groups and controls for the mean number of implantation sites and resorption sites using Fisher's exact analysis, except for the TCAA group in which a significant increase occurred (1.1 average implantation sites per litter [p = 0.0006] for TCAA exposure compared with 0.20 average implantation sites per litter for Controls, and 2.7 average resorption sites, per litter [p = 0.0001] TCAA compared with 0.70 average resorption sites, per litter for controls, [Table 2]).

Fetuses were analyzed by determining: 1) numbers of live or dead fetuses (Table 2); 2) fetal weight; 3) placental weight; 4) C/R length, and 5) external morphology. No significant difference was found when comparing treated and control fetal groups for these observations. There were no gross external or noncardiac internal congenital abnormalities found in any treated or control groups.

Cardiac anomalies. Several different cardiac malformations were found and no one lesion or grouping predominated. Variations of normal morphology similar to those found in humans were not classified as defects (for example, tricuspid valve leaflet contribution to complete coverage of a membranous ventricular defect). General types of cardiac defects were grouped in categories and listed by treatment in Table 3. Defects were as follows: secundum type atrial septal defects (n = 16); hypoplasia of the aorta or pulmonary artery (n = 9); aortic valve defects with fused leaflets (bicuspid or tricuspid) creating aortic valvular stenosis (n = 2); pulmonary valve stenosis (n = 6), including hypoplastic annulus and leaflet adhesions; hypoplastic mitral valve annulus (n = 6); tricuspid valve defects (n = 1); abnormal looping (n = 2); atrioventricular septal defect (n = 1), and both perimembranous (n = 10) and muscular (n = 8) ventricular septal defects. When these defects were divided into left and right heart abnormalities, the breakdown demonstrated was of near equal proportion: 11 left-sided versus 13 right-sided defects. Septation defects appeared to be more represented than a difference between lesions of the left and right sides of the heart.

Quantitative analysis of cardiac abnormalities. A total of 605 control fetal hearts and 941 treated fetal hearts (divided into seven groups) were examined (Fig. 1). When examining the fetuses on an individual basis the assumption was made that each fetus had an equal chance of developing a heart malformation independent of its litter mates. Because this may not be the correct assumption, fetal hearts were also evaluated

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Table 2. Maternal Parameters and Summary

Treatment	No. Mat with Abn Fet	No. Mat Rats	No. Dead Fetuses	Total No. Fetuses	Avg Fet/Lit (no. fet/no. Mat)	Avg (g) Wt Gn/Mat	No. Res	Avg Res Litter	No. Imp	Avg Imp Litter	Tot Lit w/Res	Total Hrts	Norm Hrts	Fet w/Abn Hrts	% Abn Hrts	Abn Hrt: Per Fetus Fisher Ex.	Abn Hrt: Per Litter Wilc. Test	Abn Hrt: Per Litter Exact Per. Test
																p Value	p Value	p Value
Control-Drk Water	9	55	3	605	11.3	122.1	40	0.70	13	0.20	0	605	592	13	2.15%	0.0001*	0.0004*	0.0015*
2,730 ppm TCAA	7	11	3	115†	10.5	84.6	30*	2.7*	12*	1.1*	0	114†	102	12	10.53%*	0.128	0.1673	0.2074
1,570 ppm MCAA	3	10	1	132	13.2	18.0	7	0.70	0	0.00	0	132	126	6	4.55%	0.356	0.0918	0.1841
1,249 ppm TCEth	4	10	0	121	12.1	115.6	13	1.30	4	0.40	0	121	116	5	4.13%	0.201	0.2035	0.3071
1,232 ppm TCAld	6	20	2	256‡	12.4	118.4	17	0.90	2	0.10	0	248‡	240	8	3.23%	0.453	0.1979	0.3241
174 ppm DCAld	3	9	0	101	11.1	118.5	12	1.30	1	0.10	0	101	98	3	2.97%	0.481	0.1542	0.2673
473 ppm CMC	3	8	0	92‡	11.5	114.6	10	1.30	4	0.50	0	85‡	81	4	4.71%	0.145	0.2087	0.2672
50 ppm DCVC	4	13	0	140	10.8	69.2	21*	1.6*	1	0.80	2	140	135	5	3.57%			

% Abn Hrts = the percentage of abnormal hearts compared to the total hearts. Abn = abnormal hearts, Avg = average, Avg Wt Gn (g) = weight gain during pregnancy (total weight gained/mat), Fet/Lit = average number of fetuses per litter, Hrts = number of hearts, Imp = implantation site (Avg Imp = no. Imp/no. Mat), Mat = maternal rat, Res = resorption site (Avg Res = no. Res/no. Mat), Tot Lit Res = total litter resorption, no fetuses. *Statistically significant p values ($p < 0.05$). †Discrepancies between the number of Fetuses and the number of Hearts indicates a heart was not removed from a dead fetus due to autolysis. ‡Live births in which some fetuses were cannibalized by the mother, and thus no hearts were obtained.

on a per litter basis to determine the significance of any increase in number of cardiac abnormalities. The range of cardiac abnormalities per group ranged from 2.97% to 10.53% with a control group value of 2.15%. The group imbibing 2,730 ppm TCAA (291 mg/kg/day) during pregnancy had 10.53% abnormal hearts from a total of 114 fetuses. Malformation rate for the TCAA group was significantly greater than control rats on a per fetal basis ($p = 0.0001$, Fisher's exact test) and a per litter basis (Wilcoxon and exact permutation tests ($p = 0.0004$, and a $p = 0.0015$ respectively). No other group demonstrated a significant increase in cardiac malformations compared with controls.

Discussion

Prior studies by the same investigators demonstrated an increased incidence of cardiac defects when the parent compounds TCE and DCE were administered in a similar rat model (10). The current study demonstrated that exposure to a major metabolite, TCAA administered in drinking water, resulted in increased numbers of implantation and resorption sites, and selective cardiac teratogenicity.

The logic for our initial dose selection was based on the level of the metabolite that would be expected to be produced from the breakdown of the maximum TCE dose tested (1,100 ppm) (9). Smith et al. (11) administered a gavage dose of 330 mg/kg/day TCAA, which was similar to the drinking water dose given in this study (291 mg/kg/day). In the Smith et al. study, a dose dependent increase in implantation sites and resorption sites and an increase in frequency of soft tissue malformations (mainly cardiovascular) occurred at levels of 330 mg/kg/day and above.

The types of cardiac defects found in this study (Table 3) are consistent with those in our previous studies (7–10). A variety of defects was found with no particular lesion, grouping, or syndrome predominating.

The defects observed in this study are different from those reported by Smith, et al. (11). In the Long-Evans rat they reported mainly malaligned ventricular septal defects. However, their method of examining fetuses was by section microscopy, which is quite different from the dissection method used in this study. Our method is more sensitive for lesions, such as adhered valve cusps, separating oval fossa defects from true secundum atrial defects and detection of abnormal valve dimensions.

A nonchlorinated metabolite of TCE, CMC, was included to determine if the presence or absence of the chlorine atom is involved in the production of cardiac defects. Carboxy methylcystine and all the metabolites we tested, except the TCAA, produced no cardiac abnormalities. It is of interest to note that at a high dose, MCAA produced no effect, whereas TCAA did produce cardiac defects, and increased numbers of implantation and resorption sites. Smith et al. (17) studied the effects of DCAA, and found cardiac teratogenic effects at high levels of exposure.

Although it is accepted that "no animal test and battery of

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Table 3. Types of Heart Malformations

Group	Normal	TCAA	MCAA	TCEth	TCAld	DCAld	CMC	DCVC
Heart abnormalities	Water	2,730 ppm	1,570 ppm	1,249 ppm	1,232 ppm	174 ppm	473 ppm	50 ppm
Abnormal looping	2							
AO hypoplasia		1		1		1		1
PA hypoplasia		2	1			2		
Atrial septal defects	7	3	3		2			1
Mitral valve defects (hypoplasia or ectasia)	1	1		1	2			1
Tricuspid valve defects (hypoplasia or ectasia)				1				
Ventricular septal defects:								
Perimembranous (subaortic)	2	4			3		1	
Muscular	2	1		1			2	2
Atrioventricular septal defects	1							
Pulmonary valve defects		1	3	1	1			
Aortic valve defects				1			1	

AO = Aorta; Abn = abnormal; PA = pulmonary artery.

tests will provide complete assurance in the prediction of human teratologic risk" (22), the similarities in the timing and sequencing of events during crucial periods of embryogenesis and organogenesis (particularly cardiogenesis) between humans and rats suggest that the rat is a suitable nonprimate choice for studying teratogenic effects on developing fetuses (22–25). Other advantages of the rat model include: the availability of genetically uniform strains, well documented reproductive cycles, gestational stages, multiparity, high fertility rates and resistance to surgical manipulation (26–32). The Sprague-Dawley rat model was specifically selected because of the very low incidence of spontaneous cardiovascular anomalies (25) and the general similarity to the human incidence and types of spontaneous cardiac abnormalities that occurred (22–24). All these factors contribute to the potential importance of these findings and their possible application to the human situation.

Study limitations. 1) It was not within the scope of this research to determine the mechanisms responsible for these defects, but rather, we studied the incidence and types of cardiac defects that occur. 2) Certain cardiac and great vessel abnormalities may have gone undetected, such as coarctation of the aorta, regurgitant valves and abnormal coronary artery distribution beyond the ostium. 3) As in previous studies, it was not possible to substantiate delivery of compounds to the fetal tissue due to sensitivity limitations of our gas chromatography methods. Failure to demonstrate cardiac defects associated with other metabolites in this study may be due to their lack of teratogenesis or their inability to cross the fetal membranes and/or the rapid clearance of those compounds in the maternal body. 4) This study sought a causative agent and did not address a dose response. 5) Doses in this study were higher than those typically found in contaminated wells and water supplies.

The low number of cardiac defects found in non-TCAA groups does not preclude the cardiac teratogenicity of these metabolites, but rather demonstrates that an increase could not be detected at the power level we employed. Also, these findings do not prove that human cardiac defects are caused by TCAA. However, this study raised awareness of the potential cardiac teratogenicity of some halogenated compounds.

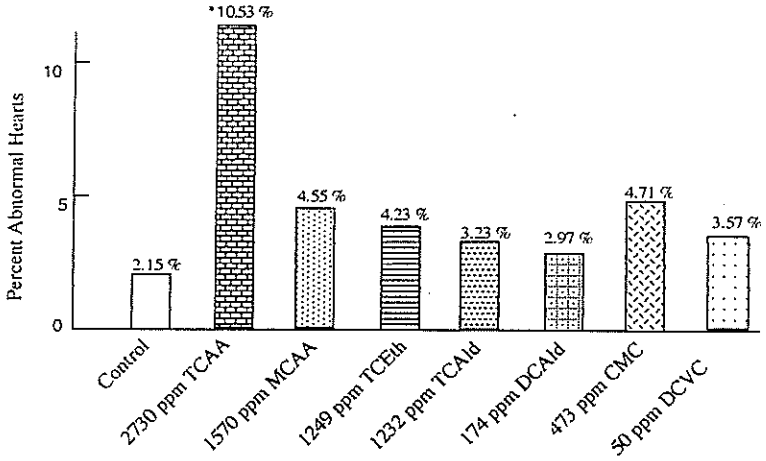

Figure 1. Percent of abnormal hearts (fetal basis). CMC = carboxy methylcystine; DCAA = dichloroacetic acid; DCAld = dichloroacetaldehyde; DCVC = dichlorovinyl cystine; MCAA = monochloroacetic acid; TCAA = trichloroacetic acid; TCAld = trichloroacetaldehyde; TCEth = trichloroethanol. *Statistical significance between the treated group and the control group.

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Conclusions. These data demonstrate that specific fetal cardiac teratogenicity occurs when TCAA is administered in drinking water to a maternal rat, in the doses studied. TCAA also results in an increased number of implantation sites and resorption sites when compared with the control animals. These data therefore support the hypothesis that not only the parent compound, but also a metabolic breakdown product of TCE may cause selective cardiac teratogenesis in a mammalian model.

Although the experimental studies discussed here cannot be extrapolated directly to humans, many processes of cell division, migration and differentiation are common to all mammals during gestation. It is anticipated that further investigation might elucidate the mechanisms relating exposure to certain halogenated hydrocarbons and cardiac defects.

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