Testicular creatine and urinary creatine–creatinine profiles in mice after the administration of the reproductive toxicant methoxyacetic acid

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It was previously observed that the acute or subchronic administration of some testicular toxicants, caused a significant raise in urinary creatine in rats. The aim of this study was to verify whether creatinuria could be detected in mice (a species with a different excretion profile of creatine) and whether it could be correlated to the levels of creatine in testis and to other parameters of testicular toxicity. The well known testicular toxicant methoxyacetic acid (MAA) was orally administered as a single dose (400 or 600 mg kg^{-1}) to male adult mice B6C3F1. Twenty-four hours after dosing, urinary creatine and creatinine showed a significant reduction with respect to the pre-treatment values. At the following times post-dosing (48 and 72 h) the creatine exceeded the control and pre-treatment values, while creatinine had not yet recovered. The ratio creatine/creatinine was significantly higher than control and pre-treatment values, at 24 and 48 h after the treatments. In testis a significant, dose-dependent, decrease of creatine was observed 24 h after dosing, with a pattern related to the histopathologic alterations observed at different times after the treatments. Creatine determination was the earlier quantitative parameter of testicular toxicity, since at this time testis weights, sperm head number and enzyme activities (LDH-C4, SDH) were less affected, their maximum decrease being reached at 14 days after the treatments. These data suggest that in mice, 2-MAA could interfere with the metabolism of creatine, both in testis and other biosynthetically active tissues.

Keywords: *creatine/creatinine, urine, testis, mouse, methoxyacetic acid.*

Introduction

Recently creatinuria determination has been proposed as a sensitive and non-invasive *in vivo* marker of acute and sub-acute testicular damage in rats (Rawcliffe *et al.* 1989). Marked creatinuria was observed in these rodents both after surgical ligation of the testicular vasculature and after exposure to testicular toxicants (2-methoxyethanol (2-ME), cadmium chloride, di-*n*-pentylphtalate, 1,3-dinitrobenzene, Gray *et al.* 1990, Moore *et al.* 1992, Nahas *et al.* 1993, Butterworth *et al.* 1995). Following treatment with 2-methoxyacetic acid, the urinary creatine/creatinine ratio in rats appeared to be more sensitive than plasma LDH-C4, testosterone or testis weight in detecting testicular damage (Timbrell *et al.* 1994). It is not yet known whether the creatine is simply released from the testis as a consequence of germ cell damage or whether the toxic compound causes a change in the synthesis or utilization of creatine.

To date the correlation between the creatinuria caused by reproductive toxicants and the levels of testicular creatine has not yet been investigated, although it is already known that rat and mouse testes contain large amounts of free creatine (most of the creatine in testes, >90% of the total, being in the extracellular fluid) and have the ability for *de-novo* synthesis (Lee *et al.* 1988). Furthermore the experimental studies in relation to creatinuria caused by testicular toxicants have, to date, been conducted only in rats, and no information is available on mice. This latter species, for its particular physiological characteristics, appeared interesting to us for the purpose of extending what is known about these biomarkers. The normal mouse excretes in urine approximately equal amounts of creatine and creatinine, a condition which is instead characteristic of an advanced muscular wasting in humans and other animals. Consequently to this physiological characteristic, the urinary creatine/creatinine ratio is considerably higher in the normal mouse (>1) than in the normal rat (<0.05) (Madison 1952, Karnofsky *et al.* 1955).

The objective of the present study was to examine whether creatinuria induced by testicular toxicants could be detected in mice and whether the creatine/creatinine excretion pattern could be correlated to the levels of creatine in testis and to other parameters of testicular toxicity. Methoxyacetic acid (MAA), the major urinary metabolite produced following oral ingestion of ethylene glycol monomethyl ether (EGME) (Miller *et al.* 1983), was chosen to induce testicular damage in B6C3F1 mice. MAA has been shown to produce testicular damage in rodents similar to that observed with the parent glycol ether (EGME) after a single oral dose (Miller *et al.* 1982), pachytene and dividing spermatocytes in stages XIII, XIV and I, being the initial, primary site of toxicity.

METHODS

Reagents

The following compounds were supplied by the Sigma Chemical Company: triethanolamine-hydrochloride, α-ketovaleric acid-sodium salt, β-nicotinamide adenine dinucleotide, reduced form from yeast grade III disodium salt (NADH), -D(–) fructose crystalline, total protein kit, protein standard (bovine serum albumin).

Tris (hydroxymethyl) amino methane, sodium chloride (NaCl), sodium dihydrogen orthophosphate ($NaH_2PO_4.H_2O$), disodium hydrogen phosphate dihydrate ($Na_2HPO_4.2H_2O$), sodium hydroxide pellets (NaOH) and sodium bicarbonate ($NaHCO_3$), were from Carlo Erba and methoxyacetic acid (MAA) was supplied by Merck-Schuchardt.

Treatments

Male, B6C3F1 type mice (2 months old, 25–30 g) from Charles-River, Italy, were used for both treatment and control groups. The animals were first acclimatized

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for at least 1 week before the treatment under standard laboratory conditions (20±2°C with 50±10% relative humidity, 12 hrs light/dark cycle).

After the acclimatization period, the animals, randomly assigned to control and treatment groups (12 mice for control groups, 12 for 400 mg kg^{-1} treated and 21 for 600 mg kg^{-1}) were placed in metabolic cages (three mice per cage), and 24 h urine collections were made for 2 days before the treatments. The animals were then treated orally with sterile water alone or with MAA in single doses of 400 or 600 mg kg^{-1} bw (equimolar with 338 and 506 mg 2-methoxyethanol per kg bw, respectively).

Urine collections were made at 24, 48 and 72 h after the MAA administration, both for treatment and control metabolic cages.

Testicular evaluation

MAA treated animals and the controls (water alone) were sacrificed by cervical dislocation at 24, 48 h and at 14, 28 and 35 days after the treatments. In each animal, testes weight, testicular sperm head number, activity levels of lactate-dehydrogenase-C4 (LDH-C4), sorbitol-dehydrogenase (SDH), creatine content, and histopathology were evaluated. In addition in a group of 10 animals, testicular creatine, sperm head number and histopathology were evaluated at 3, 6, 14 and 24 h after a single dose of MAA (400 mg kg–[1]).

Different procedures were used for right and left testes.

	Body weight		Testis weight		
	Treatment day	Sacrifice day	Right	Left	Ratio[a]
24 h after dosing					
Control (3)	28.2±0.8	27.6±0.9	113.9±5.7	106.8±1.1	3.8
400 mg (3)	28.5±0.6	27.5±0.7	94.6±1.8	92.3±4.1	3.7
600 mg (6)	27.5±0.9	24.6±0.8*	94.1±2.4	88.2±1.6*	3.7
48 h after dosing					
Control (3)	28.5±0.1	28.6±0.4	102.8±0.2	100.9±0.5	3.6
400 mg (3)	28.3±0.8	26.7±1.5	92.0±5.1	91.6±4.4	3.4
600 mg (6)	28.1±0.5	23.7±1.0**	87.1±1.8*	83.9±2.6*	3.6
14 days after dosing					
Control (3)	28.6±1.3	29.7±1.3	111.7±2.7	110.0±2.9	3.7
400 mg (3)	28.7±0.7	30.5±0.8	89.6±3.3*	89.6±2.7*	2.9
600 mg (6)	28.5±0.4	28.2±0.6	77.1±2.3**	73.2±2.6**	2.6
28 days after dosing					
Control (3)	27.9±0.7	30.6±0.3*	111.0±0.6	112.5±0.9	3.6
600 mg (3)	27.7±0.8	27.4±0.9	88.7±2.9*	87.0±3.5*	3.2
35 days after dosing					
400 mg (3)	29.0±0.7	32.8±0.5*	112.3±0.6	113.3±2.5	3.4

Table 1. Body weights and testes weights of control and 2-methoxyacetic acid-treated mice (400 mg kg^{-1} and 600 mg kg^{-1}, p.os) at different times after dosing.

The values are means ± s.e.m. of the data obtained in each group (3 or 6 mice as indicated in brackets.)

Body weight at the sacrifice day significantly different from body weight at the treatment day (paired *t*-test).

Testes weight in the treatment groups significantly different from control value (unpaired t-test, with Bonferroni correction).

*$p<0.05$ **$p<0.01$.

Body weight (x±s.e.m.) of control mice (12) at the treatment day: 28.3±0.38.

Body weight (x±s.e.m.) of treated mice (27) at the treatment day: 28.26±0.26.

[a] Ratio: testis weight (mg) body weight g.

Right testes

These were decapsulated from tunica albuginea, placed in 1.2 ml Tris buffer, 10^{-3} M, pH 7, homogenized (20 s with ultra-Turrax T25) and sonicated (2 min with soniprep). An aliquot (0.2 ml) of the testicular samples was diluted in 1.8 ml deionized H_2O (1:10) and evaluated for testicular head number according to Meistrich (1989).

The residual sample volumes (1 ml) were centrifuged for 30 min at 17000 × *g* and divided into aliquots for the determination of protein concentration and activity levels of LDH, SDH, according to Lowry *et al*. (1951), Lee *et al*. (1982) and Gerlach and Hiby (1983), respectively. The levels of creatine were measured using the enzymatic assay of Siedel *et al*. (1984) as described by Rawcliffe *et al*. (1989) and Gray *et al*. (1990).

Left testes

These were used for histopathological evaluation. The examination was performed on controls (*n*=12) and on treated mice 400 mg/3 h post-dosing (PD) (*n*=3), 400 mg/14 h PD (*n*=3), 400 mg/24 h PD (*n*=4), 600 mg/24 h PD (*n*=6), 400 mg/48 h PD (*n*=3), 600 mg/48 h PD (*n*=6), 600 mg/14 days PD (*n*=4), 600 mg/28 days PD (*n*=3), 400 mg/35 days PD (*n*=3). Testes were fixed with Carnoy liquid and embedded in 2-hydroxy-ethyl methacrylate. The sections (2–3 μm) were stained with haematoxylin–eosin.

Urine analysis

Creatine and creatinine levels were determined in the urine samples collected in the pre-treatment and post-treatment periods. All the samples collected every 24 h for each metabolic cage (pooled urine of three mice) were measured for volume, filtered (sepack) and stored (–80°C) until creatine and creatinine content were enzymatically determined.

	Sperm heads (10^6)		Creatine (μmol g^{-1}	LDH-C4	SDH
	Per testis	Per gram	testis)	(μmol mg^{-1} protein h^{-1})	
24 h after dosing					
Control (3)	21.1±3.1	197±16	21.6±0.5	121±5.4	3.63±0.21
400 mg (3)	17.0±0.4	181±5.2	14.1±0.7**	111±7.4	3.66±0.26
600 mg (6)	15.9±1.1	168±15	12.5±0.4**	119±6.5	3.73±0.26
48 h after dosing					
Control (3)	17.1±1.4	166±13	20.4±0.7	92±4.8	3.68±0.24
400 mg (3)	20.3±0.8	222±19	17.4±0.9	92±7.8	3.18±0.14
600 mg (6)	17.2±1.4	197±17	15.6±0.5*	96±4.8	4.33±0.18
14 days after dosing					
Control (3)	21.8±0.5	195±2.2	22.0±0.4	113±4.3	3.79±0.48
400 mg (3)	4.5±0.7**	50±6.9**	19.2±0.1	68±5.2**	2.11±0.55*
600 mg (6)	2.2±0.3**	20±3.1**	21.7±1.1	52±2.4**	1.65±0.12*
28 days after dosing					
Control (3)	19.8±0.9	178±8.1	19.0±1.1	100±11.5	3.25±0.64
600 mg (3)	10.5±1.5**	117±12*	20.1±0.4	71±5.7*	2.27±0.26
35 days after dosing					
400 mg (3)	20.4±0.7	181±5.5	20.2±0.3	119±8.2	3.70±0.70

Table 2. Sperm head number, creatine content and LDH, SDH activity levels in testicular preparations of control and methoxyacetic acid-treated mice (400 mg kg^{-1} and 600 mg kg^{-1}, p.os) at different times after dosing.

The values are means±s.e.m. of the data obtained in each group (3 or 6 mice as indicated in brackets).

*$p<0.05$, **$p<0.01$, significantly different from control value (unpaired *t*-test, with Bonferroni correction).

Statistical analysis

All data were subjected to statistical analysis following paired *t*-tests for body weight and unpaired-*t*-tests (with Bonferroni correction) for the other parameters evaluated in this paper (see Tables 1 and 2).

Results

Body weights and water consumption

Control animals showed an increase in body weight over the course of the experiment. The administration of MAA (400 and 600 mg kg^{-1} bw, single dose) resulted in a slight body weight decrease with respect to pre-treatment values, during the first 2 days after the treatments. This decrement (–10% and –16% at 24 and 48 h PD, respectively) was statistically significant only for the highest dose, while a slow-down in growth was observed on the following days (Table 1). Water consumption was also reduced in the groups receiving MAA, during the first 2 days following the treatments. The maximum decline observed (about 35% of the pre-treatment values) was at 48 h for both the doses of MAA administered (data not shown).

Changes in testis

As shown in Tables 1 and 2 and Figure 1, at the two doses administered MAA caused dose-related variations in testes weight and sperm head counts with respect to control values; these were particularly enhanced at 14 days after the treatments. A concurrent reduction was observed with regard to the activity levels of lactate-dehydrogenase-C4 and sorbitol-dehydrogenase: at 14 days PD, these two enzymes, associated with more mature germ cells, were both depressed to about 40% and 50% of the control values (at 400 and 600 mg kg^{-1}, respectively). The levels of these parameters tended to recover on the following days.

A significant dose-related reduction (35% and 45%, respectively) of the creatine concentration in testis, was evident 24 h after the treatments. The levels were partially recovered at 48 h and returned to the control values at 14 days after the treatments.

In the group sacrificed at 3, 6 and 14 h post-dosing (PD 400 mg kg^{-1}), sperm head number was reduced to about 30% of the control values, in one animal at 6 h PD and in two at 14 h. Creatine concentration was lowered (–15%) only in one mouse at 14 h PD (data not shown).

Histopathology

The types of histological alterations in the testes and the time course were as follows: slight (isolated cells or small clusters) degeneration and necrosis of primary pachytene spermatocytes 3 and 6 h PD; marked to severe increase in primary, pachytene spermatocyte degeneration and necrosis 14 and 24 h PD; marked depletion of spermatocytes and moderate depletion of early round spermatids 48 h PD; increased vacuolation of Sertoli cells at 600 mg, 48 h and 14 days PD; marked disorganization of the layers of seminiferous epithelium with sloughing and elongating spermatid depletion at 600 mg, 14 days PD; repopulated seminiferous tubules with residual presence of slight to moderate alterations (e.g. presence of giant, degenerating or necrotic cells) at 600 mg, 28 days PD. The severity of the lesions was dose-related. The relationship between the main kinds of alterations observed and testicular parameters are shown in Figure 1.

Urinary creatine and creatinine levels

The levels of creatine and creatinine in the urine of animals treated with MAA are shown in Table 3 and Figure 2.

In the control groups the levels of creatine and creatinine excreted in 24 h were comparable to those previously reported by Fitch *et al.* (1961) (around 0.5 mg per 24 h and 0.31 mg per 24 h, respectively). Following MAA administration, a significant decrease in the content of creatine and creatinine was observed in the samples collected 24 h after dosing, independent of the dose. At the following collections (48 h and 72 h), the levels of creatine had increased compared with controls and pre-treatment values, and returned to control values thereafter.

		Post-treatment		
	Pre-treatment	24 h	48 h	72 h
Creatine (micromoles per 24 h)				
Control	11.6±1.2 (8)[4]	10.8±1.7 (4)[4]	11.2±1.9 (3)[3]	10.6±0.05 (2)[2]
400 mg kg^{-1}	11.1±1.1 (8)[4]	6.8±1.1* (4)[4]	13.9±2.9 (3)[3]	17.0±2.1* (2)[2]
600 mg kg^{-1}	13.6±0.9 (14)[7]	7.6±1.3* (7)[7]	19.7±2.9* (5)[5]	15.8±3.2 (3)[3]
Creatinine (micromoles per 24 h)				
Control	8.2±1.1 (8)[4]	7.5±1.3 (4)[4]	8.9±0.9 (3)[3]	7.4±0.5 (2)[2]
400 mg kg^{-1}	7.8±1.0 (8)[4]	0.6±0.2** (4)[4]	4.4±1* (3)[3]	9.8±4.1 (2)[2]
600 mg kg^{-1}	7.2±0.7 (14)[7]	1.2±0.3** (7)[7]	3.3±0.6* (5)[5]	8.6±1.9 (4)[4]
Creatine/ creatinine ratio				
Control	1.4±0.1	1.4±0.2	1.3±0.2	1.4±0.07
400mg kg^{-1}	1.4±0.2	10.9±2.7**	3.2±0.4*	1.7±0.56
600mg kg^{-1}	1.8±0.1	6.5±2.1**	5.9±3.0*	1.8±0.15

Table 3. Urinary creatine and creatinine levels measured before and after the oral administration of methoxyacetic acid (single dose 400 mg kg^{-1} and 600 mg kg^{-1} bw, p.os).

The values are means±s.e.m. of all samples collected for each group and at the different times (each sample corresponds to the urine collected in 24 h from each metabolic cage=3 mice urine pooled). They are expressed as micromoles of creatine or creatinine per 24 h.

*$p<0.05$, **$p<0.01$ significantly different from pre-treatment value (unpaired *t*-test, with Bonferroni correction).

( ): Total number of urine samples analysed. Two samples were collected for each metabolic cage in the pre-treatment period, while in the post-treatment period only one sample for each cage.

[ ]: Number of metabolic cages (each containing three mice)

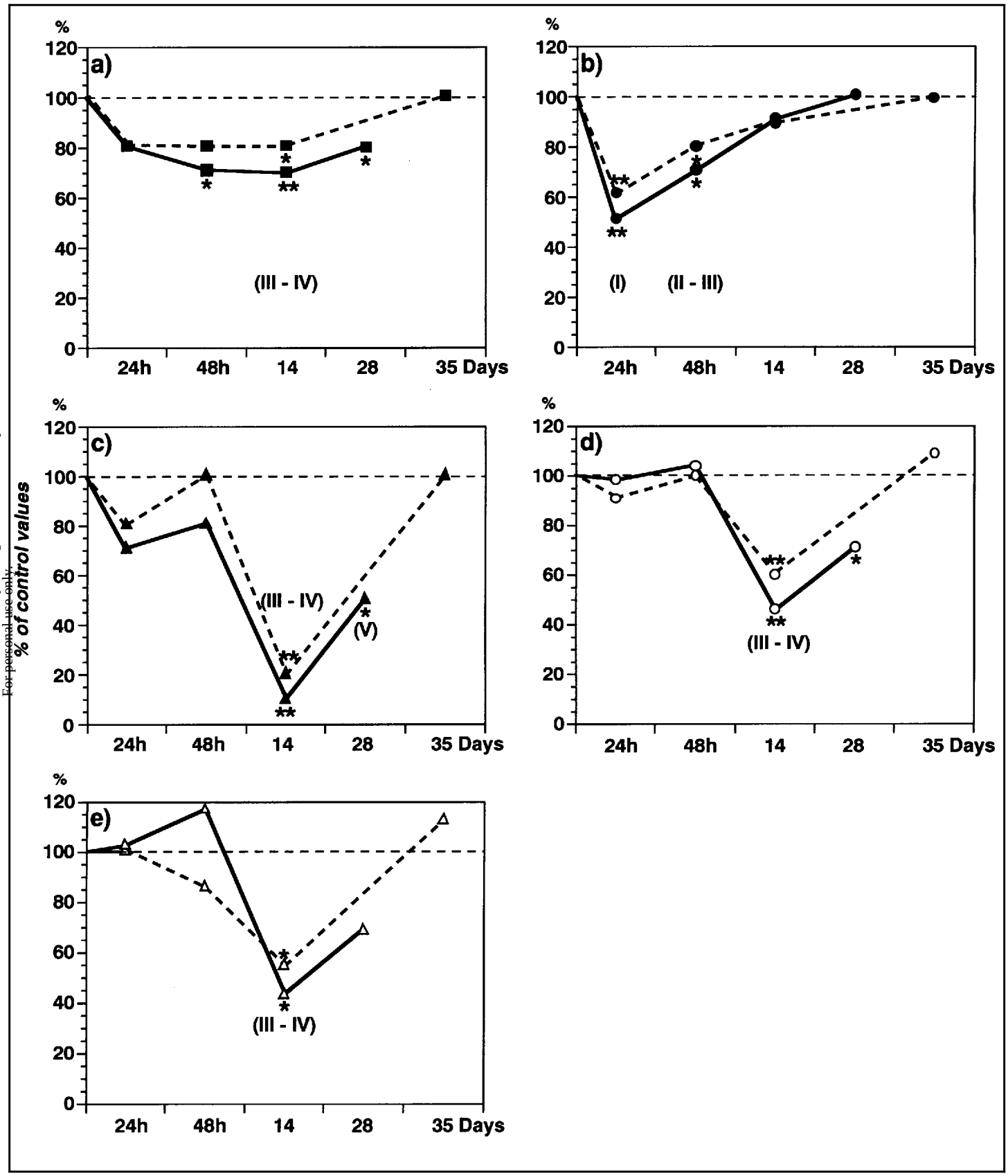

Figure 1. Percentage variation, respect to control values, of (a) testis weight, (b) testicular creatine, (c) sperm head number, (d) LDH-C4 activity, and (e) SDH activity, as a function of the 2-methoxyacetic acid treatment and the day of sacrifice. Results are means while in the interest of clarity s.e. are shown in Tables 1 and 2. – – –, 400 mg kg^{-1}; ——, 600 mg kg^{-1}. *p<0.05, **p<0.01, significantly different from control value (unpaired t-test with Bonferroni correction). Concurrent main histological alterations: (I) primary spermatocyte degeneration and necrosis; (II) depletion of spermatocytes and early round spermatids; (III) Sertoli cell vacuolation, only at 600 mg kg^{-1}; (IV) disorganization of seminiferous epithelium and depletion of elongating spermatids; (V) isolated, residual alterations in re[illegible]

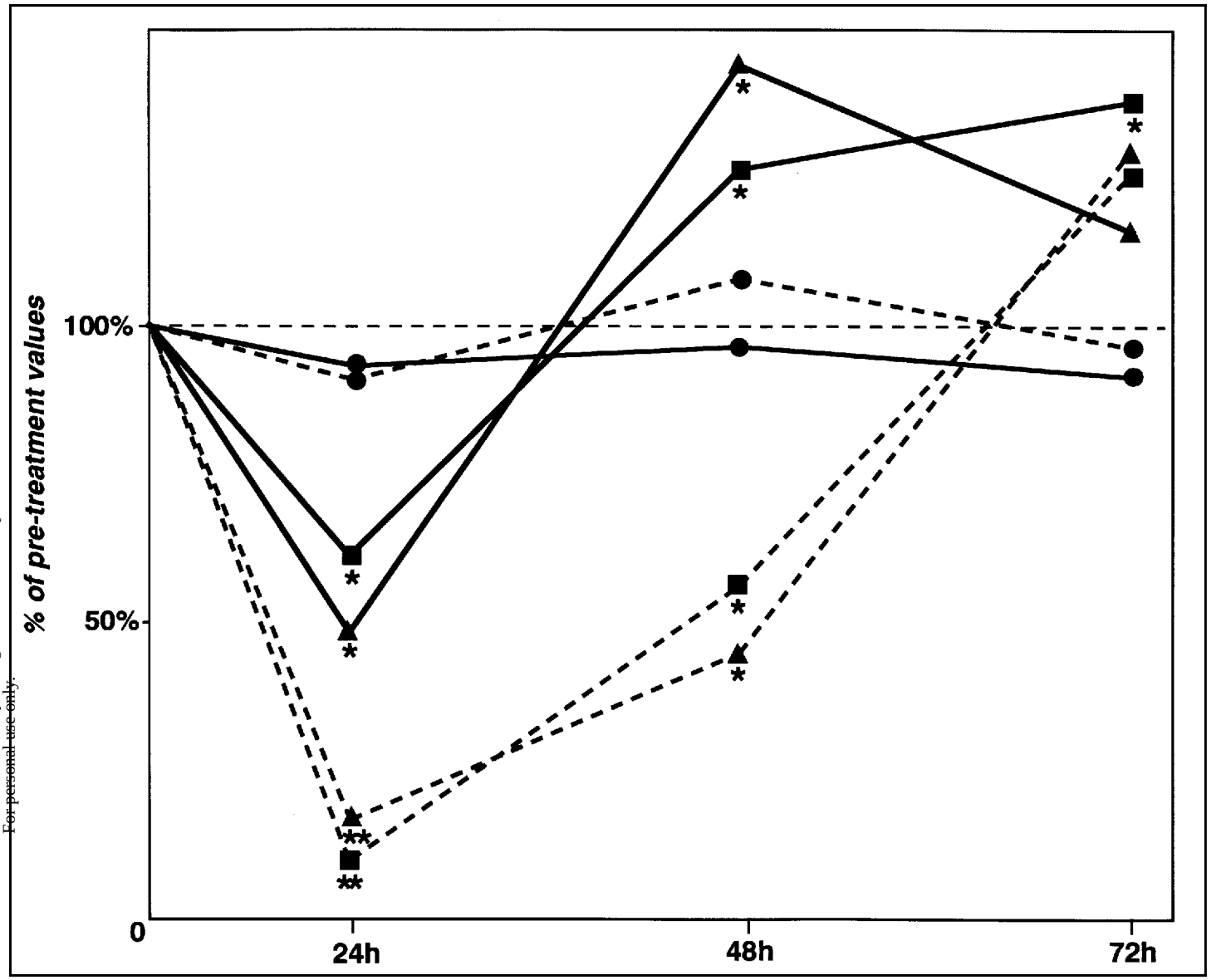

Figure 2. Percentage variation of the creatine and creatinine levels in mice urine with respect to pre-treatment values, at different times after the 2-methoxyacetic acid treatments. Results are means, s.e. are shown in Table 2. Creatine: ●—● control, ■—■ 400 mg kg^{-1}, ▲—▲ 600 mg kg^{-1}.
Creatinine: ●– – –● control, ■– – –■ 400 mg kg^{-1}, ▲– – –▲ 600 mg kg^{-1}.
*p<0.05 **p<0.01 significantly different from control value (unpaired t-test with Bonferroni correction).

The excretion of creatinine, after the initial decrease, tended to recover at 48 h after dosing and returned to pre-treatment values in the 24 h period following. When the excretion of creatine was expressed as a ratio to that of creatinine, MAA caused an increase in the ratio that was significantly different from control values, at 24 and 48 h after the treatments.

The volume of urine excreted by the treated animals showed a slight increase (around 25%) in the first 24 h after administration with respect to the pre-treatment values. This change was statistically significant (p<0.05) only for the group receiving the MAA dose of 600 mg kg^{-1} (data not shown).

Discussion

This study has shown that in the mouse testis, creatine determination resulted as the earliest quantitative indicator of testicular damage caused by MAA exposure. Twenty-four hours after the treatment the content of creatine per gram testis had significantly decreased in the two treatment groups in a more extensive manner than for testis weights and sperm head numbers. The maximum decrease in these latter parameters was reached 14 days after the treatment, as did the activity levels of LDH-C4 and SDH. This decrease in the creatine content in the testis could have been caused by a release from the testis or by an alteration in creatine synthesis. The first hypothesis was supported by Rawcliffe *et al.* (1989) and Moore *et al.* (1992): the creatinuria observed in rat urines, 24 and 48 h after dosing with 2-ME could be the consequence of the necrosis of pachytene spermatocytes. However, besides the well known cell-specificity for pachytene spermatocytes, 2-ME and MAA at high dose levels or prolonged exposure, are also seen to affect a wide range of germ cell types including spermatogonia (Lee and Kinney, 1989). In this reference pachytene spermatocytes were the o

3 day × 6 h inhalation exposure was given. Other cell types were only affected when exposure went on for 2 weeks × 6 h/day. Sertoli cell alterations, both *in vivo* and *in vitro* have been observed (Beattie *et al.* 1984, Creasy *et al.* 1986, Williams and Foster 1988); the most important ultrastructural alterations observed in Sertoli cells include cytoplasmic vacuolization, dilated intercellular space, ramified or fragmented cytoplasmic processes, and disrupted tight junctions.

The light microscopic histological alterations observed in our study were comparable to those already reported in the literature showing a dose-related severity and a time-related trend. Concurrently, when the histological alterations were correlated to the creatine levels in the testis of each mouse, a time-related decrease in creatine levels was observed up to 24 h, with a clear relationship to the occurrence of degeneration and necrosis in primary pachytene spermatocytes, possibly reflecting a steep depletion of the intracellular metabolic cycle in the target cells. This might receive further support by the findings at 48 h PD (400 and 600 mg kg^{-1}), where a relationship was observed between round spermatid depletion and lower creatine levels. At 48 h PD a generally increased Sertoli cell vacuolation after 600 mg kg^{-1} but not after 400 mg kg^{-1} was the main difference between the two dose levels with regard to histological lesions. Sertoli cell vacuolation is a non-specific finding likely to reflect phagocytosis of degenerating and necrotic cells and structural derangement of seminiferous epithelium. At 14 days PD the creatine levels in testis were not significantly different from controls; at this same time PD, marked disorganization of the layers of seminiferous epithelium, possibly related to a repopulation attempt, elongating spermatid depletion and vacuolation of Sertoli cells were observed at 400 and 600 mg kg^{-1}. The residual lesions observed in the repopulated seminiferous tubules at 28 days PD (600 mg kg^{-1}) were associated with creatine levels comparable to controls.

The synthesis of creatine has been demonstrated in the epithelia of seminiferous tubules and caput epididymis: guanidoacetate methyltransferase (GAMT), an enzyme catalysing the last step of the biosynthetic pathway to creatine and GAMT mRNA (the messenger RNA encoding GAMT), was extensively expressed in Sertoli cells of the testes and in microvilli of the epithelial cells of the epidydimis (Lee *et al.* 1994). Guanidinoacetate, the precursor of creatine, would instead be transported into the seminiferous tubules by the blood (Lee and Gerton 1995).

Therefore MAA could interfere with the metabolic pathways of creatine biosynthesis and this hypothesis is reinforced by the results obtained in mice urine: at the two doses of MAA administered, the urinary levels of creatine and creatinine were drastically decreased with respect to pre-treatment values and to control group values, 24 h after the treatments. The creatine and creatinine excreted in urine are the expression of the metabolism of creatine in the various organs where this component is synthesized (liver, pancreas, kidney, epididymis, testis) and used (approximately 95% of the total creatine pool in humans is found in skeletal muscles, while the remaining 5% is found in the heart, brain and testes; Balsom *et al.* 1994). Furthermore, when the relative distribution of EGME and its metabolite MAA were determined in mice B6C3F1, following oral and i.p. administration, by whole body autoradiographic techniques (Ahmed *et al.* 1994), the highest levels of radioactivity were detected in the liver, kidney and epididymis, all organs where creatine is synthesized.

A reduction of the creatinine content but increase in the creatine content in the urine of rats, 24 h after the administration of 2-ME and MAA was also evident, in the data reported by Rawcliffe *et al.* (1989), Moore *et al.* (1992) and Nahas *et al.* (1993). To explain this decrement it was suggested that 2-ME could disturb the interrelationship between creatine and creatinine at the biochemical level. More recently Draper and Timbrell (1996) found that both plasma and testicular creatine were significantly decreased in rat, 48 h after dosing with 2-methoxyethanol.

The data obtained in our study showed that after the intense decrease at 24 h of both creatine and creatinine, in the following period the levels of creatine tended to exceed the pre-treatment values, while this was not true for creatinine which had not completely recovered at this time. This different behaviour of the two components led to a drastic inversion of the ratio creatine/creatinine, as previously observed in rats after administration of different cell-specific testicular toxicants (Rawcliffe *et al.* 1989, Moore *et al.* 1992, Timbrell *et al.* 1994).

The increase of urinary creatine could be explained by a 'rebound effect' in the synthesis of this component. An initial but reversible inhibition occurring in the first 24 h at any step of the creatine biosynthetic pathway (for example of any enzyme involved), could cause an accumulation of the precursor and consequently more creatine could be synthesized and excreted in urine, after the inhibition recovery. Such an hypothesis could in part explain the increase of urinary creatine observed by Rawcliffe *et al.* (1989) and Moore *et al.* (1992) in rat treated with testicular toxicants. The reduction of creatine in urine might occur in rat at an earlier time than in mouse, so at 24 and 48 h after the administration of 2-ME only the increase of creatine due to the 'rebound effect' was detected. Gray *et al.* (1990) found that in the high dose cadmium-treated rats, the urinary creatine first showed a decline at 6 h after dosing and then was significantly increased at 48 h. However our hypothesis suggesting an interference of MAA with creatine synthesis, does not exclude that creatine could also be directly released by testis into blood flow and then into urine as a consequence of the testicular necrosis, as largely corroborated by the results in rat, after cadmium-chloride administration or after surgical ligation of the testicular vasculature (creatine concentration was lowered in testis, while at the same time after the treatment is raised in serum and in urine). Futhermore it is apparent from our data that MAA (especially at the highest dose) caused in mice, at 24 and 48 h PD, a significant body weight loss. Gray *et al.* (1990) showed that restricting the food to cause a significant body weight decrease resulted in an increase in urinary and plasma creatine, which was not associated with testicular damage

Therefore we cannot exclude that the increase of creatine in urine observed in mice could be partially caused by loss of weight, secondary to the general malaise of animals and reduced food intake.

Concerning sperm head number, the evaluation of this parameter affected at different treatment times, is a useful tool for both detecting the germ cell 'killing effect' of an agent and for identifying the eventual target cell in the spermatogenic process (Meistrich 1989). The maximum reduction of the elongated spermatids observed 14 days after the treatments with MAA and the tendency to recover thereafter, reflects the earlier damage to the most vulnerable testicular cell type, i.e. meiotic spermatocytes. At the same time, activity levels of LDH-C4 and SDH were more than 50% lower with respect to control values according to the decrease in sperm head numbers. It is known that these two enzymes are associated with more mature germ cells since, during the growth process, their specific activities progressively increase from late premeiotic spermatocytes to spermatids and from there to spermatozoa (Gomes and Van Demak 1974, Shen and Lee 1976). Furthermore, immunohistochemical studies (Hintz and Goldberg 1977) and studies on the synthesis of LDH-C4 in various testicular cells (Meistrich *et al.* 1977) have demonstrated the first appearance of this enzyme during the mid-pachitene stage and in spermatids.

Some considerations may be drawn from the results presented in this paper. In mice, the initial stages of germ cell damage caused by MAA, is associated with a marked decrease of creatine in testis and with a still more evident decrement of creatine and creatinine in urine. The creatinuria observed could be, in part, due to a different pattern of recovery for urinary creatine and creatinine. Further work is required to clarify whether: (1) this testicular toxicant could directly affect creatine metabolism in other biosynthetically active tissues, especially through alteration of the enzymes involved in the synthesis, (2) the effects induced by MAA on creatine and creatinine in the testis and urine of B6C3F1 mice, could be also observed with other glycol-ether derivatives. Finally this investigation may provide a basis for studying the possible utilization of the urine creatine/creatinine ratio as a biomarker, in the monitoring of workers professionally exposed to glycol ethers.

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