IDENTIFICATION OF TRICHLOROETHYLENE AND ITS METABOLITES IN HUMAN SEMINAL FLUID OF WORKERS EXPOSED TO TRICHLOROETHYLENE

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ABSTRACT:

We have investigated the potential of the male reproductive tract to accumulate trichloroethylene (TCE) and its metabolites, including chloral, trichloroethanol (TCOH), trichloroacetic acid (TCA), and dichloroacetic acid (DCA). Human seminal fluid and urine samples from eight mechanisms diagnosed with clinical infertility and exposed to TCE occupationally were analyzed. In vivo experimental studies, TCE and its metabolites were determined in epididymis and testis of mice exposed to TCE (1000 ppm) by inhalation for 1 to 4 weeks. In other studies, incubations of monkey epididymal microsomes were performed in the presence of TCE and NADPH. Our results showed that seminal fluid from all eight subjects contained TCE, chloral, and TCOH. DCA was present in samples from two subjects, and only one contained TCA. TCA and/or TCOH were also identified in urine samples from only two subjects. TCE, chloral, and TCOH were detected in murine epididymis after inhalation exposure with TCE for 1 to 4 weeks. Levels of TCE and chloral were similar throughout the entire exposure period. TCOH levels were similar at 1 and 2 weeks but increased significantly after 4 weeks of TCE exposure. Chloral was identified in microsomal incubations with TCE in monkey epididymis. CYP2E1, a P450 that metabolizes TCE, was localized in human and monkey epididymal epithelium and testicular Leydig cells. These results indicated that TCE is metabolized in the reproductive tract of the mouse and monkey. Furthermore, TCE and its metabolites accumulated in seminal fluid, and suggested associations between production of TCE metabolites, reproductive toxicity, and impaired fertility.

Trichloroethylene (C₂HCl₃; TCE) is a volatile and lipophilic compound used extensively as an industrial solvent. Because of its efficacy as a degreasing agent, TCE is used widely by workers for cleaning of metal parts, leading to a high potential for human exposure in occupational environments. Production of TCE increased from about 260,000 pounds in 1982 to 320 million pounds in 1991 (Pastino et al., 2000). The current occupational exposure limit for TCE is 50 ppm (ACIGF, 2002). The National Toxicology Program has estimated that 3.5 million workers are exposed to TCE (NTP, 1990).

The major organs affected by TCE are the liver, kidney, and lung, with severity of toxicities being dependent on species, strain, gender, physiological state, and route of exposure. The toxicities evoked by TCE are attributable to its metabolites. The major pathway of TCE metabolism is oxidation via the cytochrome P450 system, mainly by CYP2E1, which yields the primary metabolites TCE oxide, chloral, and dichloroacetyl chloride (Fig. 1; Kimmerle and Eben, 1973). TCE oxide seems to be a transient metabolite, and the evidence indicates that it does not rearrange to form chloral either in the presence or absence of cytochrome P450 (Miller and Guengerich, 1982; Cai and Guengerich, 1999). The acyl chloride subsequently decomposes to trichloroacetic acid (TCA) and trichloroacetic acid (TCA). Dichloroacetyl chloride is generated from TCE oxide and can be trapped with lysine to form N²-dichloroacetyllysine (Cai and Guengerich, 1999). The acyl chloride subsequently decomposes to form dichloroacetic acid (DCA). An additional mechanism by which DCA is formed is through dechlorination of TCA (Lash et al., 2000), and dichloroacetyl chloride (Fig. 1; Kimmerle and Eben, 1973). TCE oxide seems to be a transient metabolite, and the evidence indicates that it does not rearrange to form chloral either in the presence or absence of cytochrome P450 (Miller and Guengerich, 1982; Cai and Guengerich, 2001). It has been proposed that chloral is derived from TCE via a transition state involving an oxygenated TCE-P450 intermediate. Chloral is rapidly converted to chloral hydrate, which undergoes reduction and oxidation by alcohol dehydrogenase and aldehyde dehydrogenase to form trichloroethanol (TCA) and trichloroacetic acid (TCA), respectively (Green and Prout, 1985; Dekant et al., 1986). TCOH and TCA are major urinary metabolites and are used as markers of exposure in workers exposed to TCE (ACIGF, 2002). Glucuronidation of TCOH produces TCOH glucuronide, which is secreted into bile and then into the small intestine, hydrolyzed back to TCOH in the gut, reabsorbed, and is then available for conversion to TCA (Bull, 2000). Humans are efficient at glucuronidating TCOH relative to rodents, although impaired ability for glucuronidation reactions is relatively frequent in the human population (Templin et al., 1993; Green et al., 1997). Recent studies indicated that dichloroacetyl chloride is generated from TCE oxide and can be trapped with lysine to form N²-dichloroacetyllysine (Cai and Guengerich, 1999). The acyl chloride subsequently decomposes to form dichloroacetic acid (DCA). An additional mechanism by which DCA is formed is through dechlorination of TCA (Lash et al., 2000), and dichloroacetyl chloride (Fig. 1; Kimmerle and Eben, 1973). 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and this source of DCA is supported by studies in which DCA together with TCA, TCOH, and TCOH glucuronide were identified in children administered chloral therapeutically as a sedative-hypnotic drug (Henderson et al., 1997). Hence, TCE exposure and metabolism results in internal exposure to several metabolites, including chloral, TCOH, TCA, and DCA. The data suggested that TCA mediates the hepatotoxic effects of TCE (Bull, 2000), and DCA and TCA are responsible for hepatocarcinogenicity (Larson and Bull, 1992). On the other hand, chloral has been proposed to be the metabolite responsible for TCE-induced lung injury (Green et al., 1997), although evidence for this mechanism is lacking. Hence, it has been difficult to assign responsibility for the toxic effects produced by TCE to any specific metabolites. Nevertheless, there is general agreement that TCA, TCOH, and DCA are responsible for the toxic and/or carcinogenic effects of TCE (Pastino et al., 2000).

In our recent studies in mice, we identified the male reproductive system as a sensitive target of TCE-induced toxicity (Forkert et al., 2002). Inhalation exposure to TCE resulted in its metabolism to chloral that was produced at higher levels in the epididymis than in the testis, and is consistent with the greater amounts of CYP2E1 present in the former versus the latter. In addition, CYP2E1 was localized in the epididymal epithelium. To confirm the role of CYP2E1 in TCE metabolism, preincubation of epididymal microsomes with a CYP2E1 inhibitory antibody significantly inhibited the production of chloral from TCE (Forkert et al., 2002). These data correlated with structural damage of the epididymal epithelium, suggesting that generation of CYP2E1-mediated metabolites from TCE plays a role in the toxic response. These results led us to postulate that a similar outcome may be manifested in the human reproductive tract of workers exposed to TCE and that TCE and/or its metabolites may be detected in the seminal fluid. We have examined samples of seminal fluid from eight mechanics exposed to TCE in the workplace for an extended time period. Samples of seminal fluid and urine were analyzed for the presence of TCE and/or its metabolites. To determine whether CYP2E1, a cytochrome P450 enzyme with an important role in TCE metabolism, was available in the male reproductive tract for TCE metabolism, immunohistochemical studies were performed to identify the CYP2E1 protein in human epididymal and testicular tissues. In our previous studies in mice, we identified formation of chloral in incubations of epididymal microsomes with TCE (Forkert et al., 2002). Herein, we have extended the findings of these in vitro studies and have exposed mice to TCE by inhalation for 1 to 4 weeks. TCE and its metabolites were then determined in the epididymis of these mice. In addition, we have investigated TCE metabolism in incubations of monkey epididymal microsomes and have localized CYP2E1 in monkey epididymis and testis.

Materials and Methods

**Materials.** TCE (purity 99.9%), paraformaldehyde, hydrogen peroxide (30%, v/v), 3,3’-diaminobenzidine tetrahydrochloride, and dibromopropanol were obtained from Sigma-Aldrich (St. Louis, MO). A goat polyclonal antibody directed against rabbit liver microsomal CYP2E1 was obtained from Oxford Biomedical Research (Oxford, MI). Biotinylated horse anti-goat IgG was from Vector Laboratories (Burlingame, CA). Avidin-biotin blocking reagent and streptavidin conjugated to horseradish peroxidase were from Zymed Laboratories (South San Francisco, CA).

**Subjects.** Subjects for this study were recruited from a cohort that sought medical consultation for fertility problems. All eight subjects were mechanics that have used TCE for cleaning and degreasing purposes. The duration of TCE use was for at least 2 years for all the subjects, who still continue to use the chemical. All were nonsmokers, and alcohol consumption was limited. These subjects were classified as infertile according to criteria used by the World Health Organization. Normal fertility is defined by the following criteria: semen sperm density >20 million/ml, sperm motility >40%, and normal sperm morphology >14%. The wives were clinically assessed to lack signs and symptoms of infertility. Urine and semen samples were collected by the subjects in appropriate containers on Thursday evening of a working week and stored at 0°C. They were brought to the Ottawa Health Research Institute on Friday morning and were stored at −4°C until analysis. For controls, seminal fluid samples from five subjects that did not use TCE were analyzed. These studies were performed with the approval of the Human Research Ethics Committee of the Ottawa Health Research Institute.

**TCE Metabolites in Urine.** Urine samples were collected and stored at −20°C until analysis. Urinary concentrations of the TCE metabolites TCA and TCOH were determined by headspace gas chromatography and electron-capture detection. Analysis was performed by following procedures described by Breimer et al. (1974) for determination of TCA and TCOH in blood and urine.

**TCE and Metabolites in Seminal Fluid.** Each semen sample represented the amount obtained from a single ejaculate (2 ml). Semen samples were subjected to centrifugation (300g) at room temperature for 15 min. The seminal fluid samples were stored at −20°C until analysis. For analysis, the samples were thawed after which 1 μl of 1,3-dibromopropane diluted in ethyl acetate (1:1000) was added as an internal standard. The samples were then extracted with 0.5 ml of ethyl acetate and analyzed using an Autosystem XL gas chromatograph fitted with a PE-210 30 m × 0.25 mm i.d., 0.5-μm-thick column (PerkinElmer Instruments, Norwalk, CT) and an electron capture detector. The TCE metabolites were analyzed by injection of the ethyl acetate extracts into a split injector set at 200°C with a detector temperature of 300°C.
and a flow rate of 24.8 cm/s. The initial oven temperature was set at 35°C and maintained for 11 min. The temperature was then increased at 10°C/min to 120°C and held at this temperature for 19 min. Retention times for TCE and chloral were about 3.9 and 6.0 min, respectively.

**Immunohistochemical Localization of CYP2E1.** The immunohistochemical studies were performed with approval from the Human Ethics Committee of Queen’s University. Paraffin-embedded sections of normal testis and epididymis from four anonymous human donors and three monkeys were used for immunohistochemical identification of CYP2E1. Detection of CYP2E1 was performed by the avidin-biotin complex technique, using a goat anti-rabbit CYP2E1 polyclonal antibody (Forkert, 1995). Tissue sections were deparaffinized, cleared, and hydrated in a graded ethanol series. The sections were rinsed in phosphate-buffered saline (PBS), and treated with 5% normal horse serum to block nonspecific antibody binding. The sections were then incubated for 60 min with the CYP2E1 antibody, which was diluted in PBS containing 2.5% normal horse serum. After thorough rinsing in PBS, tissue sections were reacted for 30 min with a biotinylated horse anti-goat antibody. Endogenous peroxidase activity was inhibited by incubating tissue sections with 1% hydrogen peroxide in water for 30 min. Sections were then reacted for 10 min with streptavidin conjugated to horseradish peroxidase, and the immunoperoxidase color reaction developed by reaction with 0.05% 3,3′-diaminobenzidine tetrahydrochloride and 0.01% hydrogen peroxide. The sections were then rinsed, incubated for 5 min in 0.15 M sodium chloride containing 0.5% copper sulfate, dehydrated, cleared, and mounted. Controls for the specificity of the immunohistochemical reactions included incubations performed in the presence of preimmune serum or in the absence of the CYP2E1 antibody.

**Inhalation Exposure of Mice to TCE.** Four groups of four male CD-1 mice were exposed to TCE (1000 ppm) for 6 h/day, 5 days/week (Monday through Friday) for 1, 2, and 4 weeks. The mice were exposed to TCE at the same time each day (08:00 AM–2:00 PM). The exposures to TCE were performed using methods described in our recent study (Forkert et al., 2002). Briefly, exposures were carried out in an exposure chamber of 500 liters with the same conditions described for analysis of TCE metabolites in seminal fluid.

**TCE Metabolism and p-Nitrophenol Hydroxylase Activity in Monkey Epididymis.** A microsomal incubation system was used to examine TCE metabolism and to determine p-nitrophenol hydroxylase activity. Microsomes were prepared from epididymides of two rhesus monkeys (*Macaca mulatta*), using procedures described previously (Forkert et al., 2002). Microsomal incubations were performed at 37°C for 60 min. Reaction mixtures contained 3 mM TCE in acetonitrile (0.5%, v/v), 1 mM NADPH, and epimydial microsomes (5 mg) in 50 mM Tris-HCl buffer, pH 7.4, in a total volume of 750 μL. Control experiments consisted of incubations performed in the absence of NADPH. Microsomal p-nitrophenol hydroxylation was used as an index of CYP2E1 activity and was determined as described previously (Forkert et al., 2002).

**Statistical Analysis.** Data were analyzed by using one-way analysis of variance and the Tukey test to identify significant differences between treatment groups (P < 0.05).

**Results**

**TCE Metabolites in Urine.** The TCE metabolites TCA and TCOH were detected in urine (Table 1). Urine samples from two of the eight subjects (subjects 2 and 4) contained TCA and/or TCOH. These values suggested that TCE exposure and/or metabolism was low during the time before the urine samples were obtained.

**TCE Metabolites in Seminal Fluid.** TCE and its metabolites were detected in seminal fluid samples from all eight subjects (Table 2). Samples of seminal fluid from subjects 2, 4, and 7 contained substantial levels of the parent TCE, whereas those from the other subjects had levels that were lower. Chloral and TCOH were detected in all samples from all eight subjects, with levels that were higher for the former than the latter. Dichloroacetic acid and TCA were also detected; DCA was found in only two subjects (subjects 4 and 7), whereas TCA was identified in only subject 7. The highest chloral levels were found in the sample from subject 3, although the amount of TCE detected in this subject was not high. On the other hand, the sample from subject 4, which contained the highest level of TCE, had relatively low amounts of chloral but markedly high levels of DCA. Hence, there was relatively good correspondence between the quantities of TCE found in seminal fluid and the formation of TCE metabolites. TCE and its metabolites were not detected in any of the seminal fluid samples from the control subjects.

**Immunohistochemical Localization of CYP2E1.** The CYP2E1 protein was detected in the human epididymis and was observed in the epithelium of the caput (head), corpus (body), and cauda (tail) (Fig. 2b). In the tests, CYP2E1 labeling was found in the Leydig cells in the interstitial tissue (Fig. 2a). The distribution of CYP2E1 in monkey was similar to that in mice and was seen also in the epididymal epithelium (Fig. 3) and Leydig cells (data not shown). Specific labeling was absent in reactions in which the CYP2E1 antibody was omitted or in which the primary antibody was replaced with preimmune serum.

**TCE Metabolites in Murine Epididymis.** The parent TCE was detected at similar levels in epididymal samples from mice exposed to TCE for 1, 2, or 4 weeks (Table 3). The TCE metabolites chloral and TCOH were also detected. The levels of chloral remained similar during the entire exposure period. Although the amounts of...
TCOH were similar at TCE exposures of 1 and 2 weeks, a significant increase (3-fold) was observed after exposure for 4 weeks. DCA and TCA were not present at sufficient levels to be detected using our method of analysis.

**TCE Metabolites and PNP Hydroxylase Activity in Monkey Epididymis.** Chloral was detected in the epididymal microsomal incubations (0.12 ± 0.02 ng/mg protein). Low levels of PNP hydroxylase activity were also detectable (1.9 ± 0.2 pmol/mg protein/min).

**Discussion**

We were interested in the possibility of a link between TCE exposure and male infertility. In initial efforts to explore such a possibility, we have established an experimental model in which mice were exposed to TCE by inhalation, a common route of occupational exposure, and investigated the potential toxic effects of this exposure on the male reproductive tract (Forkert et al., 2002). Our results showed that TCE exposure produced damage to and sloughing of the epididymal epithelium, whereas such toxic effects were not apparent in the testis. In in vitro studies in which epididymal and testicular microsomes were incubated with TCE, formation of the TCE metabolite chloral was identified in both the epididymis and testis, although levels were significantly higher in the former than in the latter. These findings suggested that bioactivation of TCE to metabolites is involved in the toxic response, with negative implications for epididymal spermatozoa. These data in mice have set the stage for the present investigation in humans.

In this study, we have analyzed urine and seminal fluid samples for the presence of TCE and/or its metabolites in eight subjects exposed chronically to TCE under occupational conditions. Our results showed that low levels of urinary TCA and/or TCOH were detected in only two (subjects 2 and 4) of the eight subjects (Table 1). These metabolites are consistent with those identified in previous studies showing that TCA and TCOH are urinary TCE metabolites in human subjects exposed to TCE by inhalation (Monster and Eben, 1973; Monster et al., 1979). Urinary levels of TCA and TCOH reflect mainly TCE metabolism in the liver, and this is due, in part, to the high metabolic capacity of this tissue for drugs and other exogenous chemical agents. Analysis of seminal fluid revealed that TCE was present in samples from all eight subjects (Table 2). However, markedly higher TCE levels were found in the samples from subjects 2, 4, and 7, compared with the others. The relatively high TCE levels in the seminal fluid samples from subjects 2 and 4 coincided with detectable levels of TCA and/or TCOH in urine samples from the same subjects (Tables 1 and 2). On the other hand, the seminal fluid sample from subject 7 contained relatively higher TCE levels, whereas TCA and TCOH were not detected in the urine. It should be emphasized that the TCE detected in seminal fluid may reflect not only recent TCE exposure but also may include contributions from TCE sequestered in adipose tissue and subsequently released. This mobilization of TCE is particularly relevant in the epididymis, which is surrounded by abundant adipose tissue. The half-life of TCE in adipose tissue is estimated to

**TABLE 3**

<table>
<thead>
<tr>
<th>TCE Exposure</th>
<th>TCE Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>wk (n = 4)</td>
<td>TCE Chloral TCOH</td>
</tr>
<tr>
<td>1</td>
<td>297 ± 37 0.30 ± 0.02 50.5 ± 10.1</td>
</tr>
<tr>
<td>2</td>
<td>315 ± 25 0.33 ± 0.07 45.5 ± 22.2</td>
</tr>
<tr>
<td>4</td>
<td>357 ± 33 0.53 ± 0.09 141.0 ± 28.0</td>
</tr>
</tbody>
</table>

*P < 0.05 compared with TCE exposures for 1 or 2 weeks.
be about 3.5 to 5.0 h (Davidson and Beliles, 1991), and therefore continued slow release from body fat can lead to TCE exposure for several hours after initial exposure. Therefore, TCE and its metabolites that are identified in seminal fluid represent the effects of this cumulative exposure.

The TCE metabolites chloral and TCOH were detected in seminal fluid from all eight subjects, with chloral levels being considerably higher than TCOH (Table 2). However, the metabolites DCA and TCA were also detected but only in samples from subjects 4 and 7, which were also the ones with the highest TCE levels (Table 2). The levels of DCA (subjects 4 and 7) and TCA (subject 7), when detected, were surprisingly high. On the other hand, relatively high levels of TCE and chloral were found in the seminal fluid of subject 2, whereas DCA and TCA were below detectable levels, suggesting that TCE metabolism to chloral was the preferential pathway in this subject. Also, the amounts of chloral formed in subject 3 were the highest identified in this study, whereas neither DCA nor TCA was detected. It is evident that the types of TCE metabolites formed in individual subjects differ. Nevertheless, our findings suggested that when TCE levels are sufficiently high, formation of DCA seems to be the preferential route of metabolism. Although it is not clear which mechanisms are responsible for the variability in the levels of TCE metabolites detected, the findings suggested that the levels of metabolizing enzymes, including CYP2E1 as well as alcohol dehydrogenase and aldehyde dehydrogenase, are likely variable in the different subjects. The relative levels of other P450 enzymes, such as CYP1A1/2, CYP2B1/2, CYP2C11, which have been reported to be involved in TCE metabolism (Nakajima et al., 1990, 1992a,b, 1993), may also differ in these subjects. Differences in the levels of the metabolizing enzymes are likely responsible for the diversity in the amounts of specific TCE metabolites identified in the seminal fluid. Interestingly, there seems to be a relationship between the amounts of TCE and magnitudes of metabolite formation. These results confirmed exposure of the human male reproductive tract to TCE and its metabolites. In our studies in mice, TCE as well as its metabolites were detected in the reproductive tract of male mice exposed to TCE by inhalation (Table 3). Hence, the metabolic disposition of TCE in humans seems to be similar to that in mice, and indicated that our murine model is appropriate for investigating the effects and mechanisms of TCE-induced toxicity in the male reproductive system. Taken together, the results of our studies in mice and in humans supported the premise that TCE is metabolized in the human reproductive tract and mainly in the epididymis, resulting in the production of metabolites that cause damage to the epididymal epithelium and affect the normal development of sperm.

Evidence indicated that CYP2E1 is the predominant P450 enzyme catalyzing the hepatic metabolism of TCE in both humans and rodents (Lipscomb et al., 1998; Lash et al., 2000). In our previous studies in mice, we have used an inhibitory CYP2E1 monoclonal antibody to demonstrate that CYP2E1 is involved in TCE metabolism to chloral in both epididymis and testis (Forkert et al., 2002). A significant reduction in the formation of chloral was detected in epididymal microsomes preincubated with the CYP2E1 antibody and subsequently incubated with TCE. The extent of chloral formation was higher in epididymis than in testis, and correlated with the relative amounts of CYP2E1 activity present in the individual tissues. Immunochemical experiments confirmed the presence of CYP2E1 in the epididymis and testis of mice, and localized this P450 in the epididymal epithelium and testicular Leydig cells (Forkert et al., 2002). These findings supported the role of CYP2E1 in TCE metabolism in the male reproductive tract of mice. In the human studies described in this investigation, the presence of chloral, TCOH, TCA, and DCA in the seminal fluid samples indicated that TCE metabolism is mediated by cytochrome P450 (Table 2; Fig. 1). However, data are required to support the role of CYP2E1 in TCE metabolism in the human male reproductive tract. As a step to this end, we have performed immunohistochemical studies, using tissue samples of human epididymis and testis and the same CYP2E1 antibody applied in the murine tissues in previous studies (Forkert et al., 2002). The CYP2E1 protein was localized in the epididymal epithelium as well as in testicular Leydig cells (Fig. 2). In view of the length of the human epididymal duct, which may extend up to 6 m, total CYP2E1 activity in the epididymis may be significant. The CYP2E1 protein was also localized in monkey epididymal epithelium (Fig. 3) and testicular Leydig cells. Thus, the cellular distribution of CYP2E1 is similar in mice, monkey, and human. It is therefore plausible that the chloral and DCA detected in human seminal fluid is derived mainly from CYP2E1-mediated TCE metabolism within the epididymal epithelium, as was identified in the mouse and monkey.

Our findings have not provided direct evidence to demonstrate a link between TCE exposure and the confirmed infertility in the subjects in this study. Nevertheless, such a link is supported by localization of CYP2E1 in the male reproductive tract as well as identification of TCE and its P450-derived metabolites in seminal fluid. Moreover, neither TCE nor its metabolites were detected in seminal fluid from the control subjects who did not use TCE. Our previous studies in mice demonstrated that TCE exposure elicits epithelial damage in the epididymis (Forkert et al., 2002). The similarities between mice and humans in TCE metabolism supported a prediction that the toxic outcome will also likely be similar. Hence, bioactivation of TCE in the epididymal epithelium raises the possibility of toxic damage at this site in the human, and because the epithelium plays a central role in sperm development and maturity, the toxic effects are likely to be detrimental to production of viable sperm. However, this effect of TCE exposure on the sperm remains to be established. In addition, our results indicated that TCE and its metabolites accumulated in the male reproductive tract, and represent a marker of exposure and metabolism. In a broader context, these results have serious implications for potential toxicities in the male reproductive tract as a result of exposure to an extensive array of chemicals that may lead to the reported diminution in semen quality in humans (Carlsen et al., 1992). It should be emphasized that the studies described in this report are limited by difficulties in obtaining samples for analysis. To strengthen the link between TCE and infertility, efforts were made to obtain seminal fluid samples from workers exposed to TCE who were fertile, but these efforts were not successful. Nevertheless, we believe that the results of the present study are of interest and should set the stage for studies to address the issue of potential impairment of fertility as a result of TCE exposure.

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