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Different effects of (CIS+TRANS) 1,3-dichloropropene in renal cortical slices derived from male and female rats

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1 Nephrotoxic effects of 1,3-dichloropropene (cis and trans isomers mixture) was investigated *in vitro* by means of renal cortical slice model in male and female rats, including treatment with metabolism modifiers as an inducer of cytochrome P-450 1A class (β-naphthoflavone), a reduced glutathione depleting (DL-buthionine-[S,R]-sulfoximine), an inhibitor of γ-glutamyltransferase (AT-125) and inhibitor of cysteine conjugate β-lyase (aminooxiacetic acid).

2 Dose-dependent decrease of p-aminohippurate uptake was observed in male renal cortical slices. Only the high doses (3.0 and 4.0×10^{-4} M) caused a significant loss of organic anion uptake in females.

3 β-Naphthoflavone and α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125) partially, but significantly, reduced organic anion loss in males. In females, DL-buthionine-[S,R]-sulfoximine significantly increased in females but in males loss of organic anion accumulation caused by 1,3-dichloropropene. Aminooxyacetic acid did not ameliorate 1,3 D effects *in vivo* and *in vitro* in male rats. It appeared very toxic for female rats (all rats died) after *in vivo* injection.

4 Sensitivity to nephrotoxicity induced by 1,3-dichloropropene *in vitro* was about double in male than female rats. Reduced glutathione conjugation appeared involved in nephrotoxicity induced in males but in females, probably by means of a chloropropylcysteinylglycine-conjugate formation; slight toxicity in females is likely related to oxidative metabolism.

Keywords: 1,3-dichloropropene; nephrotoxicity; buthionine-sulfoximine; α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125); β-naphthoflavone

Introduction

1,3-Dichloropropene (1,3 D), a mixture of cis and trans isomers, is largely used in agriculture as such or as a formulation with 1,2-dichloropropane in D-D preparation as nematocide and soil fumigant. The mixture of isomers is normally in an approximate 1 : 1 ratio.

Metabolic fate of 1,3 D is via glutathione conjugation and mercapturic acid formation;[1] dose-dependent depletion of reduced glutathione content is well defined in experimental animals.[2] In addition, a specific mercapturic acid, N-acetyl-S(cis-3-chloroprop-2-enyl)cysteine (3C-NAC) was determined in urine of exposed workers, and a close relationship between exposure and urinary excretion of the specific mercapturic acid was observed.[3,4] A dose-dependent correlation was noticed in rats at lower exposure levels only; an increase of exposure was not associated with further increase of 3C-NAC excretion, suggesting the possibility that the metabolic processes may be saturated.[5]

The few reports which are available on the toxicological effects of 1,3 D show that liver and kidney are the main targets. A dose-dependent loss of organic anion accumulation was observed in rats.[6] Subclinical nephrotoxic effects in human cannot be excluded.[7]

Studies on the mutagenic properties of the solvent indicated that these were most probably due to impurities.[8] Only an isolated report on induction of haematological malignancies[9] was published.

In the present research, the effects caused by 1,3 D on renal cortical slices derived from male and female rats were studied, including treatment with oxidative metabolism inducer or glutathione pathway inhibitors.

Renal cortical slices are a suitable *in vitro* model to study the nephrotoxic effects of xenobiotic substances.[10] This model was well validated for kidney and other organs by two reports of European Centre for the Validation of Alternative Methods (ECVAM).[11,12] The model is recommended for *in vitro* studies on metabolism and toxicity of chemicals and it is reliable to define metabolic activation in the kidney and metabolism-related toxicity.

The aim of the research was to evaluate sex-related differences to nephrotoxicity caused by 1,3

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D and involvement of metabolic activation in expression of toxicity.

Methods

Chemicals

1,3 D (mixture of isomers, purity 98%) was purchased from Janssen (Geel, Belgium); DL-buthionine-[S,R]-sulfoximine (BSO), α-amino-3-chloro-4,5-dihydro-5-isoxazoleacetic acid (AT-125) and 5,5′-dithiobis-2-nitrobenzoic acid were purchased from Sigma Chemical Co. (St. Louis, USA); β-naphthoflavone (NF), aminooxyacetic acid (AOAA), lactic acid sodium salt and p-aminohippuric acid (PAH) were purchased from Fluka (Buchs, Switzerland). Other laboratory chemicals were from Merck (Darmstadt, Germany).

Animals and experimental design

Albino, male and female Wistar rats (Morini, San Polo d'Enza, Italy) two-months-old were kept in cages with natural dark-light cycle, fed with standard diet (Nuova Zoofarm, Padova, Italy) and water *ad libitum*. Oestrus cycle of female rats was not known. After 1 week of acclimatisation, rats were subdivided in four groups of three animals each and treated i.p. as follows: (1) BSO 500 mg/kg in alkaline solution (for rats weighing 200 g, 500 mg were dissolved in 3.64 ml H_2O and 1.36 ml KOH 11.2%). One ml of the solution was administered 4 h before sacrifice. Rats treated with BSO were also fasted for 24 h; (2) AT-125 10 mg/kg in saline (0.5 ml) 1 h before sacrifice; (3) AOAA 55 mg/kg in saline (0.5 ml) 1 h before sacrifice, and (4) NF 80 mg/kg in corn oil once a day for 3 days (1 ml). Ancillary groups treated with vehicles were also prepared.

At the same time, a dose-response experiment was carried out *in vitro* to obtain a suitable concentration of 1,3 D to use in further experimentation. Concentrations of 0.5, 1.0, 2.0, 3.0 and 4.0×10^{-4} M (corresponding to 0.2, 0.4, 0.8, 1.2 and 1.6 μmoles/incubation) were dissolved in ethanol. A control with ethanol alone was always prepared.

Rats were killed under ether anaesthesia and kidneys, quickly removed, placed in cold saline. Immediately after sacrifice, thin freehand renal cortical slices (100 ± 10 mg wet tissue, approximately 10–15 mg/slice, thickness less than 0.5 mm), prepared with a scalpel according to Berndt[13] were rinsed into the incubation medium prepared with NaCl 97 mM, KCl 40 mM, $CaCl_2$ 0.74 mM, and sodium phosphate buffer 7.4 mM, pH 7.4 until all slices could be prepared. All experiments were performed in triplicate. After preparation, slices were transferred to 25 ml Erlenmeyer flasks containing 4 ml of the incubation medium. The flasks were treated with $0.5 - 4.0 \times 10^{-4}$ M (0.2–1.6 μmoles/incubation, dose-dependent experiment), or 3.0×10^{-4} M (metabolism modifiers experiment) of 1,3 D, added with a 10 μl Hamilton microsyringe, stoppered, gassed with 100% O_2 for 5 min and incubated at 37°C for 90 min in a Dubnoff metabolic shaker (100 cycles/min). Other flasks were treated with 10 μl of ethanol as a solvent control. At the end of incubation period, the slices were washed into 1,3 D free medium for 15 min and then incubated in a medium (4 ml) containing additionally lactate (10 mM) and PAH (75 μM) at 25°C for 90 min in a Dubnoff metabolic shaker (100 cycle/min) under 100% O_2 to study organic anion accumulation. Immediately after incubation, the slices were homogenised with trichloroacetic acid (TCA) 3% (10 ml/100 mg of tissue). A 1 ml aliquot of the incubation medium was treated with 4 ml 3% TCA. After centrifugation the supernatant was assayed for PAH according to Smith *et al*.[14] The organic anion accumulation was expressed as the slice/medium (S/M) ratio, where PAH concentration (μg/g tissue) in the slices was divided by PAH concentration (μg/ml) into the medium. In another experiment, renal cortical slices of not pretreated male rats were preincubated *in vitro* for 30 min at 37°C in a Dubnoff metabolic shaker with AOAA 10^{-3} M before treatment with 1,3 D. The following steps were as above.

Reduced glutathione concentration as nonprotein sulfhydryl groups (NPSG) according to Sedlak and Lindsay[15] and cytochrome P-450 (CYP) content according to Omura and Sato[16] were also determined in the renal cortex. Tissue protein concentration was determined with the method of Miller.[17] Spectrophotometric determinations were carried out using a u.v.-Vis spectrophotometer Perkin-Elmer lambda 5 model.

Variance analysis was used for statistical evaluation of the results and significance was set at $P < 0.05$. The results are presented (if not otherwise indicated) as mean ± s.e.m. of percentage related to control values.

Results

A dose-dependent decrease or organic anion accumulation was observed in male but not in female rats (Figure 1). In females, only high doses (3.0 and 4.0×10^{-4} M) caused a significant decrease of PAH accumulation. At 1.9×10^{-4} M a 50% decrease in males was observed, while in females this only occurred at a 2.3 times higher dose (4.3×10^{-4} M). The use of ethanol for dilution of 1,3 D did not affect functions of renal cortical slices.

Pretreatment with NF significantly increased PAH accumulation in control renal cortical slices prepared from both sexes (Figures 2 and 3). On the contrary, BSO, AT-125 and AOAA pretreatment showed no significant effects on PAH uptake.

In the kidney, fasting and BSO reduced glutathione content in both sexes. After treatment with NF, female CYP content was induced 2.5 times more than males, leading to the same level of induction in both sexes; in not pretreated rats, males showed a CYP content 2.5 times higher than female rats (Table 1).

Pretreatment with AT-125 and NF, though partially, prevented in male (Figure 2), but not in female rats (Figure 3) loss of organic anion accumulation at a concentration of 3.0×10^{-4} M of 1,3 D. AOAA did not influence 1,3 D effects on male renal cortical slices. Similar results were obtained after *in vitro* pretreatment (data not shown). All female rats died after pretreatment with the substance. On the contrary, BSO significantly increased loss of organic anion accumulation in female renal cortical slices (Figure 3) treated with 3.0×10^{-4} M of 1,3 D, whereas males were not affected (Figure 2).

Discussion

The aim of the research was to evaluate sex and metabolic differences in the effects of 1,3 D in an *in vitro* kidney slice system after depletion of reduced glutathione, inhibition of γ-glutamyltransferase or cysteine-conjugate β-lyase and induction of oxidative metabolism.

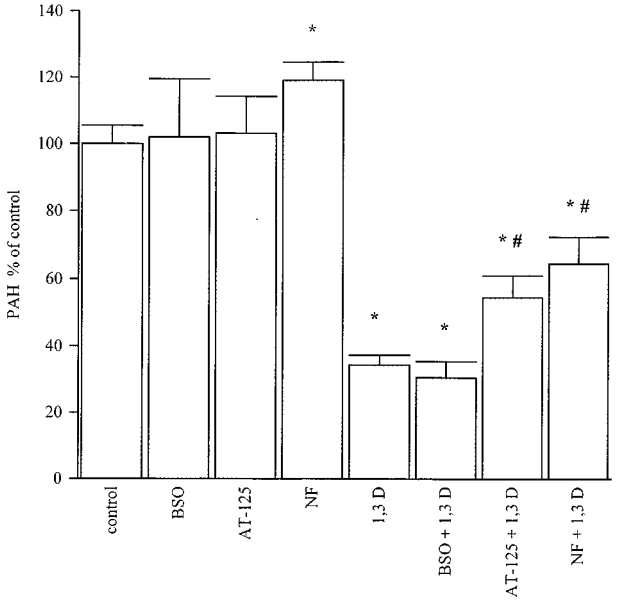

Figure 2 Effects of pretreatment with BSO, AT-125 and NF on loss of PAH accumulation induced by 1,3 D concentration of 3×10^{-4} M in male rats (mean ± s.e.m. was determined in three different triplicate proofs). *$P<0.05$ with the respect to the control; #$P<0.05$ with the respect to 1,3 D alone. Values are represented as percentage of respective controls

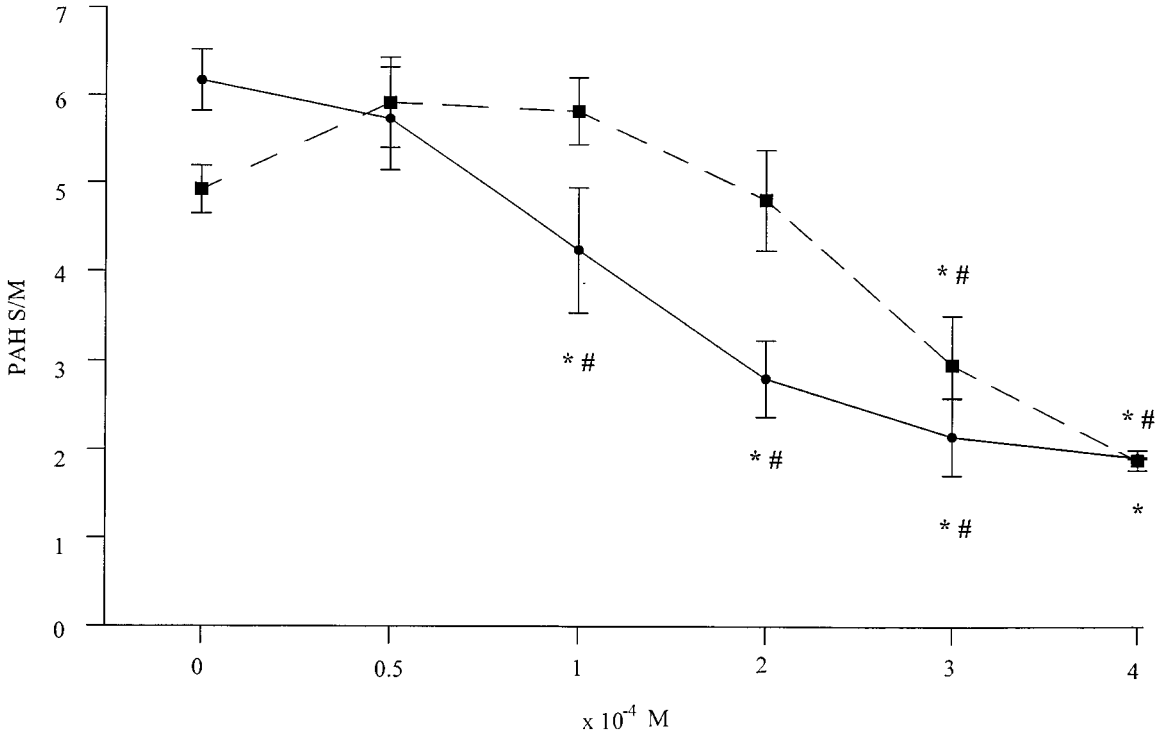

Figure 1 Dose-response relationship between 1,3 D concentration and loss of PAH accumulation in renal cortical slices of male (●, unbroken line) and female (■, dotted line) rats (mean ± s.e.m. was determined in five different triplicate proofs). *$P<0.05$ with the respect of control; #$P<0.05$ with the respect of the previous value

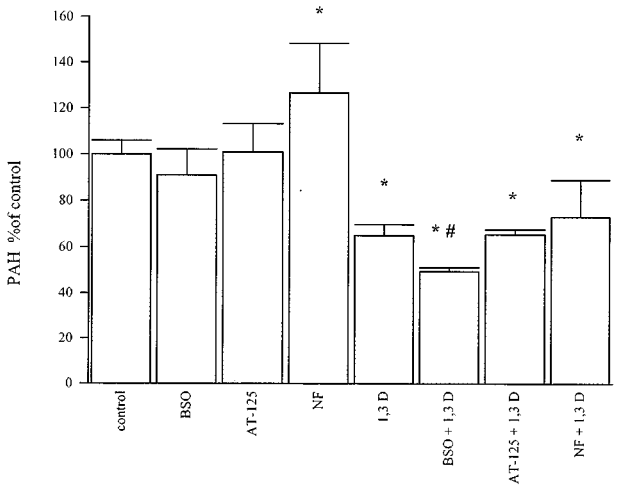

Figure 3 Effects of pretreatment with BSO, AT-125 and NF on loss of PAH accumulation induced by 1,3 D concentration of 3×10^{-4} M in female rats (mean ± s.e.m. was determined in three different triplicate proofs). *$P<0.05$ with the respect to the control; #$P<0.05$ with the respect to 1,3 D alone. Values are represented as percentage of respective controls

Table 1 NPSG and CYP content in the kidney of rats pretreated with BSO or NF (respectively). Data are expressed as mean ± s.e.m. of three rats

	NPSG nmoles/mg of proteins		*CYP* nmoles/mg of proteins	
	Males	*Females*	*Males*	*Females*
Controls	19.7 ± 0.5	22.2 ± 1.4	0.084 ± 0.002	0.034 ± 0.002
BSO	5.4 ± 0.2	5.0 ± 0.3		
NF			0.115 ± 0.009	0.116 ± 0.011

At similar concentrations, male kidney slices showed a greater sensitivity (about three times) to 1,3 D than female kidney slices. In addition, the calculated lowest nephrotoxic dose *in vitro* (1.0 for males and 3.0×10^{-4} M for females) corresponds to an *in vivo* dose of 7.2 mg/kg b.w. for male and 21.7 mg/kg b.w. for female rats, on the assumption that a kidney weighing 800 mg (rat of 200 g) receives 25% of cardiac output.[10] Jeffrey *et al*,[5] report that the lowest observed effect level of 1,3 D *in vivo* on PAH accumulation in male rats after single i.p. injection was 75 mg/kg b.w. Thus, sensitivity *in vitro* appears to be much higher than *in vivo*.

Sex differences in susceptibility may be explained by differences in metabolic activation of the solvent. In humans and rats, the major metabolism is via glutathione conjugation[1] and subsequent excretion as mercapturic acid. Parent compound may be directly conjugated with reduced glutathione or by means of an alkylating metabolite produced by oxidative metabolism. CYP and CYP subfamilies were also involved in sex-related renal toxicity of xenobiotic substances. Male mice renal CYP activity was previously shown fivefold higher than in females or in castrated males, whereas testosterone treatment of females increased the enzyme activity to levels occurring in males.[18] Henderson *et al*,[19] demonstrated that testosterone plays a pivotal role in the regulation of mouse and rat[20] renal CYP subfamilies, in particular subfamily IIE1.[21]

Use of BSO, reduced glutathione depletion by means of blocking the synthesis,[22] NF, inducer of cytochrome P-450 1A class (1A1 and 1A2 subclasses) or AT-125, selective inhibitor of γ-glutamyltransferase,[23] influences the effects of 1,3 D on organic anion accumulation in renal cortical slices according to sex. BSO increases 1,3 D effects in female but not in male rats; on the contrary, AT-125 and NF reduce 1,3 D effects in male but not in female rats. In addition, AOAA, selective renal cysteine-conjugate β-lyase inhibitor,[24] does not exert protective effects against 1,3 D toxicity in males *in vivo* and *in vitro*. It appears very toxic for female rats *in vivo*.

These results indicate that metabolism of 1,3 D plays a sex-related role to cause a different toxicity at the same dose. Conjugation with reduced glutathione appears to be a determinating factor in toxicity in male rats and may be related to the formation of a chloropropylcysteinylglycine-conjugate, since AT-125 pretreatment caused a partial, but significant, prevention of the effects and AOAA did not exert a protective role *in vivo* and *in vitro*. The protective effects of NF indicate that cytochrome P-450 1A class is fundamental in the detoxification. On the contrary, nephrotoxicity in female rats seems related to oxidative metabolism, as demonstrated by lack in protective effects after treatment with NF and by increase of toxicity after reduced glutathione depletion. Thus, the conjugation with reduced glutathione plays a detoxicant role.

In summary, 1,3 D causes a dose-dependent cytotoxicity in renal cortical slices of male rats; on the contrary, only high dose cause effects in female rats. In the male rats, the sensitivity *in vitro* appears ten times higher than *in vivo*. Conjugation with reduced glutathione appears decisive in 1,3 D nephrotoxicity in male, probably due to a cysteinylglycine-conjugate and not to a nephrotoxic thiol formed via cysteine-conjugate β-lyase. Toxic effects on female rats appears independent by reduced glutathione conjugation and may be related to oxidative metabolites.

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