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Long-term administration of toxic doses of paracetamol (acetaminophen) to rats

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ABSTRACT – The effect of dosing paracetamol, 4.25 g/kg BW, twice weekly for 18 weeks was assessed in female Wistar rats 24 h after the last dose. Hepatic function, estimated as the prothrombin index, was more depressed in rats given one paracetamol dose than in chronically treated rats. Cytochrome P-450 and protein concentrations in liver homogenate and microsomes were higher in chronically treated rats. Urinary excretion of paracetamol glucuronide and mercapturate was higher and paracetamol sulfate unchanged after the chronic treatment. Hepatic glutathione was identically depleted after one dose and chronic paracetamol treatment. Histological examination of livers from chronically treated animals showed varying degrees of centrilobular necrosis. We conclude that long-term treatment with paracetamol in toxic doses leads to partial maintenance of the well-known protective effect after a few toxic doses. Signs of chronic toxicity consisted of weight loss, progressing to death. We suggest this chronic toxicity to be due to methionine/cysteine deficiency since urinary excretion of sulfur-containing paracetamol metabolites closely corresponds to calculated dietary intake of sulfur-containing amino acids.

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Paracetamol (acetaminophen) is a commonly used analgesic and antipyretic drug that has been known for almost a century (1). It is the major metabolite of phenacetin, and has gained increasing use as an alternative to aspirin since it was recognised that phenacetin had toxic properties (2).

In large doses paracetamol can cause acute, sometimes fatal, liver necrosis, and this type of acute liver damage has been almost epidemic in some countries (3). In laboratory animals liver necrosis is also found after paracetamol overdose (4).

It has been reported that a few repeated doses of paracetamol produce complete protection against the hepatotoxic action of a further dose (5), and that 1% paracetamol in drinking water, corresponding to a daily dose of 500 mg/kg BW, induces liver cell tumors in mice after 18 months (6). In rats, paracetamol 400 mg/kg BW given for 100 days led to hepatic necrosis and inhibition of growth (7), but no tumor occurrence.

In the present paper we investigated whether the protective effect of repeated dosing of paracetamol over a long period was maintained. Further, we investigated the excretion of sulfur-containing paracetamol metabolites in order to compare the consumption of cysteine/methionine by this route to the calculated dietary intake. The extent of liver damage was estimated histologically.

Material and methods

Female Wistar rats were fed Altromin® rat pellets and tap water *ad libitum*. They were housed in cages each containing 4–5 animals, light was controlled to a 12-h

Table 1
Body weight (BW, g) after chronic administration of paracetamol

	Initial BW (g)	Final BW (g)	Time to death (days)	Max BW (g)	Time (max BW) (days)
Living (n = 9)	213 ± 2	257 ± 3	126*	261 ± 4	114 ± 2
Dying (n = 5)	211 ± 3	181 ± 10	67 ± 14	224 ± 7	37 ± 9

Paracetamol, 4.25 g/kg BW, was given by stomach tube twice weekly for 18 weeks. * indicates that the animals were sacrificed at this time. Values are mean ± SEM.

light/dark cycle, and temperature and humidity were kept constant. Fifteen rats were given paracetamol in 0.2% tragacanth gum, 4.25 g/kg BW (Merck, analytical grade), by stomach tube every Monday and Thursday for 18 weeks. After the last dose nine animals were still alive. These animals and six control animals never exposed to paracetamol were placed in individual metabolism cages and urine was collected over dry ice for the following four 24-h periods for estimation of paracetamol metabolites as described below. Ninety-six hours after the last dose the animals were anesthesized with diethyl ether and a 3-mm slice of the median anterior lobe of the liver was cut, fixed in 10% formaldehyde and embedded in paraffin. Liver cell necrosis was graded 0, +, ++, +++, by light microscopy. Six-micron sections were cut and stained with hematoxylin and eosin, van Gieson, PAS, PAS after diastase-digestion and ferrocyanide reaction for iron.

In another series treated similarly, eight animals survived. When these eight paracetamol-treated animals received the last dose, eight control animals were given the same amount of paracetamol. After 24 h, all 16 animals were anesthetized with diethyl ether. Aortic blood was collected for estimation of prothrombin index (8) and the liver was removed and homogenized in a Potter-Elvehjelm glass-teflon homogenizer in iced isotonic KCl. Liver homogenate was assayed for cytochrome P-450 (9), total glutathione (10) and protein (11). Microsomes (12) were assayed for protein (11), cytochrome P-450 (9) and para-nitro-anisole demethylase activity (13).

Paracetamol and its glucuronide, sulfate, and mercapturate metabolites were estimated in urine by high pressure liquid chromatography by a modification of the method described by Moldeus (14) using a Waters HPLC system, consisting of a WISP autoinjector, a model 721 system control, a model 730 integration module, two model 6000A pumps and a 15-cm steel column (∅ 3.2 mm) packed with 5 μ Novapack (Waters) C18 reverse phase material. Pump A delivered water/acetic acid (99/1, v/v) and pump B water/methanol/ethyl acetate (90, 30, 0.1, v/v/v). Initial conditions were 100% for pump A, and 0% for pump B. This was maintained for 17 min; then a gradient (curve 5) was started ending with 10% for pump

Table 2
Excretion of paracetamol and its metabolites (μmol) in animals treated with paracetamol 4.25 g/kg once (control) or twice weekly for 18 weeks (chronic)

Period after dosage (h)	0–24	24–48	48–72	72–96
Paracetamol (p)				
Control (n = 6)	240 ± 45	157 ± 37	133 ± 34	122 ± 23
Chronic (n = 9)	209 ± 23	460 ± 45*	453 ± 51*	376 ± 57*
p-glucuronide				
Control	496 ± 89	465 ± 112	217 ± 67	381 ± 90
Chronic	1010 ± 69*	459 ± 72	112 ± 263	25 ± 13*
p-sulfate				
Control	205 ± 25	223 ± 49	95 ± 28	157 ± 28
Chronic	202 ± 16	189 ± 20	121 ± 14	103 ± 15
p-mercapturate				
Control	79 ± 15	125 ± 26	43 ± 9	82 ± 15
Chronic	163 ± 29*	163 ± 21	83 ± 9	53 ± 14
p + metabolites				
Control	1020 ± 153	971 ± 197	428 ± 85	743 ± 144
Chronic	1587 ± 127*	1274 ± 135	771 ± 202*	558 ± 85

Values are mean ± SEM. * indicates $p < 0.05$ compared to control value (Student's t-test).

A and 90% for pump B after 32 min. This composition was maintained until 35 min and then for 5 min the initial conditions were resumed. The flow rate was 1 ml/min. Retention times of paracetamol (P), P-glucuronide, P-sulfate, P-cysteine and P-mercapturate were determined from standard metabolites (kindly donated by Sterling Winthrop, Stockholm, Sweden). Quantitation of the metabolites was accomplished using the paracetamol standard curve and 4-fluophenol as internal standard (Merck) since the molar extinction coefficient of paracetamol and the metabolites is essentially the same (15). A two-sided Student's *t*-test was used for statistical comparison, choosing 0.05 as the level of significance.

Results

After the first administration of paracetamol all animals lost weight. One of 15 animals died within the first 24 h, and was excluded. Five animals stopped gaining body weight and died after progressive weight loss. The remaining nine animals continued to gain body weight until 114 days after start of treatment and were investigated after 126 days. Data are given in Table 1.

The urinary excretion of paracetamol plus paracetamol metabolites 96 h after the last dose (36th dose) was higher in chronically treated animals than in animals of the same age given an identical dose. This was mainly due to a two-fold increase in the excretion of paracetamol glucuronide ($p < 0.05$). Paracetamol mercapturate excretion was also higher in animals treated chronically ($p < 0.05$). Data are given in Table 2.

In the second series, eight animals given the chronic paracetamol treatment (i.e. surviving out of 15) were compared to eight animals of identical weight given a single dose of paracetamol. Twenty-four hours after the dosage, hepatic function, estimated as the prothrombin index, was more severely impaired after the single dose than after the 36th dose, and hepatic glutathione was equally depleted after a single and 36 doses. Hepatic and microsomal protein content was higher in chronically treated animals, and these animals also had higher concentrations of cytochrome P-450 in homogenate and microsomes, and a higher hydroxylation capacity for para nitro-anisole. Data are given in Table 3.

In Fig. 1(a–c) is shown the varying degree of changes in histological appearance of the liver after the chronic treatment with paracetamol. The nine animals all had centrilobular liver cell necrosis, three graded as +++, three graded as ++, and three had only +. There was no difference between the amount of paracetamol mercapturate excreted by animals with necrosis graded +++, ++ and +, the values being 450±110, 411±67 and 522±140 μmol (mean±SEM), respectively.

Discussion

Strubelt et al. (5) have shown that 3 days of treatment with hepatotoxic doses of paracetamol produced complete protection against the hepatotoxic actions of a further dose of paracetamol. Buttar et al. (16) have demonstrated that the intensity of

Table 3

Hepatic glutathione, cytochrome P-450 and microsomal hydroxylation of para-nitroanisole, and prothrombin index in animals treated with paracetamol 4.25 g/kg once (control) and twice weekly for 18 weeks (chronic) after 24 h

	Chronic (N=8)		Control (N=8)	
Body weight	277±10		264±3	g
Liver weight	7.4±0.3	*	8.6±0.2	g
Liver protein	285±5	**	244±6	mg
Microsomal protein	49.9±2.4	**	30.7±1.9	mg/g liver
Prothrombin index	0.81±0.06	**	0.29±0.04	arb. units
Hepatic glutathione	15.7±0.5		22.2±3.0	nmol/mg prot.
Cytochrome P-450				
Homogenate	53.0±1.7	*	42.8±2.6	nmol/g liver
Microsomes	25.3±1.1	**	16.4±1.0	nmol/g liver
p-N-anisole demethylation	27.0±1.4	**	16.9±1.1	nmol/min/g liver

Investigation was performed 24 h after dosage. Values are given as mean±SEM. * and ** indicate $p < 0.05$ and $p < 0.01$ compared to control value.

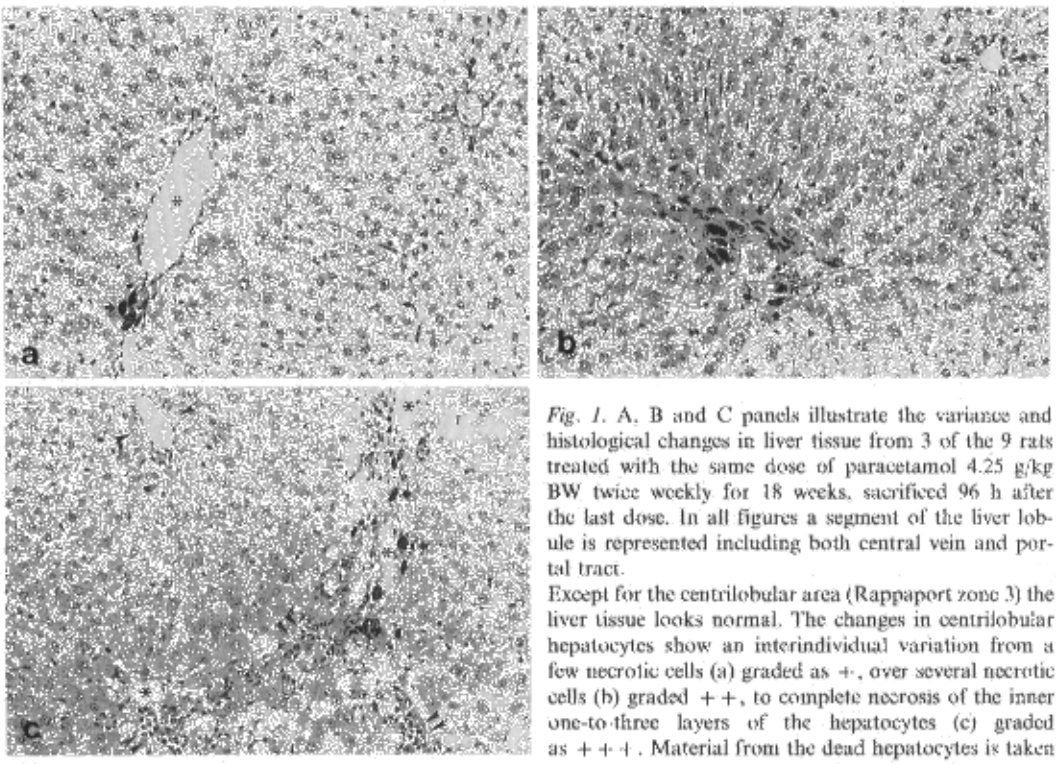

Fig. 1. A, B and C panels illustrate the variance and histological changes in liver tissue from 3 of the 9 rats treated with the same dose of paracetamol 4.25 g/kg BW twice weekly for 18 weeks, sacrificed 96 h after the last dose. In all figures a segment of the liver lobule is represented including both central vein and portal tract.

Except for the centrilobular area (Rappaport zone 3) the liver tissue looks normal. The changes in centrilobular hepatocytes show an interindividual variation from a few necrotic cells (a) graded as +, over several necrotic cells (b) graded + +, to complete necrosis of the inner one-to-three layers of the hepatocytes (c) graded as + + +. Material from the dead hepatocytes is taken up by macrophages containing ceroid (PAS-positive after diastase-digestion) and iron. In b (+ +) and c (+ + +) some collapse of the liver cell plate is observed and in c confluent necrosis and hepatocytes with severe ballooning is observed. Fibrosis was never found. (PAS-staining after diastase-digestion ×250.)

paracetamol-induced hepatotoxicity becomes less severe after repeated exposures. Apparently the protective action is only present for a few days, but some resistance remains after about a week.

In the present study we used a dose of paracetamol corresponding to LD_{10} (17), administered twice weekly, during a period of 126 days. Since only three animals out of 14 died within 90 days and two more within 126 days after start of treatment, some resistance apparently developed. Slight centrilobular necrosis of the liver was present after the last dose of paracetamol in animals surviving 126 days, demonstrating that paracetamol had some hepatotoxic effect under these conditions. After the last dose of the chronic treatment the functional impairment, evaluated by the prothrombin index, was less severe than in animals treated with a single dose of paracetamol, demonstrating that after long-term treatment hepatotoxicity is reduced.

The *in vitro* data show increased hydroxylation of para-nitroanisole after chronic treatment, suggesting an inducing effect of paracetamol on drug metabolism in the liver. A considerably increased excretion of paracetamol glucuronide and mercapturate demonstrated that the induction affected these two metabolic pathways, whereas the other major metabolic pathway of paracetamol, sulfation, was not influenced.

The amount of paracetamol and metabolites found in urine was higher in chronically treated animals. This could be due to the faster metabolism resulting in more of the dose being metabolized within the interval examined, or it could be due to residual paracetamol and metabolites from the previous dose. However, the difference is also present when calculated as per cent of total amount excreted, and is of a magnitude that can only be explained by induction of glucuronidation and some induction of cytochrome P-450, the latter in accordance with the *in vitro* findings.

Hepatic glutathione, a prerequisite for detoxif-

ication of the toxic metabolite of paracetamol formed by cytochrome P-450 oxidation (18), was depleted more, although not statistically significantly, in chronically treated animals, in accordance with the larger part of the dose excreted into urine as paracetamol mercapturate. This is suggestive of intact detoxification by glutathione conjugation in that situation.

Thus we have confirmed that long-term administration of toxic doses of paracetamol reduces its hepatotoxic effects, and demonstrated that repeated toxic doses induce glucuronidation and cytochrome P-450 activity, and we suggest that the protection under these circumstances is due to the considerably increased glucuronidation balancing out the increased cytochrome P-450 activity; the latter occurring as an isolated phenomenon should increase the hepatotoxicity.

Apart from the initial weight loss and acute death following the initial dose, most of the animals gained weight as expected (19). Some animals, however, stopped increasing their body weight, began to lose body weight and subsequently died after varying intervals, on average after 70 days. A tendency to weight loss was observed after 115 days in surviving animals. Assuming an average body weight of about 250 g during our study, the weekly food comsumption is 70 g, corresponding to about 4% of body weight per day (19). The cysteine and methionine content of the food is 3.2 and 3.4 mg per g (19), i.e. a weekly intake of 0.9 and 1.6 mmol, respectively, adding to a total of 2.5 mmol sulfur-containing amino acid intake per week under normal conditions, and presumably less in animals ill from liver damage. The formation of paracetamol sulfate and paracetamol mercapturate (Table 2) adds up to 2.2 mmol per week. Thus the major part of sulfur-containing amino acid intake may be used for paracetamol conjugation. The sulfur-containing amino acids are the only source of sulfur, and since methionine is an essential amino acid we suggest the chronic paracetamol toxicity to be a methionine/cysteine deficiency syndrome, resulting in impaired extrahepatic protein synthesis.

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