Trichloroethylene and Trichloroethanol-induced formic aciduria and renal injury in male F-344 rats following 12 weeks exposure

Noreen Yaqoob[1], Andrew Evans[1], John R Foster[2] and Edward A Lock[1]

[1]School of Pharmacy and Biomolecular Sciences,Liverpool John Moores University, Byrom Street, Liverpool, L3 3AF, UK; [2] AstraZeneca, Alderley Park, Macclesfield, Cheshire SK10 4TG

Corespondance to : Edward A Lock at address above
Telephone 44 (0) 1625 540466
e-mail e.lock@ljmu.ac.uk

Key words: Trichloroethylene; trichloroethanol; formic acid; Kim-1; NAG.

ABSTRACT

Trichloroethylene (TCE) is widely used as a cleaning and decreasing agent and has been shown to cause liver tumours in rodents and a small incidence of renal tubule tumours in male rats. The basis for the renal tubule injury is believed to be related to metabolism of TCE via glutathione conjugation to yield the cysteine conjugate that can be activated by the enzyme cysteine conjugate β-lyase in the kidney. More recently TCE and its major metabolite trichloroethanol (TCE-OH) have been shown to cause formic aciduria which can cause renal injury after chronic exposure in rats. In this study we have compared the renal toxicity of TCE and TCE-OH in rats to try and ascertain whether the glutathione pathway or formic aciduria can account for the toxicity. Male rats were given TCE (500mg/kg/day) or TCE-OH at (100mg/kg/day) for 12 weeks and the extent of renal injury measured at several time points using biomarkers of nephrotoxicity and prior to termination assessing renal tubule cell proliferation. The extent of formic aciduria was also determined at several time points, while renal pathology and plasma urea and creatinine were determined at the end of the study. TCE produced a very mild increase in biomarkers of renal injury, total protein, and glucose over the first two weeks of exposure and increased Kim-1 and NAG in urine after 1 and 5 weeks exposure, while TCE-OH did not produce a consistent increase in these biomarkers in urine. However, both chemicals produced a marked and sustained increase in the excretion of formic acid in urine to a very similar extent. The activity of methionine synthase in the liver of TCE and TCE-OH treated rats was inhibited by about 50% indicative of a block in folate synthesis. Both renal pathology and renal tubule cell proliferation were reduced after TCE and TCE-OH treatment compared to controls. Our findings do not clearly identify the pathway which is responsible for the renal toxicity of TCE but do provide some support for metabolism via glutathione conjugation.

INTRODUCTION

Trichloroethylene (TCE) has been widely used as a cleaning and degreasing agent, a de-icing chemical for aircraft and a range of other uses. TCE has been examined for carcinogenicity in rodents and in some studies shown to cause a very small number of renal tubule tumours in male rats when exposed orally at high doses of 500 or 1000mg/kg/day for typically 2 years (see Lock and Reed, 2006 for details). Over the last 20 years there has been considerable debate about the relevance of the animal findings to renal cancer in humans. Several epidemiology studies on the mortality of TCE exposed workers and one study on mortality/morbidity did not reveal an association between exposure and renal tumours (Spirtas et al., 1991; Axelson et al.,

1994; Anttila et al., 1995). Recent studies on occupation exposure to TCE and renal cancer have also reported no association (Kelsh et al., 2010; Hansen et al., 2013). Charbotel et al., (2007) examined the relationship between somatic mutation of the Von-Hippel-Landau (VHL) gene and exposure to TCE and found no VHL gene mutations in renal tumours from TCE exposed workers. In contrast, an increased incidence of renal tubule tumours was reported in a retrospective study by Henschler et al., (1995) in workers exposed to very high concentrations of TCE over a prolonged period of time. Recent studies have also reported an association between TCE exposure and an increased incidence of renal tubule tumours (Karami et al., 2012, Scott and Jinot, 2011). While Moore and coworkers (2010) have shown that workers exposed to TCE and expressing glutathione transferase T1(GSTT1) were more susceptible than GSTT1 nulls which they suggest is consistent with the hypothesis that metabolism by the reductive (glutathione) pathway can lead to renal injury.

Bioactivation of TCE is believed to be responsible for the injury to the kidneys. This occurs following glutathione conjugation of TCE by glutathione S-transferases, followed by further metabolism by enzymes of the mercapturic acid pathway to form S-1,2-(dichlorovinyl)-L-cysteine (DCVC) which accumulates in proximal renal tubule cells. DCVC is then cleaved by the enzyme cysteine conjugate β-lyase to produce a reactive thioketene (se Anders, 2008). This causes marked toxicity to the proximal renal tubules in rats and mice administered DCVC (Gandolfi et al., 1981; Darnerud et al., 1988; Vaidya et al., 2003; Green et al., 1997). DCVC is also weakly mutagenic in bacteria, induces mitochondrial toxicity and perturbation of intracellular calcium homeostasis (Green and Odum, 1985; Groves et al., 1990; Stonard and Parker, 1971). DCVC can also undergo metabolism by cytochrome P450 to form a sulphoxide, which is also nephrotoxic and mutagenic to bacteria (Lash et al., 1994; Irving et al., 2013; Irving and Elfarra, 2013). Bioactivation of TCE, by a mechanism currently not full understood, can perturb folate metabolism resulting in formic aciduria in rats (Green et al., 1998; Dow and Green, 2000; Yaqoob et al., 2013). It has been postulated that a marked and sustained excretion of formic acid, over a prolonged period of time, may explain the renal toxicity in long term studies after TCE exposure (Green et al., 1998). The major metabolites of TCE formed by the oxidative pathway of metabolism, namely trichloroethanol (TCE-OH) and trichloroacetic acid are also able to perturb formic acid excretion in rats (Dow and Green, 2000; Yaqoob et al., 2013). In the rat, TCE metabolism via cytochrome P450 metabolism becomes saturated at doses of 500mg/kg/day and above, allowing

metabolism via reductive pathways (Prout et al., 1985). Metabolism of TCE to TCE-OH is a major pathway of elimination with about 54 % of the dose excreted in the urine as the glucuronide conjugate, there being little if any free TCE-OH (Prout & Green, 1985).

The aim of this study was to expose rats to TCE at 500mg/kg/day which has the opportunity to undergo metabolism to DCVC and TCE-OH and to TCE-OH alone, (which cannot form DCVC) at a dose of 100mg/kg/day to see if we could determine which pathway of metabolism leads to renal injury after oral exposure over 12 weeks. The dose of TCE-OH was based on an estimate of the likely exposure to free TCE-OH and more importantly was a dose that produces the same amount of formic acid excretion to that seen with TCE alone. It should be noted that these doses are very high and many fold above occupational exposure to TCE

MATERIALS AND METHODS

Materials

Trichloroethylene (TCE), reagent grade, 98%, inhibited with about 1% 1-2-epoxybutane, maleic acid disodium salt hydrate and Dowex 1X8-200 ion exchange resin were from Sigma Aldrich, Poole, UK. Deuterium oxide (D, 99.9%) from Cambridge Isotope Laboratories, Inc. Andover, Massachusetts, USA. 5-[^{14}C] methyl-tetrahydrofolic acid, barium salt 55µCi, 1.85 M Bq from GE Healthcare, Amersham, UK. Pure corn oil, low in saturates and high in polyunsaturates from Tesco Supermarket, Liverpool, UK. Norell 5mm NMR tubes with round bottom and 178mm length from Glass Precision Engineering Scientific Limited, Leighton Buzzard, UK. All other chemicals were of the highest purity available commercially.

Animal and treatment

Male F-344 rats were from the breeding colony at the Life Science Support Unit, Liverpool John Moores University. All rats were housed in North Kent Plastic cages on Beta bed sawdust (Grade 5, Datesand Ltd., Manchester, UK) which was changed daily. The animal room was maintained at a constant temperature of 20ºC ± 2ºC and humidity of 50% ± 5% with a 12h light-dark cycle starting at 04.00h. Rats were allowed rat expanded diet (Bantin and Kingman, Hull, UK.), and water *ad libitum*. Fifteen male F-344 rats weighing 236-312g (13-14 weeks of age) were used, with 5 rats per cage. The rats

were dosed daily by oral gavage for 12 weeks with either trichloroethylene (TCE; 500mg/kg/day), or trichloroethanol (TCE-OH; 100mg/kg/day) in corn oil at 5ml/kg body weight. Controls received corn oil alone at 5ml/kg/day. On week 11 cell proliferative responses in the kidney were evaluated via bromodeoxyuridine (BrdU) immunohistochemistry. Five days prior to scheduled necropsy, rats were implanted subcutaneously with osmotic pumps (Alzet® 2ML1, Durect Corporation, Cupertino, CA, USA); each pump contained 2 ml of 15 mg/ml BrdU solution in phosphate buffered saline at pH 7.4. Dosing solutions was stored in sealed glass containers, covered in aluminium foil at 4℃, with fresh dosing solutions made each week. All animal procedures were performed in accordance with a license issued under the UK, Scientific Procedures Act, 1986.

Urine, blood plasma and tissue collection

Rats were placed in metabolic cages for the collection of urine overnight (16h) after dosing on day 1, weeks 1, 2, 5, 6, 8, 10 and 12. Urine was collected from 4 rats of each dosing group into a container which had 0.1ml of 10% sodium azide to prevent bacterial growth. Urine volume and pH was measured and a sample taken for ^{1}H NMR analysis, the remainder being frozen -80°C for subsequent analyses.
Twenty four hours after the last dose the rats were killed by exposure to a rising concentration of carbon dioxide. Blood (4-5ml) was collected from the heart by cardiac puncture into heparinised tubes. The blood was then centrifuged at 330g for 10 min at 4°C for separation of plasma. The plasma was carefully removed and stored frozen at -80°C for subsequent analysis. The liver and kidneys were removed, weighed and a cross section of kidney fixed in buffered formal saline for histological examination. The remaining liver and kidney were stored at -80°C for subsequent analysis.

Measurement of metabolic changes in rat urine and plasma

^{1}H-NMR spectroscopy was used to measure metabolic changes in urine and plasma. Aliquots of urine (500µl) were mixed with 0.2M phosphate buffer pH 7.4 in D_2O (250µl) containing sodium-3(trimethylsilyl) propionate-2,2,3,3-d_4 (TSP; 0.5mg TSP/ml buffer) and then centrifuged at 14,000g for 10 min at 4℃. Aliquots of plasma (500µl) were mixed with 0.2M phosphate buffer pH 7.4 in D_2O (250µl) containing maleic acid disodium salt hydrate (0.5mg/ml) and centrifuged at 14,000g for 10 min at 4°C. The supernatants (600µl) were placed into a 5mm NMR tube and ^{1}H-NMR spectra acquired using a Bruker 300MHz instrument (Bruker Analytik GmbH, Germany). The standard ‘noesypr1d’ pulse sequence was utilised for data acquisition on urine, which efficiently suppresses the large water signal while the standard pulse sequence ‘cpmgpr1d’ was utilised for data acquisition on plasma samples. The Bruker software quantitates the signal intensities and spectra were baseline corrected using Mestrec software and normalised to TSP or maleic acid. Urinary metabolites were quantified with reference to TSP, the peak height of TSP being set at 0ppm, while plasma metabolites were quantified with reference to the maleic acid signal at 6ppm. The accuracy of the determination of formic acid was measured by spiked addition and

found to be 98%. The detection of formic acid using the 3σ method (Miller and Miller, 1993) gave an LOD of 0.004 mg/ml and using the 10σ method an LOQ of 0.04 mg/ml.

Clinical chemistry

Urinary glucose concentration was measured using glucose hexokinase assay reagents from Randox Laboratories Ltd, UK. Urinary protein concentration determined using Bio-Rad Detergent Compatible from Bio-Rad Laboratories, UK. While urine and plasma urea and creatinine assays were performed using Quantichrome urea assay kit, and creatinine assay kit from Bioassay Systems, Hayward, USA.

Measurement of Kidney injury molecule (Kim-1) and N-acetyl-β-D-glucosaminidase (NAG) in rat urine

Urinary Kim-1 protein was measured using microsphere-based Luminex xMAP technology with monoclonal antibodies raised against rat Kim-1 as described by Vaidya *et al*., (2006). Urinary NAG was measured spectrophotometrically according to the manufacturer's protocols (Roche diagnostics, Basel, Switzerland).

Measurement of methionine synthase activity in rat liver

The activity of hepatic methionine synthase was determined using radiolabelled ^{14}[C] methyl tetrahydrofolate as described by Banerjee *et al.,* (1997). Radioactivity was measured using a liquid scintillation counter (Packard liquid scintillation counter, Model 2100, UK). Specific activity is reported as picomol of methionine formed per minute per mg protein.

Histopathology

One kidney was fixed in buffered formal saline then dehydrated through a series of alcohols and embedded in paraffin wax. Tissue sections (5µm) were then cut processed and stained with haematoxylin and eosin. Microscopic examination of the kidney was performed by an experienced pathologist. The extent of cell proliferation was evaluated with BrdU incorporation via indirect BrdU labelling assay (Wijsman *et al.,* 1992). For the estimation of the proliferation index, the number of BrdU positive cells was counted and expressed as a percentage of the total number of the proximal tubule cell nuclei in 10 fields at 40× objective lens in a light microscope.

Statistical Analysis

The data are expressed as mean ± standard error of the mean (SEM). The time course data was analysed using two way analysis of variance (ANOVA) with repeated measures. Factors considered were time and treatment and then any possible interaction. Multiple comparisons between treatments were made using Tukey's method and analysed using SPSS version 21. End point measurements were analysed using one way ANOVA followed by Tukey's correction. A corrected p-value < 0.05 was considered statistically significant.

RESULTS

The effect of TCE and TCE-OH on body weight, liver and kidney weight, urine volume and pH in male F-344 rats over 12 weeks

No signs of toxicity were observed following 12 weeks daily oral administration of TCE (500mg/kg/day) or TCE-OH (100mg/kg/day) to male F344 rats compared to corn oil treated controls. Neither treatment had any effect on body weight gain over the 12 week period (data not shown). In contrast, liver and kidney weights, expressed as % of body weight were statistically significantly increased in rats dosed with TCE compared to corn oil treated controls, while TCE-OH had no effect on the liver or kidney weight (Figure 1). No increase in urine volume compared to controls was observed with either chemical over time (data not shown). Daily dosing with corn oil reduced urinary pH by about 0.5 of a pH unit; however, treatment with either TCE or TCE-OH produced further acidification of the urine compared to corn oil alone (Table 1). Statistical analysis showed a significant effect of time ($P< 0.05$) with TCE being significantly different from control ($P<0.05$) while TCE-OH was not quite significant (P, 0.056).

The urinary excretion of formic acid and methylmalonic acid in male F-344 rats following daily oral doses of TCE and TCE-OH over 12 weeks

The concentration of formic acid in the urine of male F-344 rats was determined using ^{1}H-NMR spectroscopy (Figure 2). Both TCE and TCE-OH produced a marked and sustained increase in formic acid over the 12 week period (Table 1). The urinary concentration of formic acid was elevated 1 week after dosing, peaked after 5 weeks and then remained elevated for the remainder of the study (Figure 3). Statistical analysis showed a significant effect of time and treatment ($P< 0.001$) with TCE ($P<0.01$) and TCE-OH ($P<0.001$) being significantly different from control. The amount of formic acid excreted was not statistically different between TCE and TCE-OH treatment, the only difference being observed at 6 weeks when for some unexplained reason the formic acid values for TCE exposed rats was rather low. Comparative analysis of the urine ^{1}H-NMR spectra showed treatment-related metabolic changes between the control and TCE-treated group with increases in TCE-glucuronide, creatine, taurine, with trimethylamine N-oxide and dimethylglycine being elevated 2-fold 10 weeks after dosing. Following 2 weeks exposure to TCE there was a small increase in the urinary excretion of methylmalonic acid (MMA), which from 5 weeks to the end of the study was increased in both the TCE and TCE-OH treated rats (Table 1). Statistical analysis showed a significant effect of treatment ($P<0.001$) but not time, while both TCE ($P<0.001$) and TCE-OH ($P<0.05$) where significantly different from control but not from one another. Difference between control and TCE-OH treated rats were less marked and were mainly due to TCE-glucuronide (Figure 2). These findings are in agreement with Principal Component Analysis (PCA) which showed clear separation of the three dose groups (data not shown).

The urinary excretion of markers of renal injury: total protein, glucose, Kim-1 and NAG in male F-344 rats following daily oral doses of TCE and TCE-OH over 12 weeks

Mild proteinuria was observed in rats treated with TCE one and two weeks after dosing but not after TCE-OH treatment (Table 2). Statistical analysis showed the proteinuria was treatment related ($P<0.05$) with TCE being significantly different from control ($P<0.05$) but not quite significant from TCE-OH (P,0.054), while TCE-OH was not significantly different from control. Glucosuria was only seen during the first 2 weeks of exposure to TCE and then returned to normal (data not shown). Kidney injury molecule 1 (Kim-1) was increased in the urine of TCE-treated rats after 1 and 5 weeks exposure (Table 2) however neither time nor treatment over the 12 week period was statistical significant from control for both TCE and TCE-OH. The urinary excretion of N-acetyl-β-D-glucosaminidase (NAG) showed a small increase 1, 5 and 10 weeks after TCE (Table 2). This showed both a time and

treatment dependent increase (P<0.05) with TCE being significant from control (P<0.05) indicating very mild renal tubular injury. In contrast, no increase in excretion of NAG was seen after TCE-OH treatment compared to control (Table 2).

The effect of TCE and TCE-OH on plasma urea, creatinine and formic acid in male F-344 rats over 12 weeks

Plasma urea and creatinine concentrations were determined using assay kits and no significant increase was seen with either chemical compared to the control (Table 3). The findings for plasma creatinine were confirmed using ^{1}H NMR spectroscopy. Plasma formic acid concentration was determined using ^{1}H-NMR spectroscopy following 12 weeks exposure to TCE and TCE-OH (Table 3) and was statistically significantly increased following exposure to both chemicals (Table 3). However the concentration of formic acid in plasma was statistically significant higher in the TCE-OH dosed group compared to TCE (Table 3).

The effect of TCE and TCE-OH on the activity of hepatic methionine synthase in male F-344 rats following 12 weeks exposure

The hepatic activity of methionine synthase was statistically significantly reduced by about 45% of the control value after 12 weeks treatment with either TCE or TCE-OH (Table 3).

The effect of TCE and TCE-OH on renal pathology following 12 weeks exposure

No evidence of marked renal tubule necrosis or cytomegaly was observed in either TCE or TCE-OH treated rat kidneys compared to corn oil treated controls There was however some indication of more basophilic staining in the renal proximal tubule cells in TCE and TCE-OH treated rats suggesting there may have been increased cell proliferation (Table 3). Also some mild degeneration in the proximal tubules with some hyaline inclusions was seen in the control and TCE-OH group but not in the TCE treated group (Table 3). Cell proliferation was monitored over the last 5 days prior to termination by inserting mini-pump containing BrDU. The number of BrDU positive cells was counted in approximately 400 proximal tubule nuclei, in 5 different regions and the labelling index expressed as a percentage (Table 3). The labelling index in renal proximal tubule cells was statistically significantly

lower in the TCE-OH and TCE treated rats compared to corn oil treated controls (Table 3). There was no statistical difference between TCE-OH and TCE-treatment.

DISCUSSION

Rats were given daily oral doses of TCE at 500mg/kg, a dose which would saturate the cytochrome P450 pathway of metabolism (Prout et al., 1985) such that TCE could undergo glutathione (GSH) conjugation, while another group were given TCE-OH at 100mg/kg/day which could not undergo conjugation with GSH. About 50% of a dose of TCE given orally to rats at 500mg/kg is excreted as the glucuronide conjugate of TCE-OH in the urine with very little excreted as free TCE-OH (Prout and Green, 1985). Conjugation of TCE-OH occurs primarily in the liver and hence it is difficult to know exactly how much free TCE-OH the kidney receives, we therefore selected a dose that produced a similar extent of formic aciduria as TCE. The current view is that conjugation of TCE via GSH is responsible for the renal injury, due to the formation of DCVC or its sulphoxide (Anders 2008; Green et al., 1997; Irving et al., 2013; Lash et al., 1994). In contrast, the production of formic aciduria following TCE or TCE-OH has also been proposed as a possible mechanism for causing long term renal injury (Green et al., 1998; 2003). We therefore compare the toxicity of TCE and TCE-OH to the kidney at doses which produced a similar urinary concentration of formic aciduria.

Renal injury following either TCE or TCE-OH

We found that both these dosing regimens were well tolerated by the rats over 12 weeks of daily dosing. No statistical differences for treatment or time were seen in urine volume compared to controls given corn oil alone. However, the excretion of total protein in urine was mildly elevated after 1 day, 1, and 2 weeks exposure to TCE, and statistically significantly increased with treatment (Table 2). The biomarkers of kidney injury Kim-1 and NAG were increased after 2 and 5 weeks exposure to TCE, but only NAG was statistically significantly with time and treatment (Table 2). However, these changes were small, total protein and NAG activity increasing maximally 2-fold after 2 weeks exposure, while Kim-1 was increased 5-fold after 5 weeks exposure. Mild glucosuria was seen over the first two weeks of exposure to TCE. These findings suggest a very mild insult to the renal tubules while the increase in Kim-1 may reflect renal tubule regeneration. In contrast, following TCE-OH exposure no consistent pattern of increase was seen in urinary

protein, Kim-1 or NAG compared to control. No statistically significant increase in either plasma creatinine or urea was observed after 12 weeks exposure to TCE or TCE-OH. The mild nature of these changes is reflected in the renal tubular pathology which was within the normal range, with the control animals showing slightly more minor pathological changes than those treated with TCE-OH, while the TCE treated rats showed the least morphological changes (Table 3). Cell turnover in the kidney was measured during the last 5 days of treatment and showed a similar profile to the renal pathology with the labelling index in the controls being higher than after either TCE-OH or TCE (Table 3). Renal proximal tubular cell turnover in the control male F344 rats was 4.8 % is similar to that previously reported (Lock et al., 2004). It appears that after 12 weeks daily exposure to either TCE or TCE-OH renal cell proliferation is decreased, this was also observed in rats and mice treated with 50mg/kg/day for 5 days/week with bromodichloromethane (Lock et al., 2004). Overall, our findings indicate mild renal tubular injury, as judged by urinary biomarkers, after TCE but not TCE-OH following 12 weeks of exposure. Similar findings to ours with evidence of only minor changes in biochemical markers of kidney injury and no morphological damage to the kidney has been reported following very high dose levels of TCE at 2000mg/kg for 42 days or following inhalation exposure to TCE at 500ppm for 6h/day for 28 days (Green et al., 1997; 1998). Others have similarly failed to find evidence of kidney damage or increased renal tubule cell proliferation in rats dosed with 1000mg/kg TCE for up to 3 weeks (Goldsworthy et al., 1988; Stott et al., 1982). Thus in TCE treated rats there is evidence of mild renal tubular injury which does not result in any renal tubular morphological changes and appears to suppress renal tubular cell proliferation after exposure to 500mg/kg/day TCE for 12 weeks.

We found no evidence of increased urinary markers of renal damage or of any renal pathology following treatment with TCE-OH at 100mg/kg/day for 12 weeks. However, others giving lower doses of TCE-OH(average of 18 and 54mg/kg/day) to rats in their drinking water for up to 52 weeks, reported small increases in urinary NAG and total protein after 4 weeks exposure and then intermittent small increases up to 52 weeks.(Green et al., 2003). These workers also measured renal tubule cell proliferation in the kidney after 29 and 40 weeks exposure to TCE-OH and found a significant focal increase in cells in S-phase in the outer cortex at the high dose after 29 weeks, with nothing at the low dose after 29 weeks or at the low or high dose after 40 weeks. Renal pathology showed an increase in tubular basophilia between 12 and 28 weeks, which by week 40 had progressed to tubular degeneration (Green et al., 2003).

Thus it is clear that chronic exposure of rats to TCE-OH or TCE can cause renal injury, but continued exposure typically for longer than 12 weeks is required to see renal tubule degeneration.

Formic aciduria following either TCE or TCE-OH

The major urinary changes observed following both TCE and TCE-OH exposure was acidification of the urine which was associated with a large increase in the excretion of formic acid which was both time and treatment related. Urine pH dropped by about 0.5 of a unit in control rats treated with corn oil alone, while those given TCE or TCE-OH dropped even further. The basis for the urinary acidification in the controls is not clear but could be related to metabolism of corn oil to acidic metabolites. There was a small increase in formic acid in urine after 1 week of exposure to either chemical, which was statistically significant after 2 weeks exposure and them remained elevated for the length of the study, being about 50-fold above control after 12 weeks exposure. These findings are consistent with that reported by Green et al., 1998; 2003; Dow and Green 2000; Yaqoob et al., 2013. Thus under the conditions of our study the extent of formic aciduria was similar with both chemicals. We also observed an increase in the excretion of methylmalonic acid with both chemicals as reported previously, supporting the hypothesis that these chemicals may be targeting the two enzymes that require vitamin B12 as a cofactor (Dow and Green, 2000). Regulation of tissue levels of tetrahydrofolate, the folate involved in the metabolism of formic acid, is largely controlled by the vitamin B_{12}-dependent methionine salvage pathway (Eells et al., 1982). Recovery of tetrahydrofolate is facilitated by methionine synthase and vitamin B_{12} acts as a co-enzyme in the transfer of the methyl group from 5-methyltetrahydrofolate to homocysteine resulting in the formation of methionine and tetrahydrofolate. Dow and Green, (2000) reported that TCE-OH caused an increase in N-methyltetrahydrofolate in the plasma indicating a blockage of the methionine salvage pathway. We report that the activity of methionine synthase in the liver of both TCE and TCE-OH treated rats following 12 weeks exposure is reduced by about 50% supporting a reduction in the activity of the methionine salvage pathway. The other B_{12} containing enzyme methylmalonyl CoA mutase, is involved in the regulation of odd chain fatty acids, several amino acids as well as gluconeogenesis and haem synthesis. The elevation in urinary methyl malonic acid is indicative of an effect on this pathway, suggesting TCE and TCE-OH are targeting B_{12} dependent metabolism.. In many ways the action of TCE and TCE-OH on these pathways resembles that of nitrous oxide (Kondo et al., 1981).

Conclusion

Our findings have not been able to clearly identify which pathway of metabolism leads to renal injury following exposure to TCE, although it gives some support to the GSH conjugation pathway, based on the finding of mild renal tubular injury after TCE but not TCE-OH. We know that the amount of TCE metabolised via the GSH conjugation pathway is very small, 4.6μg of N-acetyl DCVC being detected in a 24h urine sample following ten daily doses of 500mg/kg TCE (Green et al., 1997). This shows the amount of S-1,2-dichlorovinyl-L-cysteine (DCVC) undergoing N-acetylation, but does not tell us the amount of DCVC metabolised by cysteine conjugate β-lyase, although it is likely to be small. Whether this very small amount of DCVC formed could account for the very mild renal tubule injury after TCE exposure is currently unclear. The fact that TCE-OH produced a similar degree of formic aciduria to that seen after TCE but without renal injury, suggests that the perturbation of folate metabolism does not cause renal injury over that time period of exposure. However, a more sustained exposure to TCE-OH, which cannot lead to GSH conjugation, does lead to renal tubule injury and degeneration after exposure for 40 plus weeks (Green et al., 2003). So the situation is still not clear as to whether the kidney injury seen after chronic exposure to TCE is due to metabolism via the glutathione pathway or perturbation of folate metabolism by TCE-OH leading to formic aciduria or a combination of both resulting in renal tubule cell degeneration and chronic progressive nephropathy accounts for the very small increase in renal tubule tumours in male rats only.

Acknowledgements

The authors would like to thank: Dr Vishal Vaidya, Renal Division of Harvard Institutes of Medicine, Harvard, US for Kim-1 and NAG analysis on the urine samples; Dr Gordon Hard for histopathological examination of the kidneys; and Dr Phillip Rowe for help with the 2-way ANOVA with repeated measures. This work was supported in part by a grant from Halogenated Solvent Industry Alliance Inc to EAL.

References

Anders, M.W. (2008) Chemical toxicology of reactive intermediates formed by the glutathione-dependent bioactivation of halogen-containing compounds. Chem. Res. Toxicol. 21, 145-59

Anttila, A., Pukkala, E., Sallmén, M., Hernberg, S., and Hemminki, K. (1995) Cancer incidence among Finnish workers exposed to halogenated hydrocarbons. J. Occup. Environ. Med. 37, 797-806

Axelson, O., Seldén, A., Andersson, K., and Hogstedt, C. J. (1994) Updated and expanded Swedish cohort study on trichloroethylene and cancer risk. Occup. Med. 36, 556-62

Banerjee, R., Chen, Z. and, Gulati, S. (1997) Methionine synthase from pig liver. Methods. Enzymol. 281, 189-96

Charbotel, B., Gad, S., Caïola, D., Béroud, C., Fevotte, J., Bergeret, A., Ferlicot, S.,and Richard, S.(2007) Trichloroethylene exposure and somatic mutations of the VHL gene in patients with renal cell carcinoma. J. Occup. Med. Toxicol. 12, 2-13

Darnerud, P.O., Brandt, I., Feil, V.J., and Bakke, J.E. (1988) S-(1,2-dichloro-[14C]vinyl)-L-cysteine (DCVC) in the mouse kidney: correlation between tissue-binding and toxicity. Toxicol. Appl. Pharmacol. 95, 423-34

Dow, J.L. and Green, T. (2000) Trichloroethylene-induced vitamin B_{12} and folate deficiency leads to increased formic acid excretion in the rat. Toxicology, 146, 123-136

Eells, J.T., Black, K. A. Makar, A.B. Tedford, C.E. and Tephly, T.R. (1982) The regulation of one-carbon oxidation in the rat by nitrous oxide. Arch. Biochem. Biophys. 219, 316-26

Gandolfi, A.J., Nagle, R.B., Soltis, J.J., and Plescia, F.H. (1981) Nephrotoxicity of halogenated vinyl cysteine compounds. Res. Commun. Chem. Pathol. Pharmacol. 33, 249-61

Goldsworthy, T.L., Lyght, O., Burnett, J.A. and Popp, J.A. (1988) Potential role of alpha 2_u-globulin, protein droplet accumulation and cell replication in the renal carcinogenicity of rats exposed to trichloroethylene, perchloroethylene and pentachloroethane. Toxicol. Appl. Pharmacol. 96, 367-379

Green, T., and Odum, J. (1985) Structure/activity studies of the nephrotoxic and mutagenic action of cysteine conjugates of chloro- and fluoroalkenes. Chem. Biol. Interact. 54, 15-31

Green, T., Dow, J., Ellis, M.K., Foster, J.R. and Odum, J. (1997) The role of glutathione conjugation in the development of kidney tumours in rats exposed to trichloroethylene. Chem.Biol. Interac. 105, 99-117

Green, T., Dow, J., Foster, J.R., and Hext, P.M. (1998) Formic acid excretion in rats exposed to trichloroethylene: a possible explanation for the renal toxicity in long term studies. Toxicology, 127, 39-47

Green, T., Dow, J., and Foster, J.R. (2003) Increased formic acid excretion and the development of kidney toxicity in rats following chronic dosing with trichloroethanol, a major metabolite of trichloroethylene. Toxicology, 191, 109-119

Groves, C.E., Lock, E.A., and Schnellmann, R.G. (1990) The effects of haloalkene cysteine conjugates on cytosolic free calcium levels in suspensions of rat renal proximal tubules. J. Biochem. Toxicol. 5, 187-92

Hansen, J., Sallmén, M., Seldén, A.I., Anttila, A., Pukkala, E., Andersson, K., Bryngelsson, I.L., Raaschou-Nielsen, O., Olsen, J.H., and McLaughlin, J.K. (2013) Risk of cancer among workers exposed to trichloroethylene: analysis of three Nordic cohort studies. J. Natl. Cancer Inst. 105, 869-77.

Henschler, D., Vamvakas, S., Lammert, M., Dekant, W., Kraus, B., Thomas, B., and Ulm, K.(1995) Increased incidence of renal cell tumors in a cohort of cardboard workers exposed to trichloroethene. Arch. Toxicol. 69, 291-9

Irving, R.M., Pinkerton, M.E., and Elfarra, A.A. (2013) Characterization of the chemical reactivity and nephrotoxicity of N-acetyl-S-(1,2-dichlorovinyl)-L-cysteine sulfoxide, a potential reactive metabolite of trichloroethylene. Toxicol. Appl. Pharmacol. 267,1-10

Irving, R.M.,and Elfarra, A.A.(2013) Mutagenicity of the cysteine S-conjugate sulfoxides of trichloroethylene and tetrachloroethylene in the Ames test. Toxicology 306, 157-61

Karami, S., Lan, Q., Rothman, N., Stewart, .PA., Lee, K.M., Vermeulen, R., and Moore, L.E.(2012) Occupational trichloroethylene exposure and kidney cancer risk: a meta-analysis. Occup. Environ. Med. 69, 858-67

Kelsh, M.A., Alexander, D.D., Mink, P.J., and Mandel, J.H (2010) Occupational trichloroethylene exposure and kidney cancer: a meta-analysis. Epidemiology 21, 95-102

Kondo, H., Osborne, M., Kolhouse, J., Binder, M., Podell, E., Utley, C., Abrams, R and Allen, R. (1981) Nitrous oxide has multiple deleterious effects on cobalamin metabolism and causes decreases in activities of both mammalian cobalamin dependant enzymes in rats. J. Clin. Invest. 67, 1270-1283

Lash, L.H., Sausen, P.J., Duescher, R.J., Cooley, A.J., and Elfarra, A.A.(1994) Roles of cysteine conjugate beta-lyase and S-oxidase in nephrotoxicity: studies with S-(1,2-dichlorovinyl)-L-cysteine and S-(1,2-dichlorovinyl)-L-cysteine sulfoxide. J. Pharmacol. Exp. Ther. 269, 374-83

Lock, E.A and Reed, C.J. (2006).Trichloroethylene: Mechanisms of renal toxicity and renal cancer and relevance to risk assessment. Toxicol. Sci. 91, 313-331.

Lock, E.A. Cottrell, L., Soames, A., Jacobsen, M and Williams, R. (2004) Formic acid excretion in rats and mice exposed to bromodichloromethane: a possible link to renal tubule cell proliferation in long-term studies. Arch. Toxicol. 78, 410-417.

Miller, J.C. and Miller J.N. (Eds.), Statistics for Analytical Chemistry, PTR Prentice Hall/Ellis Horwood, New York, (1993)

Moore, L.E., Boffetta, P., Karami, S., Brennan, P., Stewart, P.S., Hung, R., Zaridze, D., Matveev, V., Janout, V., Kollarova, H., Bencko, V., Navratilova, M., Szeszenia-Dabrowska, N., Mates, D., Gromiec, J., Holcatova, I., Merino, M., Chanock, S., Chow, W.H., and Rothman, N. (2010) Occupational trichloroethylene exposure and renal carcinoma risk: evidence of genetic susceptibility by reductive metabolism gene variants. Cancer Res. 70, 6527-36.

Prout, M.S. and Green, T. (1985) Species differences in response to trichloroethylene, II. Biotransformation in rats and mice. Toxicol. Appl. Pharmacol. 79, 401-4011.

Prout, M.S., Provan, W.M., and Green, T (1985) Species differences in response to trichloroethylene. I. Pharmacokinetics in rats and mice. Toxicol. Appl. Pharmacol. 79, 389-400

Scott, C.S., and Jinot, J. (2011) Trichloroethylene and cancer: systematic and quantitative review of epidemiologic evidence for identifying hazards. Int. J. Environ. Res. Public Health. 8, 4238-72

Spirtas, R., Stewart, P.A., Lee, J.S., Marano, D.E., Forbes, C.D., Grauman, D.J., Pettigrew, H.M., Blair, A., Hoover, R.N., and Cohen, J.L.(1991) Retrospective cohort mortality study of workers at an aircraft maintenance facility. I. Epidemiological results. Br. J. Ind. Med. 48, 515-30

Stonard, M.D., and Parker, V.H.(1971) 2-Oxoacid dehydrogenases of rat liver mitochondria as the site of action of S-(1,2 dichlorovinyl)-L-cysteine and S-(1,2-dichlorovinyl)-3-mercaptopropionic acid. Biochem. Pharmacol. 20, 2417-27

Stott, W.T. Quast, J.F., and Watanabe, P.G. (1982) The pharmacokinetics and macromolecular interactions of trichloroethylene in mice and rats. Toxicol. Appl. Pharmacol. 62, 137-151

Vaidya, V.S., Shankar K., Lock, E.A., Bucci, T.J., and Mehendale, H.M.(2003) Renal injury and repair following S-1, 2 dichlorovinyl-L-cysteine administration to mice. Toxicol. Appl. Pharmacol. 188, 110-21

Vaidya, V.S., Ramirez, V., Ichimura, T., Bobadilla, N.A., and Bonventre, J.V. (2006) Urinary kidney injury molecule-1: a sensitive quantitative biomarker for early detection of kidney tubular injury. Am. J. Physiol. Renal Physiol.290, F517-29

Wijsman, J.H., Van Dierendonck, J.H., Keijzer, R., van de Velde, C.J., and Cornelisse, C.J. (1992) Immunoreactivity of proliferating cell nuclear antigen compared with bromodeoxyuridine incorporation in normal and neoplastic rat tissue. J Pathol. 168, 75-83.

Yaqoob, N., Evans, A.R., and Lock, E.A. (2013) Trichloroethylene-induced formic aciduria: Effect of dose, sex and strain of rat. Toxicology 304, 49-56.

Legends to Figures

Figure 1. Liver and kidney weights of male F-344 rats given daily doses of corn oil or TCE-OH or TCE for 12 weeks

Three groups of male F-344 rats were given oral doses of either corn oil, TCE-OH (100mg/kg/day) or TCE (500mg/kg/day) daily for 12 weeks. The rats were killed 24h after the last dose and liver and kidneys removed and weighed. Liver and kidney weights are expressed as a percentage of bodyweight. Values are mean ± SEM, n=5
*P<0.05 statistically significantly different from corn oil alone.

Figure 2. Urine ^{1}H NMR spectra of male F-344 rat given daily doses of corn oil, TCE-OH or TCE for 12 weeks

Three groups of male F-344 rats were given oral doses of either corn oil, TCE-OH or TCE daily for 12 weeks. Urine was collected at 8 time points during 12 weeks of dosing and analysed using ^{1}H NMR spectroscopy. TSP was used as the standard reference its signal appears at 0ppm.

Figure 3. Time dependent effect of exposure to TCE and TCE-OH on the urinary excretion of formic acid over 12 weeks

Urinary excretion of formic acid was measured by ^{1}H NMR at 8 time points during 12 weeks exposure. The results were analyzed by 2-way analysis of variance with repeated time measures. Both chemicals caused a statistically significant increase in formic acid excretion which was time $P<0.008$ and treatment $P<0.001$ related.

Table 1

The effect of daily oral dosing of TCE or TCE-OH on urinary pH and the excretion of formic acid and methylmalonic acid over 12 weeks

Time after dosing	Control	TCE-OH	TCE	Control	TCE-OH	TCE	Control	TCE-OH	TCE
	Urinary pH			Urinary formic acid (mg/16h)			Urinary methylmalonic acid (mg/16h)		
1 day	7.05 ± 0.02	6.99 ± 0.03	6.90 ± 0.02	0.66 ± 0.13	0.93 ± 0.27	1.12 ± 0.26	1.93 ± 0.35	1.03 ±0.16	1.83 ± 0.27
1 week	6.52 ± 0.05	6.34 ± 0.04	6.25 ± 0.02	0.56 ± 0.10	3.4 ± 1.6	4.00 ± 1.3	1.33 ± 0.38	0.83 ± 0.31	2.16 ± 0.26
2 weeks	6.57 ± 0.11	6.30 ±0.05	6.24 ± 0.07*	0.63 ± 0.14	23.2 ± 2.7*	13.9 ± 4.3*	1.13 ± 0.23	2.16 ± 0.76	2.35 ± 0.34
5 weeks	6.39 ± 0.06	5.95 ± 0.06*	6.16 ± 0.10	0.43 ± 0.03	55.2 ±12.2*	39.7 ± 3.3*	1.12 ± 0.08	1.88 ± 0.27*+	3.01 ± 0.10*
6 weeks	6.40 ± 0.07	6.21 ± 0.13	6.23 ± 0.12	0.50 ± 0.22	36.8 ± 7.7*+	9.1 ± 3.5	0.82 ± 0.22	2.13 ± 0.27*	2.66 ± 0.19*
8 weeks	6.47 ± 0.02	6.20 ± 0.12	6.23 ± 0.12	0.38 ± 0.06	35.5 ± 10.2*	23.5 ± 7.4	1.08 ± 0.24	1.81 ± 0.54	2.39 ± 0.62
10 weeks	6.64 ± 0.28	6.42 ± 0.05	6.17 ± 0.04	0.61 ± 0.18	54.8 ± 10.8*+	26.0 ± 5.6	0.97 ± 0.26	3.14 ± 0.77*	2.35 ± 0.38
12 weeks	6.54 ± 0.16	6.30 ± 0.09	6.06 ± 0.05*	0.96 ± 0.29	45.6 ± 9.1*	48.9 ± 10.6*	1.14 ± 0.20	2.24 ± 0.26*	2.72 ± 0.34*

Male F344 rats were dosed orally with corn oil (control) or TCE or TCE-OH in corn oil at 500mg/kg/day or 100mg/kg/day respectively and returned to their home cage. During late afternoon they were transferred to metabolism cages and urine collected for 16h overnight at the times shown and then returned to their home cage. Results are Mean ± SEM n=4/group.* statistically significant from control at the same time point. + statistically significant from TCE at the same time point. Statistical analysis was also made using 2-way ANOVA with repeated measures for time and treatment and the degree of significance is reported in the text.

Table 2 The effect of daily oral dosing of TCE or TCE-OH on urinary excretion of protein, Kim-1 and N-acetyl-β-D-glucosaminidase over 12 week

Time after dosing	Control	TCE-OH	TCE	Control	TCE-OH	TCE	Control	TCE-OH	TCE
	Urinary protein (mg/16h)			Urinary Kim-1 (pg/16h)			Urinary NAG (mU/16h)		
1 day	5.3 ± 0.8	4.5 ± 0.51^{+}	7.58 ± 0.31*	689 ± 188	475 ± 150	709 ± 83	22.8 ± 4.6	14.0 ± 2.7	18.9 ± 1.8
1 week	4.80 ± 1.07	4.85 ± 0.8	7.25± 0.77	811 ± 97	601 ± 85^{+}	1548 ± 142*	19.6 ± 3.8	17.1 ± 4.4^{+}	44.0 ± 3.3*
2 weeks	3.22 ± 0.36	7.53 ±1.26^{+}	14.28±1.38*	542 ± 43	458 ± 20	1025 ± 262	22.3 ± 3.5	40.1 ± 8.9	46.4 ± 11.4
5 weeks	5.88 ± 1.34	5.43 ± 0.63	8.18 ± 0.17	344 ± 172	582 ± 120^{+}	1487 ± 286*	20.7 ± 1.1	21.7 ± 3.4^{+}	35.6 ± 3.3*
6 weeks	3.09 ± 1.55	5.05 ± 0.78	6.13 ± 1.83	576 ± 125	414 ± 53	413 ± 101	27.2 ± 3.9	32.3 ± 8.8	36.7 ± 5.4
8 weeks	4.45 ± 0.39	5.75 ± 1.48	6.50 ± 1.69	581 ± 95	1095 ± 166	1337 ± 644	29.3 ± 3.3	32.0 ± 7.6	42.1 ±9.7
10 weeks	6.98 ± 2.14	6.08 ± 1.66	5.80 ± 0.83	750 ± 205	560 ± 90	1100 ± 400	25.4 ± 6.4	30.5 ± 2.9^{+}	59.1 ± 6.5*
12 weeks	6.78 ± 0.68	6.05 ± 0.85	8.57 ± 1.96	745 ± 310	400 ± 110	405 ± 285	27.3 ± 3.2	30.4 ± 2.4	35.4 ± 4.8

Male F344 rats were dosed orally with corn oil (control) or TCE or TCE-OH in corn oil at 500mg/kg/day or 100mg/kg/day respectively and returned to their home cage. During late afternoon they were transferred to metabolism cages and urine collected for 16h overnight at the times shown and then returned to their home cage. Results are Mean ± SEM n=4/group.* statistically significant from control at the same time point. $^{+}$ statistically significant from TCE at the same time point. Statistical analysis was also made using 2-way ANOVA with repeated measures for time and treatment and the degree of significance is reported in the text.

Table 3

The effect of daily oral dosing of TCE or TCE-OH on plasma creatinine, urea and formic acid and hepatic methionine synthase activity 12 weeks after exposure

Measurement	Control	TCE-OH	TCE
Plasma creatinine (mg/dl)	0.89 ± 0.09	1.02 ± 0.04	1.22 ± 0.24
Plasma urea (mg/dl)	32.8 ± 2.6	32.4 ± 3.0	36.8 ± 2.6
Plasma formic acid (µg/ml)	10.0 ± 0.3	101.0^{*+} ± 2.4	62.4* ± 16.2
Hepatic methionine synthase activity (pmol/min/mg protein)	2.78 ± 0.23	1.58* ± 0.17	1.50* ± 0.11
Renal pathology (severity grade/animal)			
Incidence of basophilic tubules	1, 2, 0, 2, 2	2, 2, 2, 2, 2	1, 0,1, 0, 0
S2 tubular degeneration/intra-cytoplasmic hyaline inclusions	2, 2, 2, 2, 2	2, 2, 2, 2, 2	0, 0, 0, 0, 0
Luminal tubular dilatation with protein	1, 2, 0, 1, 0	0, 0, 0, 1, 1	0, 0, 0, 0, 2
Renal proximal tubule labelling index (% BrDU positive cells)	4.76 ± 0.29	2.61* ± 0.45	1.39* ± 0.18

Male F344 rats were given orally either corn oil (control) or TCE or TCE-OH in corn oil at 500mg/kg/day or 100mg/kg/day respectively for 12 weeks. Five days before the last dose Altzet mini pumps containing BrDU were inserted under the skin at the nape, then 24h after the last dose killed by a rising concentration of carbon

dioxide. Blood was collected by cardiac puncture into heparinised tubes and a sample of liver rapidly removed and frozen at -80C and a kidney removed and fixed as described in the Methods. Light microscopic changes were graded 0, **No abnormality** detected; 1, **Minimal** (Very Slight): A histopathologic change ranging from inconspicuous to barely noticeable but so minor, small, or infrequent as to warrant no more than the least assignable grade 2. **Mild** (Slight): A histopathologic change that is a readily noticeable but not a prominent feature of the tissue and/or may be considered to be of no functional consequence.

Results are Mean ± SEM with 5rats/group. *Statistically significantly different from control $P<0.05$. $^{+}$ Statistically significantly different from TCE, $P<0.05$