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*S*-(1,2,2-Trichlorovinyl)-L-Cysteine Sulfoxide, a Reactive Metabolite of *S*-(1,2,2-Trichlorovinyl)-L-Cysteine Formed in Rat Liver and Kidney Microsomes, is a Potent Nephrotoxicant

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Running title:  Nephrotoxicity of TCVC and its corresponding sulfoxide

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Abbreviations:  AOAA, aminooxyacetic acid; DTNB, 5,5'-dithiobis(2-nitrobenzoic acid); TFA,

trifluoroacetic acid; HPLC, high pressure liquid chromatography; BUN, blood urea nitrogen; β-

lyase, cysteine conjugate β-lyase; FMO, flavin-containing monooxygenase; TCVC, $S$-(1,2,2-

trichlorovinyl)-L-cysteine; TCVCS, $S$-(1,2,2-trichlorovinyl)-L-cysteine sulfoxide; TCVG, $S$-

(1,2,2-trichlorovinyl)glutathione; ACN, acetonitrile; NPT, nonprotein thiol; GGTP, γ-glutamyl

transpeptidase; SGPT, serum glutamic pyruvic transaminase; SGOT, serum glutamate-

oxalacetate transaminase; TETRA, tetrachloroethylene or tetrachloroethene.

## ABSTRACT

Previously, we have provided evidence that cytochrome P450s and flavin-containing monooxygenases (FMOs) are involved in the oxidation of *S*-(1,2,2-trichlorovinyl)-L-cysteine (TCVC) in rabbit liver microsomes to yield the reactive metabolite TCVC sulfoxide (TCVCS). Because TCVC is a known nephrotoxic metabolite of tetrachloroethylene, the nephrotoxic potential of TCVCS in rats and TCVCS formation in rat liver and kidney microsomes were investigated. At 5 mM TCVC, rat liver microsomes formed TCVCS at a rate nearly five times higher than the rate measured with rat kidney microsomes, whereas at 1 mM TCVC only the liver activity was detectable. TCVCS formation in liver and kidney microsomes was dependent upon the presence of NADPH and was inhibited by the addition of methimazole or 1-benzylimidazole, but not superoxide dismutase, catalase, KCN, or deferoxamine consistent with the involvement of both FMOs and P450s. Rats given TCVCS (230 µmol/kg i.p.) exhibited acute tubular necrosis at 2 and 24 h after treatment and had elevated blood urea nitrogen levels at 24 h, whereas TCVC was a much less potent nephrotoxicant than TCVCS. Furthermore, pretreatment with aminooxyacetic acid enhanced TCVC toxicity. In addition, reduced non-protein thiol concentrations in the kidney were decreased by nearly 50% 2 h after TCVCS treatment compared to saline treated rats whereas the equimolar dose of TCVC had no effect on kidney non-protein thiol status. No significant lesions or changes in non-protein thiol status were observed in liver with either TCVC or TCVCS. Collectively, the results suggest that TCVCS may play a role in TCVC-induced nephrotoxicity.

## INTRODUCTION

Tetrachloroethylene (TETRA), a widely used dry cleaning solvent and metal degreaser, is a common surface and ground water pollutant, air pollutant, and Superfund site contaminant. TETRA is a known rodent liver and kidney toxicant. Long-term exposure studies have also identified TETRA as a liver carcinogen in mice (Anna et al., 1994; NTP 1986) and as a kidney carcinogen in rats (Green et al., 1990; Dekant et al., 1986; NTP, 1986).   The International Agency for Research on Cancer (IARC) considers TETRA as "probably carcinogenic to humans (group 2A)" (IARC, 1995).  Increases in leukemias and cancers of the bladder, esophagus, and cervix have been observed in workers in the dry-cleaning industry (IARC, 1995).  Additional epidemiological studies have shown increases in kidney cancers (Antilla, et al, 1995; Katz and Jowett, 1981; Duh and Asal, 1984). Studies of workers exposed to low concentrations of TETRA have reported increased urinary levels of lysozyme, albumin, β-glucuronidase, and the brush border membrane antigens B50, BBA, and HF5 (Franchini et al., 1983; Mutti et al., 1992).  Another study of Dutch workers exposed to TETRA at levels well below the Dutch occupational exposure limit (7.9 mg/m$^3$ versus 240 mg/m$^3$) showed increased levels of retinol-binding protein compared to controls (Verplanke, et al., 1999).  Collectively, these results indicate that exposure to TETRA may cause renal tubular toxicity in humans.

Whereas TETRA is metabolized by both cytochrome P450s and glutathione *S*-transferases, only the latter enzymes have been implicated in the formation of nephrotoxic and nephrocarcinogenic metabolites (Dekant et al., 1986, 1987; Lash et al., 1998; Lash and Parker, 2001).  The glutathione conjugation of TETRA occurs primarily in the liver and results in the

formation of $S$-(1,2,2-trichlorovinyl)glutathione (TCVG), which has been shown to be more toxic to isolated kidney cells from male rats than TETRA (Lash et al., 2002). TCVG can be translocated to the kidneys via the circulation. In kidney cells, TCVG can be cleaved to the corresponding cysteine $S$-conjugate, $S$-(1,2,2-trichlorovinyl)-L-cysteine (TCVC, Fig. 1) by γ-glutamyl transpeptidase (GGTP) and dipeptidase. Hepatic TCVG may also be excreted in bile and be converted to TCVC by enzymes present in biliary epithelial cells and intestinal microflora before it can be reabsorbed and concentrated in kidney cells. The relevance of this metabolic pathway to humans was evidenced by the detection of the mercapturic acid $N$-acetyl-$S$-(1,2,2-trichlorovinyl)-L-cysteine in the urine of humans exposed to TETRA (Birner et al., 1996; Völkel, et al., 1998). In this regard, the mercapturic acid of TCVC was presumably formed from TCVC by hepatic and/or renal $N$-acetyltransferases.

Two additional TCVC metabolic reactions have been characterized. The first results in cleavage of TCVC by cysteine S-conjugate β-lyases (β-lyases), present in kidney cytosol and mitochondria, to produce a sulfur species (Fig. 1) that readily loses chloride ion to form dichlorothioketene. Formation of dichlorothioketene has been implicated in TETRA-induced nephrotoxicity and nephrocarcinogenicity, because of its reactivity toward renal macromolecules (Dekant et al., 1988; Vamvakas et al., 1989a; Vamvakas et al., 1989b; Pähler et al., 1999). In the second metabolic reaction, TCVC is oxidized by flavin-containing monooxygenases (FMOs) or cytochrome P450s to form $S$-(1,2,2-trichlorovinyl)-L-cysteine sulfoxide (TCVCS) (Ripp et al., 1997). In rabbit liver microsomes, the TCVC $S$-oxidase activity was dependent on the presence of NADPH, was stereoselective (diastereomer 2 comprising 65-100% of the combined sulfoxide peak area), and was inhibited by the inclusion of methimazole, an alternative FMO substrate, or

1-benzylimidazole, a generic cytochrome P450 inhibitor.  Using cDNA-expressed rabbit FMO

isozymes, TCVC has been shown to be a substrate for FMO3 but not for FMO1, FMO2 or

FMO5.  Because TCVCS reacts readily with glutathione ($t_{1/2}$=20 min) in vitro whereas TCVC is

unreactive (Ripp et al., 1997), TCVCS formation has been implicated in TCVC-induced

nephrotoxicity.


        In the current study, we characterized the ability of rat liver and kidney microsomes to

metabolize TCVC to TCVCS in vitro. We also investigated TCVCS nephrotoxicity in rats in

vivo and determined the effect of aminooxyacetic acid (AOAA), a potent β-lyase inhibitor, on

TCVC toxicity.  Furthermore, the effects of TCVC and TCVCS on hepatic and renal NPT status

were also examined.  Collectively, these experiments were designed to clarify the potential roles

of *S*-oxidases and β-lyases in TCVC-induced nephrotoxicity.

## METHODS

**Materials:** TETRA, L-cysteine, AOAA, catalase, superoxide dismutase, deferoxamine, and 5,5'-dithiobis(2-nitrobenzoic acid) were purchased from Sigma Chemical Co (St Louis, MO). Kits to determine serum glutamate-pyruvate transaminase (SGPT), serum glutamate-oxalacetate transaminase (SGOT), and urinary GGTP activities, and serum and urinary glucose and blood urea nitrogen (BUN) levels were also obtained from Sigma. Methimazole, 1-benzylimidazole, trifluoroacetic acid (TFA), and NaBH$_4$ were purchased from Aldrich Chemical Co. (Milwaukee, WI). HPLC-grade acetonitrile (ACN) was obtained from EM Science (Gibbstown, NJ). TCVC and TCVCS were prepared as previously described (Ripp et al., 1997) and purity was >95% as determined by HPLC. All other chemicals and reagents were of the highest grade commercially available.

**Microsomal preparations and incubations:** Unless otherwise stated, the buffer used in all experiments was 0.1 M KH$_2$PO$_4$, 0.1 M KCl, and 5 mM EDTA, pH 7.4. "Washed" microsomes were prepared as described previously (Sausen and Elfarra, 1990) from livers and kidneys of male Sprague-Dawley rats (225-250 g) obtained from Sasco (Omaha, NE). The microsomal protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard.

Microsomal incubations (the final incubation volume was 0.5 ml) typically were carried out as follows: microsomal suspensions (0.3 - 0.7 mg protein suspended in the incubation buffer described above) were pre-incubated in the presence or absence (controls) of 1.5 mM NADPH

for 5 min at 37°C in a Dubnoff shaking water bath (Precision Scientific, Chicago, IL).  TCVC

was dissolved in incubation buffer with the aid of a few drops of 0.1N NaOH (pH 8.0). TCVC

(150 µl) was added to the enzymatic incubation to start the metabolic reaction; the pH of the

reaction mixture was approximately pH 7.6 after the addition of TCVC solution (final

concentration - 5 mM). The kidney microsomal experiments were carried out in the presence of

0.1 mM AOAA in order to inhibit any β-lyase-mediated reaction (Lash et al., 1994; Werner et

al., 1996).  To terminate the reaction, ice-cold 0.75% perchloric acid (0.15 ml) was added.  The

samples were centrifuged for 20 min at 4°C at 3000 rpm to remove precipitated proteins in a

Beckman model TJ-6R bench top centrifuge (Beckman Instruments, Palo Alto, CA).  The

supernatants were filtered using 0.2 µm Acrodisc filters and analyzed directly by HPLC

(described below).


When microsomal incubations were carried out in the presence of enzyme inhibitors,

alternative substrates or other reaction modifiers, these compounds were pre-incubated with

NADPH and microsomes for 5 min before 5 mM TCVC (final concentration) was added.  The

reaction time was 30 min. The modifier final concentration in the assay was as follows: 1 mM,

1-benzylimidazole; 1 mM, methimazole; 1 mM, KCN; 10 µM, deferoxamine; 2,800 U/mL,

catalase; 504 U/mL, superoxide dismutase.


**HPLC analyses:** HPLC analyses of supernatants were carried out on a gradient-controlled

Gilson Solvent Module (Gilson Medical Electronics, Inc., Middleton, WI) and Gilson 117

Detector using a Beckman ODS 5 µm reverse phase C-18 column (4.6 mm x 25 cm; Beckman

Instruments, Inc., Fullerton, CA) and a Brownlee Newguard guard column (Rainin Instruments,

Woburn, MA).  The flow rate was 1 ml/min with 20 µl injections made by a Gilson 234 auto

injector.  Pump A had a mobile phase of 1% ACN in water, pH adjusted to 2.5 with TFA, and

pump B had a mobile phase of 75% ACN, pH 2.5 adjusted with TFA.  The detection wavelength

used was 220 nm.  The gradient was as follows: initial 15% B for 5 min then increased to 40%

B over a 3 min period and maintained for 5 min after which it was returned to 15% B over 2.5

min and kept for the remainder of the 20 min analysis period.  This method allowed detection of

the two sulfoxide diastereomers, although their exact chiral identity was not determined.  The

earlier eluting peak of the two diastereomers will be referred to as "diastereomer 1" and the later

eluting peak as "diastereomer 2." Retention times for the compounds of interest were: TCVC,

11.5 min; the two TCVCS diastereomers, 4.5 min and 5.0 min.  Quantitation of enzymatically

produced TCVCS was done by comparing peak areas produced to the corresponding areas of

known concentrations of synthetic TCVCS standards ($r > 0.99$).  The limit of detection of

TCVCS was 25 pmol/20 µl injection .  Data was corrected for non-enzymatic activity (trace

amounts) by subtracting the peak areas of the 0 min and/or -NADPH controls.

**In vivo toxicity studies:** Male Sprague-Dawley rats (175-220 g; Sasco, NE) were housed in

individual plastic metabolic cages (Nalge Company, Rochester, NY) in a controlled room on a

12-hr light-dark cycle and were given food (Rodent laboratory chow) and water *ad libitum*.  Rats

were given a single i.p. injection (0.6 mL) of isotonic saline (control),  TCVC or TCVCS (115 or

230 µmol/kg body weight, dissolved in saline along with a few drops of 1 N NaOH and pH

adjusted to approximately 8).  The second series of experiments required a pre-treatment (single

dose i.p.) of saline or AOAA (500 µmol/kg body weight) 30 min prior to the administration of

TCVC (230 μmol/kg).  Two groups of four rats each (total = 8) were used in this study. All rats were sacrificed 24 h after treatment.  Serum was analyzed for BUN levels, a marker for nephrotoxicity, and for SGPT and SGOT activities as markers for liver injury.   Urine samples were also collected and analyzed for GGTP activity as another indicator of renal damage (Scherberich et al., 1974). Furthermore serum and urine samples were analyzed for glucose levels.

In a third series of experiments designed to provide insight into the mechanism of TCVC and TCVCS-induced toxicity, renal and hepatic reduced NPT and NPT disulfide concentrations were measured.  A total of twelve animals divided into three treatment groups of four rats each were used. Briefly, a single injection of saline, TCVC or TCVCS (230 μmol/kg) was given i.p. and the animals were sacrificed 2 h after treatment.  Portions of the kidneys and livers were removed for NPT and NPT disulfide determination according to the method described below.

Evaluation of renal and hepatic damage by histopathological analysis was carried out at the Histopathology facility of the Veterinary Medical Teaching Hospital, University of Wisconsin - Madison. Tissues were fixed in 10% buffered formalin.  After fixation, the tissues were embedded in paraffin, cut, and stained with hematoxylin and eosin (H & E) for qualitative assessment of nephrotoxicity or hepatotoxicity without prior knowledge of the protocol to which each section belonged.  For renal damage, the following criteria were used:  minimal (0-25% of tubules showing necrosis); mild (25-50% of the tubules showing necrosis); moderate (50-75% of tubules showing necrosis); severe (75-100% of tubules showing necrosis).

**Determination of hepatic and renal reduced NPT and NPT disulfides:** Reduced NPT and

NPT disulfides were determined by minor modifications of a previously described method

(Sausen and Elfarra, 1991; Elfarra, et al., 1994).  Briefly, renal or hepatic tissue (1 g) was

homogenized in 10 or 20 volumes of a solution of 0.15 M KCl, 30 mM EDTA, pH 4.3,

respectively.  An aliquot of the homogenate (2 ml) was deproteinized by adding 3 ml of a

solution containing 0.3 g/ml NaCl, 0.017 g/ml metaphosphoric acid and 0.002 g/ml EDTA. The

solution was then centrifuged for 20 min at 3,000 rpm and 4°C, using a Beckman TJ-6 tabletop

centrifuge.  The supernatants were used to determine reduced NPT and NPT disulfides as

described previously (Elfarra et al., 1994).

**Statistics:** All data are expressed as means ± S.D. of measurements made on the indicated

number (n) of microsomal experiments or animals as indicated in the table or figure legends.

Statistical analyses were carried out using SigmaStat (SPSS Inc., Chicago, IL).   Data was

analyzed using t-test or Mann-Whitney rank sum test as appropriate, using $p < 0.05$ as the

criterion for significance.

RESULTS

**Characterization of TCVC oxidation in rat liver and kidney microsomes:**

Incubations of TCVC (5 mM) with male rat liver or kidney microsomes at 37°C and pH 7.4

resulted in detection of two peaks by HPLC, corresponding to the two TCVCS diastereomers;

the late-eluting peak (diastereomer 2) comprised 60-100% of the total TCVCS peak areas with

both rat liver and kidney microsomes.  TCVCS formation was dependent upon incubation time

and the presence of both protein and NADPH (1.5 mM). The results presented in Fig. 2 show

that TCVCS formation was linear for at least 30 min with the rate exhibited by liver microsomes

being nearly five times higher than the rate exhibited by kidney microsomes. At a lower TCVC

concentration (1 mM), TCVCS formation was detectable only with the liver microsomes (data

not shown).  Addition of superoxide dismutase, catalase, KCN (peroxidase inhibitor) or

deferoxamine (iron chelator) did not significantly inhibit DCVCS formation, whereas inclusion

of 1-benzylimidazole (cytochrome P450 inhibitor) or methimazole (FMO alternate substrate)

almost totally inhibited TCVC oxidation (Fig. 3), consistent with involvement of P450s and

FMOs in the TCVC *S*-oxidase activity of rat liver microsomes. Methimazole or 1-

benzylimidazole also inhibited (by nearly 80-90%) TCVC oxidation in rat kidney microsomes

(data not shown).

**Acute in vivo toxicity of TCVC and TCVCS:**

*Physical Observations and Urine Output:* Rats given TCVC or TCVCS at the 115 µmol/kg dose

remained physically active throughout the 24 hr study, where rats treated with TCVC or TCVCS

at the 230 µmol/kg dose appeared lethargic within 1 hr after dosing. The TCVC-treated rats

exhibited improved physical condition with time until they were sacrificed 24 hr post treatment,

whereas the physical condition of the TCVCS-treated rats worsened with time (they exhibited

recumbency and their hind limbs appeared paralyzed) until 3 hr post treatment when their

physical condition improved until they were sacrificed 24 h post treatment. Rats given 230

µmol/kg TCVC excreted significantly less urine ($9.9 \pm 6.3$ ml; mean $\pm$ SD) over the 24 h time

period relative to control rats ($18.9 \pm 7.1$ ml). However, the urine volumes of all other treatment

groups were not statistically different from that of control rats.

*Morphology:*  The liver and kidneys of rats given 115 µmol/kg TCVC or TCVCS, the livers and

kidneys of rats given 230 µmol/kg TCVC, and the liver of rats given 230 µmol/kg TCVCS

appeared morphologically normal. However, the kidneys of rats given 230 µmol/kg TCVCS

showed visible signs of necrosis.  Morphologically, the latter kidneys appeared normal in size

compared to control but were tan colored and when cut, there were bloody lesions in the

medulla.

*Histology:*  Histological examination of the liver sections from rats treated with both TCVC and

TCVCS were normal and did not exhibit any lesions at the two doses given.  In contrast, kidneys

from rats treated with the 115 µmol/kg dose of TCVC or TCVCS showed slight to mild acute

tubular necrosis (data not shown).  Kidneys taken 2 h post treatment from rats given TCVC (230

µmol/kg) exhibited scattered foci of mild acute tubular necrosis (Fig. 4B) as compared to saline-

treated animals (Fig. 4A), whereas kidneys from rats given 230 µmol/kg TCVCS showed

widespread acute tubular necrosis, intratubular casts, and interstitial congestion and hemorrhage

(Fig. 4C). At 24 h post treatment, rats given TCVC (230 µmol/kg) showed mild to moderate

acute tubular necrosis whereas rats given the equimolar dose of TCVCS showed severe tubular

necrosis (data not shown). Collectively, these histopathological results show that TCVCS

nephrotoxicity occurs faster than that of TCVC and suggest TCVCS is also a more potent

nephrotoxicant.

*Clinical Chemistry:* All the biochemical parameters examined from rats given 115 µmol/kg

TCVC or TCVCS were similar to the corresponding parameters from control rats, except for the

SGPT levels in the TCVCS-treated animals that were significantly lower than in the saline-

treated controls (Table 1). Consistent with the histopathological results described above, rats

given 230 µmol/kg TCVCS exhibited a nearly 4-fold significant increase in BUN levels relative

to saline-treated rats, whereas rats treated with the equimolar dose of TCVC did not exhibit a

significant elevation in BUN levels (Table 1). Serum glucose levels were unaffected in rats

treated with TCVC and TCVCS whereas urine glucose levels in treated rats varied greatly

among the treated animals. Three of the eight rats treated with 230 µmol/kg TCVC and four of

eight rats treated with the equimolar dose of TCVCS excreted >50 mg glucose within 24 h post

treatment whereas control animals excreted only $0.9 \pm 0.7$ mg glucose within 24 h. Large

variability was also observed in GGTP levels with five of eight rats given 230 µmol/kg TCVC

and four of eight rats given the equimolar dose of TCVCS showing GGTP levels that were more

than 3-fold higher than the average control value ($3.4 \pm 1.4$ U). Interestingly, most of the TCVC

and TCVCS-treated animals that excreted >50 mg glucose also had elevated GGTP levels and

exhibited moderate to severe kidney lesions whereas the animals that did not excrete high levels

of glucose and GGTP exhibited minimal to mild tubular necrosis. The SGOT activities were not significantly increased with either TCVC or TCVCS treatment; however there was a significant decrease in SGPT levels in rats given TCVCS, which was similar in magnitude to that observed with the 115 μmol/kg dose.  Collectively, these clinical chemistry results show that kidney injury has occurred at the high dose of both TCVC and TCVCS.  However, the results suggest TCVCS is the more potent nephrotoxicant.

*Hepatic and Renal Thiol Status:*  To help elucidate the mechanism of TCVC and TCVCS toxicity, we examined the effect of TCVC or TCVCS (230 μmol/kg) administration on liver and kidney NPT status. TCVCS treatment significantly decreased the reduced NPT concentration in kidneys to approximately 52% of control values 2 h post-treatment (Table 2).  The equimolar dose of TCVC had no effect on the kidney NPT concentration.  No effect was observed on the NPT disulfides in the kidney and there was no significant alteration of NPT status in liver by either TCVC or TCVCS.

*AOAA Pre-Treatment:*  The effect of the β-lyase inhibitor, AOAA, on TCVC toxicity at the 230 μmol/kg dose was examined.  Pre-treatment of rats with AOAA 30 min before TCVC treatment resulted in increased toxicity as one of the AOAA-TCVC treated rats died and another had to be sacrificed before the 24 hr time point whereas animals treated with the same dose of TCVC only showed minimal discomfort.

DISCUSSION

To our knowledge, the results presented in this manuscript are the first to characterize

TCVCS formation in rat liver and kidney microsomes. In this regard, both rat liver and kidney

microsomes were able to produce TCVCS from TCVC at physiological conditions (pH 7.4,

37ºC). However, both of these activities diminished as the TCVC concentration was lowered

from 5 mM to 1 mM, suggesting any role for TCVCS in TCVC-induced toxicity will likely

increase as the hepatic and renal TCVC concentrations are increased. Because of its ability to

concentrate tubular fluid and the presence of active transport carriers for cysteine $S$-conjugates

along the nephron, any TCVC or TCVCS present in the circulation is likely to get concentrated

in tubular cells to selectively predispose these cells to TCVC and TCVCS-induced toxicities.

Evidence for this hypothesis is provided by the selective nephrotoxicity of both TCVC and

TCVCS after i.p.administration (Table 1 and Fig. 4), and the selective depletion of renal NPT

after rats were given TCVCS (Table 2). The findings that TCVC oxidation was NADPH-

dependent, stereoselective, and was inhibited by methimazole or 1-benzylimidazole are

consistent with the involvement of FMO3 and P450s in this sulfoxidation reaction (Ripp et al.,

1997; Ripp et al., 1999; Krause et al., 2003). $N$-Acetyl-$S$-(1,2,2-trichlorovinyl)-L-cysteine, the

mercapturic acid of TCVC, is metabolized to its corresponding sulfoxide by the isozymes of the

cytochrome P450 3A family (Werner et al.,1996).

The results presented in this manuscript are also the first to characterize the in vivo

toxicity of TCVCS in any species. The clinical chemistry, urine output, and histopathological

results (Table 1 and Fig. 4) revealed a dose-and time-dependent toxicity for TCVCS. The results

also show the kidney was the primary target organ of TCVCS toxicity and that TCVCS was a

more potent nephrotoxicant than TCVC as demonstrated by the significant increase in BUN levels in the TCVCS-treated animals. Increases in BUN levels are consistent with a drop in glomerular filtration rate secondary to either vasoconstriction or direct tubular damage or both. Urine glucose and GGTP levels were not significantly elevated in either TCVC or TCVCS-treated animals (230 µmol/kg) but a large variability in the animal response was observed. Four of four TCVCS-treated rats and three of three TCVC-treated rats that excreted >50 mg glucose had elevated GGTP levels. These increases in glucose excretion and GGTP levels are consistent with tubular damage as was observed histopathologically. Collectively, these results show that whereas both TCVC and TCVCS can induce nephrotoxicity, TCVCS is a more potent nephrotoxin than TCVC. When rats were pre-treated with AOAA before TCVC, they exhibited severe toxicity and two rats out of four died during the course of the 24 hr experiment whereas all rats given the equimolar dose of TCVC did not exhibit severe toxicity signs or death during the course of the experiment. This was unexpected as this AOAA dosing protocol had been found previously protective with other nephrotoxic cysteine $S$-conjugates (Elfarra 1997; Lash et al., 1994; Krause et al., 2002). Because of the potent nephrotoxicity of TCVCS compared to TCVC, the data suggest that inhibiting the β-lyase pathway results in increased toxicity, possibly due to increased formation of TCVCS in rats given AOAA and TCVC. This finding also suggests that even a small flux through the S-oxidase pathway may account for the increased toxic effects seen with TCVC. Interestingly, sulfoxidation was previously shown to increase the toxicity of $N$-acetyl-$S$-(1,2,2-trichlorovinyl)-L-cysteine, the mercapturic acid of TCVC, in rat renal proximal tubular cells in vitro (Werner et al., 1996).

Although TCVCS administration was not associated with any histopathological effects on the liver, a significant decrease in SGPT levels was observed with both the high and low TCVCS doses (Table 1). As TCVCS readily reacts in vitro with GSH ($t_{1/2}$ = 20 min) while TCVC is not reactive (Ripp et al., 1997), TCVCS may have reacted with a reactive nucleophilic group on the enzyme causing inhibition of the SGPT activity. A similar decrease in SGPT activity was reported after rats were treated with methidathion (Altuntas et al., 2002). The temporary loss of motor activity in the lower limbs of rats treated with TCVCS could also have been mediated through direct effects of TCVCS on the lower part of the spinal cord, ion channels or cholinergic receptors in muscles secondary to diffusion of some TCVCS after its i.p. administration.

The in vivo results observed with TCVCS are for the most part similar to the results that we have previously reported with *S*-(1,2-dichlorovinyl)-L-cysteine sulfoxide (DCVCS, Sausen and Elfarra, 1991; Lash et al., 1994). While TCVCS was a potent nephrotoxin similar to DCVCS (Lash et al., 1994), TCVCS depleted kidney NPT and had no effect on liver NPT status, whereas DCVCS depleted NPT in both the liver and kidney. These differences between TCVCS and DCVCS could be explained by the lower reactivity of TCVCS towards biological nucleophiles in comparison with DCVCS (Ripp et al., 1997) which may have allowed TCVCS to be more selective for the kidneys.

Although we did not characterize TCVC metabolism by human FMOs, we have previously shown that human FMOs exhibit kinetic values with L-methionine, *S*-benzyl-L-cysteine and *S*-allyl-L-cysteine similar to those exhibited by rat and rabbit FMOs (Duescher et

al., 1994; Ripp et al., 1997, Ripp et al., 1999). This suggests human FMO3 may also convert TCVC to TCVCS. FMO3 is the major FMO isoform expressed in adult human liver (Ripp et al., 1999) and FMO3 expression has also been shown to vary 5-6-fold in human kidney as determined from a sample size of twenty-six human kidneys (Krause et al., 2003). Because β-lyase activity in human kidney is present at only 10% of the levels present in rat kidney (Lash et al., 1990), bioactivation of TCVC by the β-lyase pathway may be less important in humans, thereby allowing more TCVC to be available for sulfoxidation which would ultimately result in TCVCS possibly playing a larger role in TCVC toxicity in humans.

In conclusion, the data presented in this study demonstrate that TCVCS is a much more potent nephrotoxin than TCVC. Although only a small amount of TCVCS may be formed in the kidney itself after exposure to TETRA, any TCVCS formed in liver could also be translocated to the kidney and cause damage because of its potency as a nephrotoxicant. Collectively, the data provides evidence for a potential role for TCVCS in TCVC–induced nephrotoxicity in rats. As human enzymes have recently been shown to convert DCVC to DCVCS (Krause et al., 2003) and as little as 10 μM DCVCS has been shown to induce cytotoxicity in primary cultures of human renal proximal tubular cells (Lash et al., 2003), TCVC could also be oxidized in human tissues to yield TCVCS, which could then contribute to TCVC toxicity in human cells.

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## ACKNOWLEDGEMENTS

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FOOTNOTES

JPET #120444

## LEGENDS FOR FIGURES

Fig. 1.  Possible pathways for the bioactivation of *S*-(1,2,2-trichlorovinyl)-L-cysteine.

Figure 2. Representative results from time-dependent oxidation of TCVC to TCVCS by male rat liver and kidney microsomes.  Experiments were carried out as described in the Methods section, using 5 mM TCVC and 1.5 mM NADPH.

Figure 3.  Effect of inhibitors, alternative substrates, and oxygen scavengers on TCVCS formation.  All activities were determined as described in the Methods section using 5 mM TCVC, 1.5 mM NADPH and male rat liver microsomes for 30 min.  All activities are corrected for non-NADPH dependent TCVCS formation.  Values (means ± S.D., n = 3) marked with asterisks were significantly different from control (p<0.001).

Figure 4.  Representative histopathological kidney sections (all 200X) from rats treated with A) saline, B) TCVC (230 µmol/kg) or C) TCVCS (230 µmol/kg) and killed 2 h after dosing.  Note the scattered foci of mild acute tubular necrosis (arrows) in B and the widespread acute tubular necrosis (regionally severe in deep cortex and outer stripe of the outer medulla), intratubular casts (arrows), and interstitial congestion and hemorrhage in C.

TABLE 1. Effects of TCVC and TCVC sulfoxide on renal and hepatic function in male Sprague-Dawley rats[a].

| Treatment | Number of animals (n) | BUN (mg/dL) | Serum Glucose (mg/dL) | SGOT (SF U/mL) | SGPT (SF U/mL) | Total Urinary GGTP (U/24 h) | Total Urinary Glucose (mg/24 h) |
|---|---|---|---|---|---|---|---|
| Saline | 7 | 23.1 ± 2.9 | 150.2 ± 22.4 | 135.7 ± 46.4 | 45.5 ± 12.6 | 3.4 ± 1.4 | 0.9 ± 0.7 |
| TCVC | | | | | | | |
| 115 µmol/kg | 4 | 23.0 ± 4.0 | 126.8 ± 10.5 | 111.0 ± 15.9 | 39.0 ± 8.4 | 5.5 ± 1.6 | 1.1 ± 0.8 |
| 230 µmol/kg | 8 | 28.7 ± 10.8 | 148.2 ± 21.2 | 193.6 ± 74.7 | 55.1 ± 20.0 | 13.4 ± 6.7 | 56.0 ± 73.7 |
| TCVCS | | | | | | | |
| 115 µmol/kg | 4 | 25.2 ± 5.0 | 132.7 ± 7.8 | 122.5 ± 23.5 | 21.8 ± 7.1** | 5.1 ± 2.8 | 2.3 ± 3.1 |
| 230 µmol/kg | 8 | 96.1 ± 52.7*** | 153.7 ± 23.7 | 204.8 ± 105.6 | 28.0 ± 10.3* | 10.3 ± 12.0 | 46.2 ± 54.1 |

[a]Male Sprague-Dawley rats (175-225 g) were given a single i.p. injection of isotonic saline, TCVC, or TCVCS.  Animals were killed 24 h later and serum and urine was analyzed as described in the Methods section.  Values presented are means ± S.D. (n = 4-8).

*Significantly different from saline-treated rats; $p < 0.05$ as determined by t-test or Mann-Whitney Rank sum test as appropriate.

**Significantly different from saline-treated rats; $p < 0.01$ as determined by t-test or Mann-Whitney Rank sum test as appropriate.

***Significantly different from saline-treated rats; $p < 0.001$ as determined by t-test or Mann-Whitney Rank sum test as appropriate.

U = One unit is equal to 1 µmole product/min at 25°C.

One SF Unit will form $4.82 \times 10^{-4}$ µmol glutamate/min at pH 7.5 and 25°C.

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TABLE 2. Effects of TCVC and TCVCS (230 µmol/kg) on NPT status in rats 2 h post-treatment[a].

| Treatment | Liver | | Kidney | |
|---|---|---|---|---|
| | Reduced NPT | NPT Disulfides | Reduced NPT | NPT Disulfides |
| | (µmol/g tissue) | (µmol/g tissue) | (µmol/g tissue) | (µmol/g tissue) |
| Saline | $7.9 \pm 1.4$ | $0.4 \pm 0.1$ | $3.4 \pm 0.2$ | $0.1 \pm 0.1$ |
| TCVC | $7.9 \pm 0.8$ | $0.4 \pm 0.2$ | $4.2 \pm 0.8$ | $0.0 \pm 0.0$ |
| TCVCS | $6.5 \pm 1.3$ | $0.3 \pm 0.2$ | $1.8 \pm 0.6^{**}$ | $0.0 \pm 0.0$ |

[a]Male Sprague Dawley rats (160-175 g) were given a single i.p. injection of either saline, TCVC, or TCVCS. Animals were sacrificed 2 h later and NPT concentrations were determined as described in Materials and Methods. Values presented are means ± S.D. (n = 4).
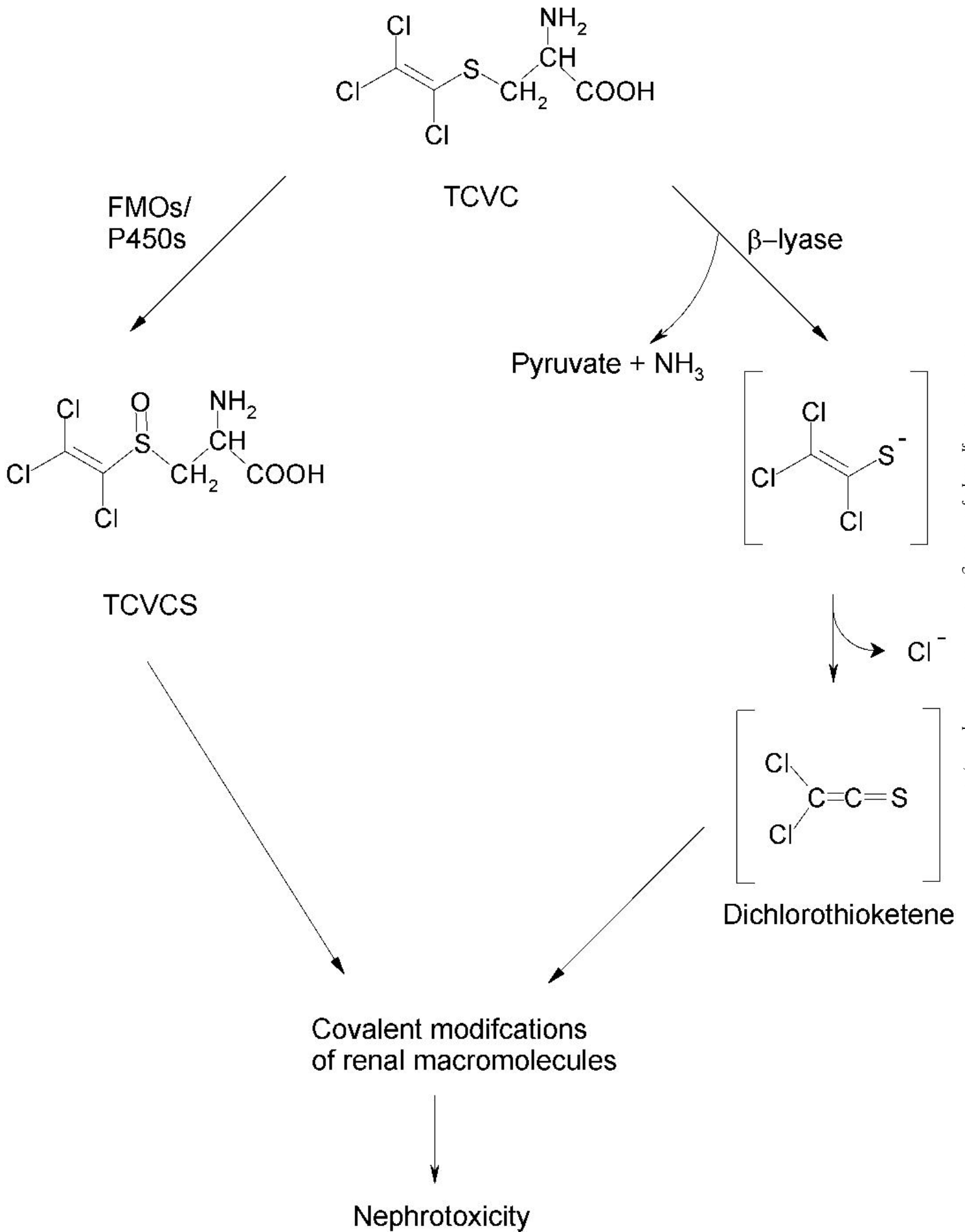**Statistically significant from saline treated rats ($p < 0.01$).

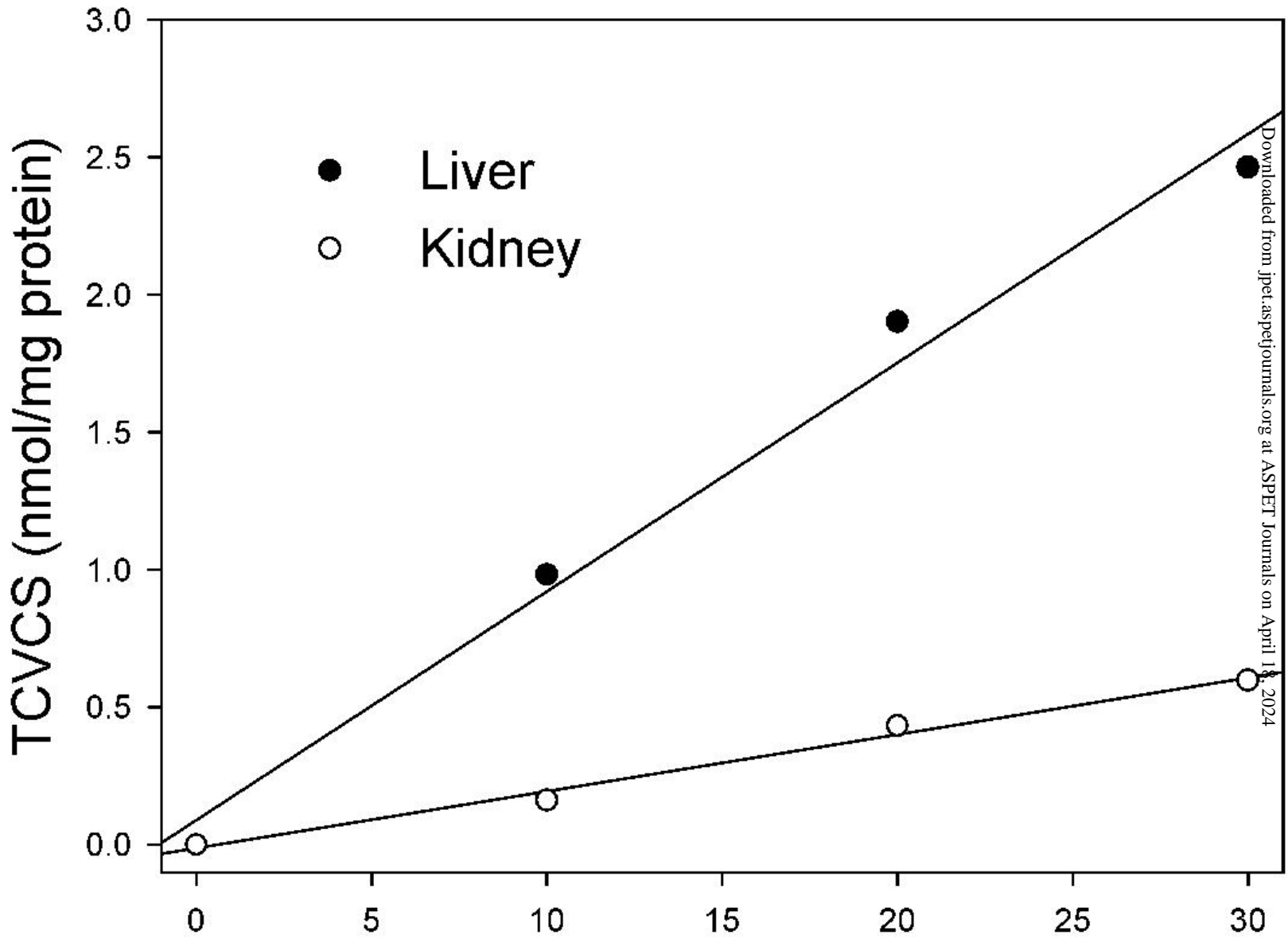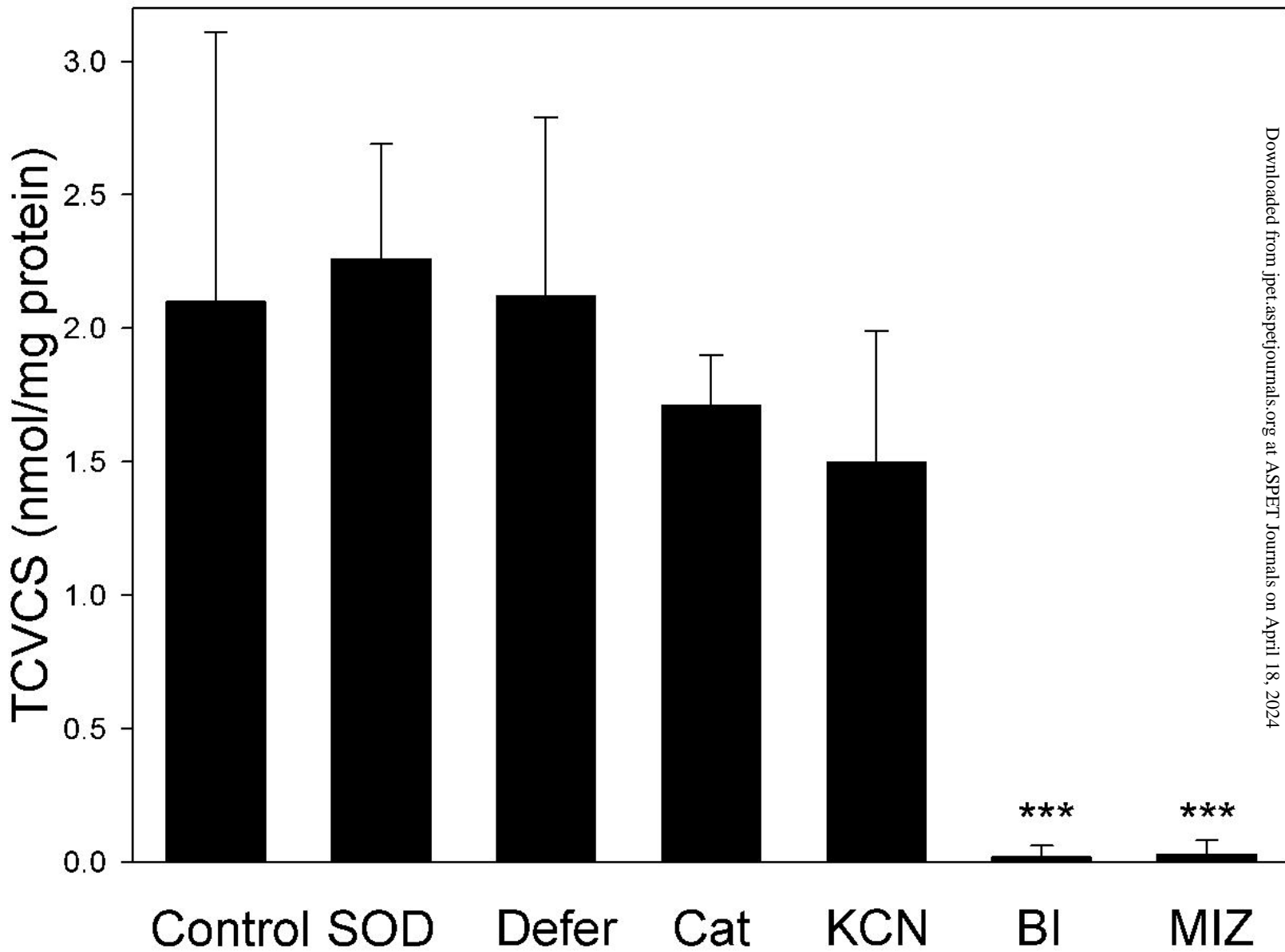# Figure 1

# Figure 2

## Figure 3

# Figure 4