Comparison of urinary creatine with other biomarkers for the detection of 2-methoxyethanol-induced testicular damage

Ruth P. Draper, Dianne M. Creasy and John A. Timbrell

This study has compared different biomarkers of testicular damage, in particular evaluating urinary creatine as a non-invasive marker. Male rats were exposed to various doses of 2-methoxyethanol, a known testicular toxicant. Pathological damage, testis weight, urinary creatine and creatinine, serum lactate dehydrogenase, isozyme C4 (LDH-C4), and serum testosterone were determined. 2-Methoxyethanol caused dose-dependent pathological damage to the testes which was detectable at the lowest dose (100 mg kg^{-1}). Urinary creatine excretion was significantly raised at all doses but testis weight was only significantly decreased at the highest two doses (500, 750 mg kg^{-1}). Serum testosterone was only significantly decreased at 500 mg kg^{-1} and LDH-C4 was not significantly increased at any dose. Therefore urinary creatine was the most sensitive marker of 2-methoxethanol-induced testicular damage and dysfunction.

Keywords: *creatine, urinary biomarker, testicular toxicity, 2-methoxethanol.*

Introduction

The ideal biological marker of male reproductive dysfunction should detect early manifestations as well as the later stages of damage caused by a variety of male reproductive toxicants with different mechanisms of action. It has previously been shown that urinary creatine levels are markedly elevated in rats following testicular damage caused by a variety of toxicants (Timbrell *et al.* 1994) such as 2-methoxyethanol (Rawcliffe *et al.* 1989, Nahas *et al.* 1993), cadmium (Gray *et al.* 1990), 2-methoxyacetic acid, 1,3-dinitrobenzene and di-n-pentylphthalate (Moore *et al.* 1992) and more recently in mice exposed to 2-methoxyacetic acid (Traina *et al.* 1995). Creatine is, therefore, a potentially useful marker of male reproductive dysfunction due to chemicals. Furthermore ischaemic necrosis of the testis caused by ligation of the vasculature also elevates urinary creatine (Gray *et al.* 1990). However, dosing female animals with 2-methoxyethanol (Rawcliffe *et al.* 1989) or $CdCl_2$ (Nicholson *et al.* 1989) or orchidectomized male rats with $CdCl_2$ (Gray *et al.* 1990) does not significantly raise creatine levels in urine. The data from these studies are consistent with the hypothesis that the creatine is derived from the testes. However urinary creatine has not been systematically compared with other markers of testicular damage.

In this study the sensitivity of urinary, creatine was assessed by comparing its ability to detect pathological damage in the testes with two other markers, namely serum lactate dehydrogenase, isozyme C4 (LDH-C4) and testosterone, and with the standard measure of testis weight. LDH-C4 is a testis-specific isozyme whose presence in the serum is indicative of damage to mature spermatozoa (Reader *et al.* 1991). Testosterone is produced by the Leydig cells under the influence of luteinizing hormone (LH) and gives an indication of the hormonal status of the individual and any effects on the Leydig cell. 2-Methoxyethanol, a solvent which has been used widely in industry, is a well studied testicular toxicant, which we have shown previously to cause creatinuria in male rates (Rawcliffe *et al.* 1989). It is believed to be toxic to the testes following metabolism to 2-methoxyacetic acid (Moss *et al.* 1985). 2-Methoxyethanol has a stage specific effect on pachytene spermatocytes such that tubules in some stages will be totally depleted of spermatocytes, while other stages will only be partially depleted or unaffected (Foster *et al.* 1983; Creasy *et al.* 1985). The degree of depletion is also dependent on the dose of 2-methoxyethanol (Creasy *et al.* 1985). Because it is highly specific for the testis in general and the spermatocytes in particular it is an ideal compound to investigate with regard to biomarkers of testicular damage.

The aim of these studies was to validate the use of urinary creatine as a marker of testicular damage by comparing its ability to detect pathological damage to the testes caused by 2-methoxyethanol with two serum markers, serum LDH-C4 and testosterone, and with testis weight.

Ruth P. Draper *is now in the Clinical Chemistry Department, Southmead Hospital, Westbury on Trim, Bristol BS10 5NB, UK;* **Dianne M. Creasy** *is at Pharmaco LSR Ltd, Eye, Suffolk IP23 7PX, UK; and* **John A. Timbrell** *(corresponding author) is in the Department of Toxicology, School of Pharmacy, Brunswick Square, London WC1N 1AX, UK.*

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METHODS

Materials

2-Methoxyethanol (2-ME; 95.5% purity) was obtained from BDH Ltd (Poole, Dorset, UK). The Creatinine PAP kit used for measurement of creatine and creatinine was obtained from Boehringer Mannheim UK Ltd (Lewes, East Sussex, UK). The testosterone/dihydrotesterone [^{3}H] assay system used to measure total testosterone was obtained from Amersham International plc (Aylesbury, Bucks, UK). All other chemicals used were of Analar grade or of the highest purity commercially available.

Animals and treatment

Outbred, male Sprague-Dawley rats (Glaxo Research and Development) were housed in individual metabolism cages and allowed to acclimatize for 2–3 days before the start of the study. Dose ranges were designed with reference to the published literature to include doses causing both minimal and easily detectable toxic effects.

Groups of four animals, weighing 192–233 g (6 weeks old), were given a single i.p. injection of 2-ME in ultra high quality (UHQ) water (5.5 ml kg^{-1}) at doses of 100, 250, or 500 mg kg^{-1} body weight (Study 1). Control groups were given the vehicle (water) by the same route (i.p.). A second study over a larger dose range was carried out with doses of 250, 500 and 750 mg kg^{-1} with an additional control group (Study 2). The data was combined with that from the first study where appropriate.

Body weight, food and water intake were measured daily for the period of the study.

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Urine collection

Urine was collected in ice cooled vessels for periods of 24 h. The volume and, in the second study, the pH of urine samples were measured.

Experiment termination

Animals were killed 48 h after dosing by exsanguination under ether anaesthesia and blood collected from the abdominal aorta for the preparation of serum. The testes, seminal vesicles, liver and kidney were excised, weighed and processed for histology and/or biochemical analyses, as described below.

Histology

Testes were fixed in Bouin's fixative for at least 24 h before storing in 10.5% neutral buffered formalin prior to processing. After dehydrating in graded alcohols and embedding in paraffin wax blocks, 5 μm sections were cut and stained with haemotoxylin and eosin (H & E) and periodic acid Schiff stain (PAS) to identify the stages of spermatogenesis in individual tubules.

The overall severity of testicular damage was estimated using a combined scoring system. The severity of seminiferous tubule damage (judged largely on the extent of spermatocyte necrosis and/or depletion) and the proportion of affected tubules were each assigned a score out of 5 giving a 'combined pathology score' out of 10 for each testis section. This score was used to correlate the severity of histopathological damage with the other markers of testicular damage.

To gain a more accurate numerical estimate of overall spermatocyte loss for correlation purposes, the testes from Study 1, using 0, 250 and 500 mg kg^{-1} of 2-ME were assessed on a stage by stage basis:

For each testicular section, tubules in each of the 14 stages of the spermatogenic cycle were examined and the percentage of pachytene spermatocytes missing from each stage was estimated. (Since the frequency of occurrence of tubules in each of the 14 stages (stage frequency) in a section of testis is constant for a given species and strain, the percentge of spermatocytes lost from each stage, multiplied by the percentage frequency of that stage could be used to calculate the approximate percentage of pachytene spermatocytes lost from the entire testis.) Stage frequency values for Sprague Dawley rats were used (Russell *et al.* 1990).

Creatine and creatinine extraction and determination

Creatine was extracted and protein precipitated from the testes and seminal vesicles by a modification of the method of Lee *et al.* (1988). Serum and tissue creatine and urinary creatine and creatinine were determined by the enzymatic method of Siedel *et al.* (1984), using a Creatinine PAP kit obtained from Boehringer as previously described (Gray *et al.* 1990).

LDH-C4 determination

Tissue extracts were prepared and serum and testicular LDH-C4 were assayed by the methods of Reader *et al.* (1991), as described (Draper and Timbrell 1996).

Testosterone radioimmunoassay

Serum and testicular testosterone were measured using a commercially available single-antibody radioimmunoassay kit (Testosterone/dihydrotestosterone [^{3}H] assay system, Amersham International plc, Amersham, UK) as described (Draper and Timbrell 1996).

Results

Clinical effects of 2-ME administration

A statistically significant ($p < 0.001$), dose dependent weight loss was seen at doses greater than 100 mg kg^{-1} kg 0–24 h post-dose (Figure 1). At 24–48 h postdose, the animals were gaining weight normally. Food intake was significantly decreased ($p < 0.01$, 100 mg kg^{-1}; $p < 0.001$, 250–750 mg kg^{-1}) at all doses 0–24 h post-dose when compared with pre-dose values. At 24–48 h postdose, food intake had increased, but was still significantly lower ($p < 0.05$, 100 mg kg^{-1}; $p < 0.001$, 750 mg kg^{-1}) than pre-dose values (data not shown). Water intake only showed a significant decrease at doses of 250 and 500 mg kg^{-1} 0–24 h post-dose ($p < 0.01$, $p < 0.001$ respectively; data not shown).

Urine volume was significantly increased ($p < 0.05$) above the predose value at the highest dose (750 mg kg^{-1}) 0–24 h postdose (data not shown). Urinary pH showed a significant ($p < 0.05$) dose related reduction in treated animals 24 h after dosing. However this change was small, with a maximal decrease from 7.3 to 6.8 (data not shown).

The effects of 2-ME on organ weights

A significant dose-related decrease ($p < 0.01$) in actual and relative testis weight was observed at doses of 500 and 750 mg kg^{-1} (Table 1). There was a significant loss ($p < 0.05$) of liver weight relative to body weight but this was not dose dependent (data not shown).

The effects of 2-ME on the histopathology of the testis

At 100 mg kg^{-1} 2-ME, there was no obvious effect on the testis apart from a few necrotic late pachytene and dividing spermatocytes showing cytoplasmic eosinophilia and granularity (stages XII–XIV). At doses of 250 mg kg^{-1} and above, there was a depletion in numbers of early and late pachytene spermatocytes (stages I–IV, IX–XIV) that extended to mid-pachytene spermatocytes (stages VII–VIII) at the highest doses. These effects were dose-related with almost complete loss of pachytene spermatocytes in all stages of spermatogenic cycle at the highest dose.

Table 2 shows the 'combined pathology score' for the frequency and severity of the pathological damage caused by 2-ME in the rat testis.

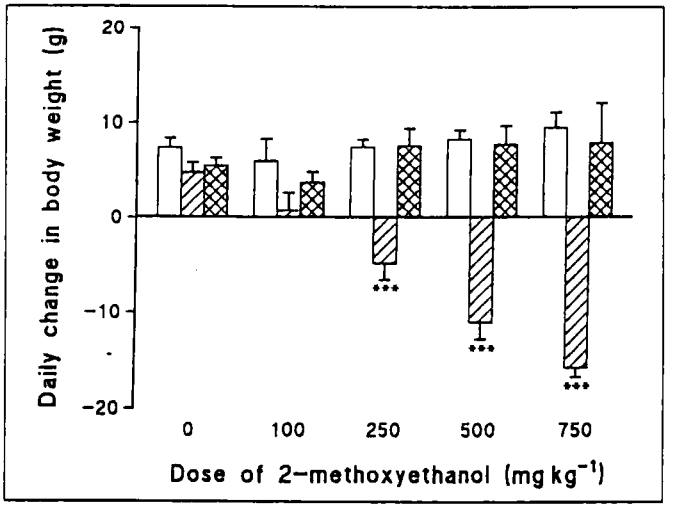

Figure 1. Effect of various doses of 2-methoxyethanol on the change in body weight in rats. Results are mean±SEM where $n = 7$ (control); $n = 8$ (250, 500 mg kg^{-1}), $n = 4$ (100 and 750 mg kg^{-1}). □ pre-dose; ▨ 0–24 h post-dose; ⊠ 24–48 h post-dose. *** $p < 0.001$, paired t-test.

Dose of 2-ME (mg kg^{-1})	Actual testis weight (g)	Relative testis weight (g 100 g^{-1} body wt)
0 (n = 7)	2.29±0.08	0.94±0.03
100 (n = 4)	2.53±0.13	1.05±0.05
250 (n = 8)	2.13±0.06	0.88±0.03
500 (n = 8)	1.88±0.04**	0.80±0.02**
750 (n = 4)	1.79±0.06**	0.76±0.02**

Table 1. Effect of 2-ME on testis weight in the rat.

Data are mean±SEM.
Significant difference from control (Dunnett's test): **$p < 0.01$.

Creatine and creatinine levels in the urine, serum and tissues after dosing with 2-ME

There was a maximal, dose-related increase in urinary creatine 0–24 h after dosing with 2-ME that was significant (p < 0.05–0.001) at all doses (Figure 2). Creatine excretion was still elevated above pretreatment values 24–48 h after dosing, although this was only significant at the two highest doses ($p < 0.05$–0.01) (Figure 2). Urinary creatinine excretion was significantly decreased 0–24 h after dosing at 500 and 750 mg kg^{-1} ($p < 0.01$ and 0.001 respectively; Figure 3). The creatine:creatinine ratio in the urine (Figure 4) shows the same dose-related effects as seen with creatine excretion, although the rise at 500 and 750 mg kg^{-1} 2-ME 0–24 h postdose is more marked due to the decrease in urinary creatinine excretion (Figures 3 and 4). There was a significant decrease ($p < 0.01$) in serum creatine at all doses of 2-ME which was not dose-related (Table 3). There was no change in the concentration of creatine in the testes. In contrast, there was a significant increase ($p < 0.05$) in the creatine concentration in the seminal vesicles (SV) at the highest dose.

Effects of 2-ME on serum and testicular LDH-C4 and testosterone levels

There was a significant decrease ($p < 0.01$) in serum testosterone at 500 mg kg^{-1} but no effect on LDH-C4 at any dose (Figure 5). Testicular testosterone showed a dose-related decrease that was significant ($p < 0.05$) at the highest dose (Table 3) but 2-ME had no effect on testicular LDH-C4 (data not shown).

Correlation of parameters indicative of testicular damage after dosing with 2-ME

Actual and relative testis weights showed a significant negative correlation with the combined 'pathology score' (r = 0.766 and r = 0.742 respectively, $p < 0.001$). Urinary creatine excretion at 0–24 h and 24–48 h post dosing showed a significant negative correlation with actual testis weight (r = 0.5698, $p < 0.05$; r = 0.6646, $p < 0.01$, respectively) and a significant positive correlation with the 'combined pathology score' (r = 0.596, $p < 0.001$; r = 0.429, $p < 0.05$, respectively).

Serum testosterone showed a significant negative correlation with urinary creatine 24–48 h after dosing (r = 0.5219, $p < 0.05$) and with the 'combined pathology score' (r = 0.602, $p < 0.001$). Serum LDH-C4 showed no correlation with any of the other parameters.

Dose of 2-ME (mg kg^{-1})	Average combined score
0 (n = 7)	1.14
100 (n = 4)	1.50
250 (n = 8)	6.25
500 (n = 8)	8.88
750 (n = 4)	9.50

Table 2. 'Pathology score' for 2-ME-induced testicular damage in the rat testis.

The 'combined pathology score' was determined as described in Materials and Methods.

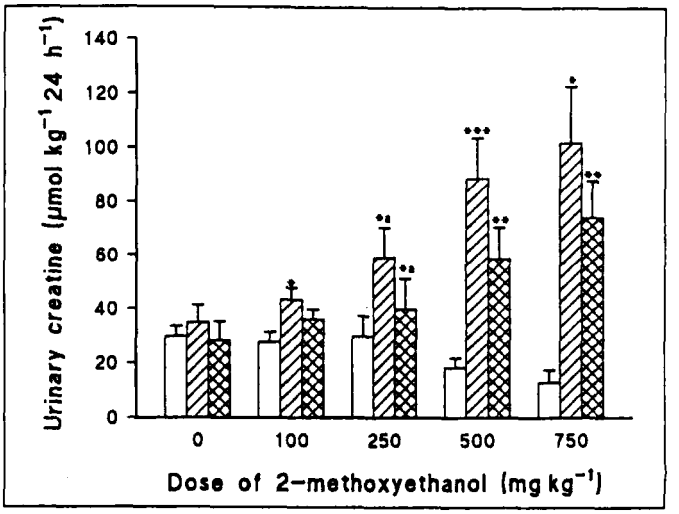

Figure 2. Effect of various doses of 2-methoxyethanol on urinary creatine excretion by rats. Results are mean±SEM, where n = 7 (control); n = 8 (250, 500 mg kg^{-1}), n = 4 (100 and 750 mg kg^{-1}). ☐ pre-dose; ▨ 0–24 h post-dose; ⊠ 24–48 h post-dose. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, paired t-test. *a, significant for combined data (Studies 1 and 2) if compared with mean pre-dose creatine value for all animals by t-test; significant for data from Study 1 by paired t-test.

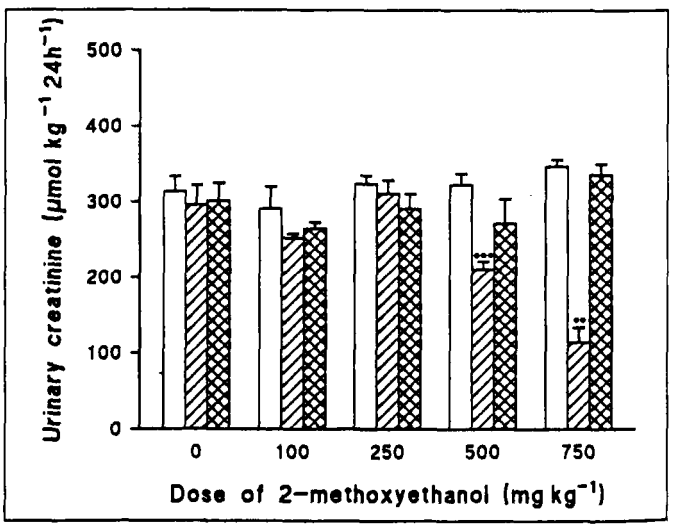

Figure 3. Effect of various doses of 2-methoxyethanol on urinary creatinine excretion by rats. Results are mean±SEM, where n = 7 (control); n = 8 (250, 500 mg kg^{-1}), n = 4 (100 and 750 mg kg^{-1}). ☐ pre-dose; ▨ 0–24 h post-dose; ⊠ 24–48 h post-dose. **$p < 0.01$; ***$p < 0.001$, paired t-test.

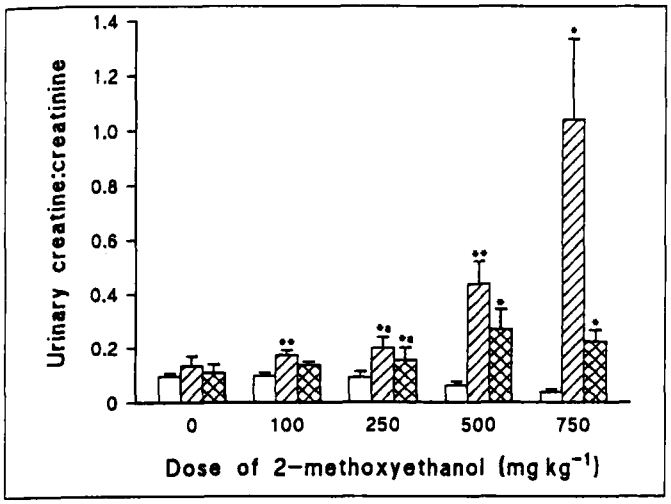

Figure 4. Effect of various doses of 2-methoxyethanol on the urinary creatine:creatinine ratio in rats. Results are mean±SEM, where $n = 7$ (control); $n = 8$ (250, 500 mg kg^{-1}), $n = 4$ (100 and 750 mg kg^{-1}). ☐ pre-dose; ▨ 0–24 h post-dose; ▩ 24–48 h post-dose. $^{*}p < 0.05$; $^{**}p < 0.01$; paired t-test, ^{a}, not significant for combined data but significant for data from Study 1 by paired t-test.

Dose of 2-ME (mg kg^{-1})	Serum creatine (μmol ml^{-1})	Testicular creatine (nmol mg^{-1} tissue)	SV creatine (nmol mg^{-1} tissue)	Testicular testosterone (μg g^{-1} testis)
0 ($n = 7$)	0.213±0.012	18.67±1.81	15.46±1.41	13.24±2.22
100 ($n = 4$)	0.144±0.012**	18.77±2.55	12.23±1.81	13.55±5.98
250 ($n = 8$)	0.156±0.009**	19.52±0.73	16.70±1.22	12.61±3.98
500 ($n = 8$)	0.159±0.015**	17.58±0.69	17.72±1.09	10.75±3.27
750 ($n = 4$)	0.127±0.009**	18.47±0.60	21.39±1.40*	8.56±3.24*

Table 3. Serum, testes and seminal vesicles creatine content in 2-ME-treated rats.

Data are mean±SEM.

Significant difference from control (Dunnett's test): $^{*}p < 0.05$; $^{**}p < 0.01$.

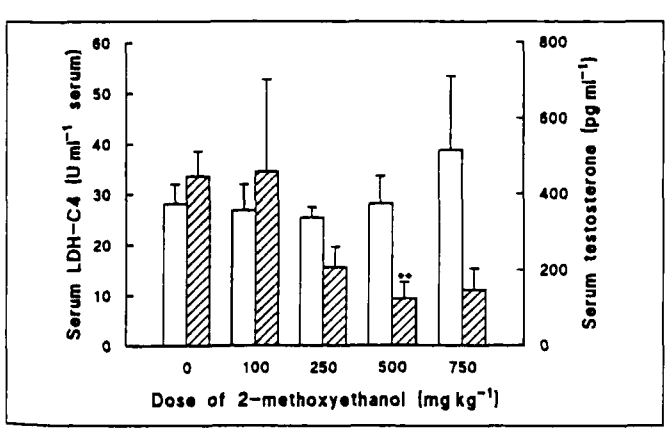

Figure 5. Effect of various doses of 2-methoxyethanol on serum levels of testosterone and LDH-C4 in the rat. ☐ LDH-C4; ▨ testosterone. Results are mean±SEM, where $n = 7$ (control); $n = 8$ (250, 500 mg kg^{-1}), $n = 4$ (100 and 750 mg kg^{-1}). $^{**}p < 0.01$ Dunnetts test.

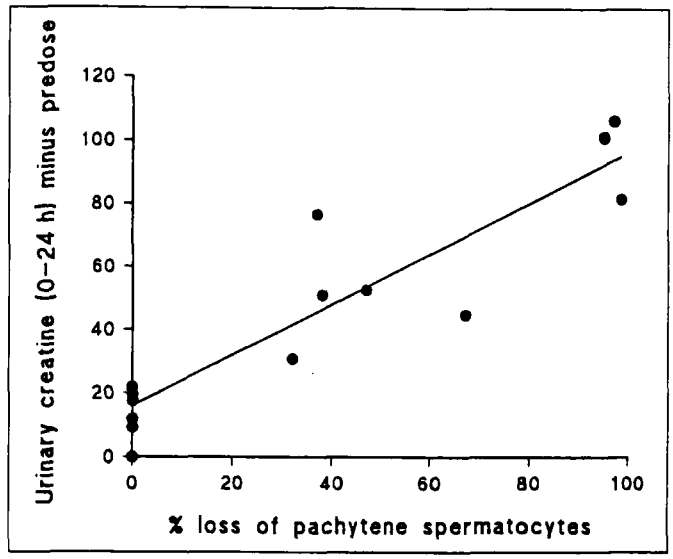

Figure 6. Linear correlation between excess creatine excreted and loss of pachytene spermatocytes in the testes of rats given various doses of 2-methoxyethanol (0, 250 or 500 mg kg^{-1}). Each data point represents an individual rat. $r = 0.92$, $p < 10^{-7}$.

There was a highly significant correlation ($r = 0.92$, $p < 0.001$) between the total excess creatine excreted and the percentage of pachytene spermatocyes damaged (Figure 6).

Discussion

As previously reported (Foster *et al.* 1983, Creasy *et al.* 1985, Rawcliffe *et al.* 1989, Nahas *et al.* 1993), doses of 250 mg kg^{-1} of 2-ME or greater caused obvious dose-related testicular damage (Table 2), characterized by necrosis of early and late pachytene spermatocytes in particular. This was reflected by a decrease in actual testis weights, which seemed to be a better indicator of testicular damage than relative testis weights (Table 1) due to the loss in body weight (Figure 1).

All doses of 2-ME caused an increase in urinary creatine that was maximal at 0–24 h after dosing (Figure 2). This differs from data presented by Rawcliffe *et al.* (1989), where the maximal increase was at 24–48 h but is similar to that of Nahas *et al.* (1993). Therefore, the peak in urinary creatine excretion probably occurs about 24 h after dosing, but whether this is measurable in the first or second 24 h urine collection will depend on individual variation in excretion profiles and the exact times of the collections. Interestingly in mice dosed with the testicular toxicant 2-methoxyacetic acid there is an initial decrease in creatine 24 h after dosing followed by a peak urinary excretion at 72 h post-dosing (Traina *et al.* 1995).

Results showing that 500 mg kg^{-1} 2-ME caused no increased creatine excretion in female rats (Rawcliffe *et al.* 1989) and measurement of creatine excretion in male rats after restricting food intake (Draper and Timbrell, unpublished observations) supports the theory that the 2-ME-induced creatinuria in this study is not due to a loss in body weight and that its probable source is the testes.

The slight drop in urinary pH also would not account for the rise in urinary creatine as indicated by studies on the stability of creatine and creatinine under different conditions (Draper and Timbrell, unpublished observations). 2-ME does not cause any observable liver or kidney damage as indicated both by serum biochemistry and histopathology (Draper and Timbrell 1996, Draper unpublished observations). Serum creatine was significantly decreased at all doses 48 h after dosing with 2-ME (Table 3). As increased creatinuria was maximal at 0–24 h post-dosing, any peak in serum creatine would be prior to this time. The significant decrease might, therefore, be due to a diminished total testicular creatine content. Although a decrease in testis weight, probably due to cell loss, was associated with a decrease in the total creatine content of the testes at the two highest doses (data not shown), the creatinuria observed cannot be accounted for by simple leakage from the testes. In addition, there was significant creatinuria 0–24 h after dosing with 100 mg kg^{-1} 2-ME. This could not be accounted for by body weight loss, and there was no decrease in testis weight (Table 1) or total testicular creatine content (data not shown) and little pathological damage (Table 2). This suggests the possible presence of a toxicant-induced biochemical lesion affecting creatine synthesis, metabolism and/or secretion. Work by Lee *et al.* (1991) on seminal vesicular creatine and phosphocreatine suggests that phosphocreatine is actively secreted into the seminal vesicular fluid, which also contains high concentrations of creatine (Lee *et al.* 1988) by the epithelial cells of the gland under the influence of testosterone.

Therefore the significant increase seen in seminal vesicular creatine concentration after 750 mg kg^{-1} of 2-ME (Table 3) is of interest, as a reduced testosterone level might be expected to impair secretion of creatine from the epithelium leading to accumulation in the cells of the seminal vesicles.

In this study, no significant changes in serum (Figure 5) or testicular (data not shown) LDH-C4 were observed after any dose of 2-ME. Reader *et al.* (1991) have previously shown a dose-dependent increase in serum LDH-C4 preceding a reduction in testicular LDH-C4 in male Wistar-deprived Alpk: APfSD strain rats. The increase in serum LDH-C4 was maximal at 48 h and remained elevated for 96 h after dosing with 2-ME. Ward *et al.* (1989) have shown major differences in the effect of procarb-azine on both the size and direction of response of several testicular parameters in different strains of rat. Such strain differences may be a possible explanation for the lack of a significant effect of 2-ME on serum and testicular LDH-C4 in this study.

The dose-dependent decrease in serum testosterone, at doses of 250 mg kg^{-1} 2-ME or greater (Figure 5), was accompanied by a concomitant decrease in testicular testosterone (Table 3). However 2-ME is not reported to affect Leydig cells, the site of testosterone production. Therefore, this decrease is probably due to an indirect effect on the complex signalling system between the Leydig cells and the cells of the seminiferous tubules, that ensures an adequate supply of testosterone to the developing germ cells.

This study shows that urinary creatine excretion is able to detect acute 2-ME-induced testicular damage at doses of 100 mg kg^{-1} and above in male Sprague-Dawley rats. In contrast serum LDH-C4 is not effective as a marker in this case and serum testosterone was only significantly decreased after a dose of 500 mg kg^{-1}. Urinary creatine seems to be more effective than serum testosterone as it correlates with the decrease in testis weight, as well as the 'pathology score'. The close correlation between loss of spermatocytes and excess creatine excreted reveals how specific and sensitive urinary creatine is as a marker (Figure 6). Testis weight was only significantly affected by doses of 500 and 750 mg kg^{-1}. Urinary creatine was significantly raised 0–24 h after 100 mg kg^{-1} 2-ME, when histopathological damage in the testis was barely, if at all, visible. This may be an indication of biochemical disturbances caused by 2-ME prior to visible pathological damage.

Testis weight is a rough measure of testicular damage but can only be measured after termination and is consequently only useful in experimental animal studies, although testis size may be an alternative in man. Weight and size reduction can also be masked by an increase in interstitial fluid volume. Serum LDH-C4 is known to be specific to the testes and should, therefore, be an ideal marker of testicular damage, as increases in serum LDH-C4 are then a clear indication of damage to cells in the testes resulting in leakage into the general circulation. Thus, it will detect obvious damage, but may show no change if there are subtle effects, such as changes in the biochemical status of the testicular cells. However, in this study LDH-C4 was not significantly increased by any dose of 2-ME.

Serum testosterone indicates the hormonal status of the testes and may be important in the detection of specific toxic effects on the Leydig cells or on the hypothalamic-pituitary-testicular axis. However, results often show great variation, because of the cyclical nature of testosterone production and will also show changes as a secondary effect of spermatogenic disturbances. In this study, serum testosterone was decreased at most doses of 2-ME but this was only significant at 500 mg kg^{-1}.

Both LDH-C4 and testosterone are measured in the blood. This involves an invasive technique and is not ideal in routine clinical or industrial monitoring or in studies in animals where repeated sampling is required.

Urinary markers are preferable because, urine is easier to collect, safer to handle and provides larger volumes for analysis than blood or plasma. In this study, urinary creatine was raised in a dose-dependent manner at all doses of 2-ME. Thus in conclusion:

(a) Urinary creatine is more sensitive than testis weight, serum LDH-C4 and serum testosterone at detecting acute 2-ME-induced testicular damage;
(b) Urinary creatine is able to detect the germ-cell specific damage caused by 2-ME;
(c) Urinary creatine may be able to detect subtle, toxicant-induced effects on the biochemical status of the testicular cells present prior to the appearance of pathological damage.

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