

# Autoprotection in Acetaminophen Intoxication in Rats: The Role of Liver Regeneration

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**Abstract:** Autoprotection by acetaminophen, i.e. increased resistance to toxic effects caused by pretreatment, is a well-known phenomenon. The purpose of the present work was to identify mechanisms for increased acetaminophen tolerance induced by pretreatment of rats. One group of female Wistar rats (pretreated rats) received acetaminophen orally in increasing doses (1 to 4.3 g/kg) twice a week for 3 weeks, one group (naïve rats) received the vehicle. At time zero pretreated rats received a toxic dose of 7.5 g/kg (100% lethal in naïve rats), and naïve rats received a toxic dose of 4.3 g/kg. Blood and liver tissue were collected before and 12, 24, 36, and 48 hr after the toxic dose and were analysed for hepatic glutathione and cysteine contents, hepatic glutathione-S-transferase and blood alanine aminotransferase activity, as well as acetaminophen concentration in plasma. Steady-state mRNA levels of proteins involved in acetaminophen detoxification, cell division and acute phase response were measured, liver tissue was examined for proliferating cell nuclear antigen and degree of hepatocyte necrosis. Six naïve rats not receiving acetaminophen served as controls. The mortality was the same in pre-treated and naïve rats (33 percent). Thus, pretreatment increased the tolerance twice. Before the toxic dose pretreated rats compared to control rats had higher activity of glutathione-S-transferase (liver) and alanine aminotransferase (serum), higher hepatic mRNA level of glutathione-S-transferase and  $\gamma$ -glutamylcysteine synthetase heavy and light chain subunits, and lower hepatic concentration of glutathione, cysteine and mRNA of CYP1A2 than control rats. After the toxic dose, the mRNA levels of glutathione-S-transferase,  $\gamma$ -glutamylcysteine synthetase heavy and light chain subunits, and CYP1A2 in naïve rats rose, approaching those of pretreated rats. Proliferating cell nuclear antigen labelling was high in pretreated rats, while only slightly increased in a few of the naïve rats. Necrotic hepatocytes were found at all time intervals in pretreated rats, and in naïve rats they appeared after 12 hr, peaking after 36 hr. Pretreatment increased the tolerance to acetaminophen toxicity twice, as estimated by mortality. The data indicate that pretreatment may reduce the relative production of toxic metabolites, but it primarily enhances the protection against these metabolites by regenerating hepatocytes.

Acetaminophen overdose is the most common cause of acute liver failure in many countries. The toxic effects of acetaminophen have been intensively investigated in animals. A key factor in toxicity appears to be the exhaustion of the hepatic stores of glutathione, which is used to detoxify the reactive metabolite N-acetylbenzoquinoneimine (NAPQI). NAPQI forms adducts with several cellular proteins, thereby inactivating them (Nelson 1990). The importance of this mechanism is demonstrated by the beneficial effect of treatment with N-acetylcysteine which serves to restore the amount of hepatocellular glutathione (Massey & Racz 1981; Lauterburg *et al.* 1983; Pratt & Ioannides 1985; Corcoran & Wong 1986). It is also known that other factors may modulate the toxic effects of acetaminophen on the liver and other organs, such as alcohol consumption (Sinclair *et al.* 1998) intake of other drugs (Jørgensen *et al.* 1988), dietary habits (Whitcomb & Block 1994) and inhibition of

inducible nitric oxide synthase (iNOS) (Gardner *et al.* 1998). Also preceding exposure to acetaminophen influences toxicity, so-called autoprotection, is well-known (Strubelt *et al.* 1979; Poulsen & Thomsen 1988; Shayiq *et al.* 1999). Several mechanisms for this effect have been suggested, such as decreased bioactivation due to down-regulation of microsomal enzymes (Shayiq *et al.* 1999), increased detoxification (Poulsen & Thomsen 1988), uncoupling of covalent binding from toxicity, change in type of arylated proteins (Shayiq *et al.* 1999), zonal redistribution of microsomal enzymes (Shayiq *et al.* 1999), and stimulated cell repair. Most studies have compared changes in pretreated and naïve animals receiving the same dose of acetaminophen, which raises the question whether differences in effective toxic dose are the cause of or the result of differences in observed toxicity.

The aim of the present study was to analyse responses in pretreated and naïve rats suffering a similar level of acetaminophen toxicity, as evidenced by comparable mortality. A dose causing significant (33%), but limited mortality in naïve rats, i.e. 4.3 g/kg b.wt. given intragastrically has been documented (Tygstrup *et al.* 1997). Twice that dose was

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found to cause 100% mortality in naïve rats. Pretreated rats were challenged with 7.5 g/kg, a dose that is only 33% lethal to them, but 100% to naïve rats.

### Materials and Methods

Female Wistar rats, weighing about 200 g, were kept at a 12 hr light/dark cycle and fed Altromin® pellets *ad libitum* with free access to water. Acetaminophen suspended in tragacanth was given by gastric tube in the morning after over-night fasting. Pretreated rats (N=43) received 1 g/kg of acetaminophen on day -20, 2 g/kg on day -16, 3 g/kg on day -12, 4.3 g/kg on day -10, day -6, and day -2, and 7.5 g/kg on day 0. Two rats died on day -12, 2 on day -5, and 12 after the last dose (3 before 6 hr, 7 between 6 and 12 hr, and 2 between 12 and 24 h). Five rats were killed before, 6 at 12 hr, 4 at 24 hr, 5 at 36 hr, and 5 48 hr after the 7.5 g/kg dose. Naïve rats: 43 rats received 4.3 g/kg on day 0, 13 rats died (8 between 6 and 12 hr, 5 between 12 and 24 hr). Six rats were killed before (serving as controls), and 6 at hr, 24 hr, 36 hr, and 48 hr after the 4.3 g/kg dose. The experiments were approved by the Danish Council for Supervision with Experimental Animals.

**Analysis.** Alanine aminotransferase activity (IFCC method with pyridoxal phosphate activation (Roche)) and acetaminophen concentration in serum from heart puncture were measured at the Department of Clinical Biochemistry by routine clinical chemical methods. Glutathione-S-transferase activity in plasma was measured with 1-chloro-2, 4-dinitrobenzene as a substrate in zero time samples as described by Habig *et al.* (1974). Liver tissue was collected in fluid nitrogen and stored at -80° until analysis. Glutathione and cysteine concentration in liver tissue was measured as described by Boesgaard *et al.* (1993).

Part of the tissue was fixed in paraformaldehyde, paraffin imbedded and 4 µm sections were stained with haematoxylin & eosin. Deparaffinated sections were incubated with mouse anti-proliferating cell nuclear antigen (Dako-PCNA, PC10, M879), then with biotinylated rabbit anti-mouse immunoglobulin (Dako E354). The sections were incubated with ABCComplex-HRP (Dako K355) and the peroxidase complex reaction was visualised by use of 3-amino-9-ethylcarbazole (AEC) chromogen. The number of proliferating cell nuclear antigen positive nuclear profiles was estimated, without knowledge of the coding, on each section in an Aristoplan microscope at 256 X magnification in four counting frames measuring 0.12 mm<sup>2</sup> (approximately 800 cells in each frame). The counting frames were positioned in the areas where the staining was most intensive, normally in the centrilobular zone. The number of proliferating cell nuclear antigen positive nuclear profiles was classified as grade 0: no hepatocyte stained, grade 1: >0 to <2% of hepatocytes stained, grade 2: 2 to <5% of hepatocytes stained, grade 3: 5 to <20% of hepatocytes stained, grade 4: 20 to <50% of hepatocytes stained, and grade 5: > 50% of hepatocytes stained. On the haematoxylin & eosin stained sections the degree of necrosis was graded as follows: 0: no necrotic hepatocytes, 1: scattered single cell necrosis, 2: small foci of cell necroses, 3: large foci of cell necroses, and 4: necrotic areas bridging centrilobular and periportal spaces.

About 200 mg of liver tissue was taken from the same site and stored in liquid nitrogen until total RNA was isolated using a Promega kit Z 5110 as previously described (Tygstrup *et al.* 1997).

The following cDNA probes were used: 1. *Related to acetaminophen toxicity:* glutathione-S-transferase (GST π subunit 7, X02904 (Genbank) PCR amplified cDNA with the following primers: sense 23-43, antisense 602-621, cloned via the TaqA overhang (pMOS kit, Amersham), identity verified by sequencing), γ-glutamylcysteine synthetase, light chain (GCSlc) (Huang *et al.* 1998), γ-glutamylcysteine synthetase, heavy chain (GCShc) (Mulcahy *et al.* 1997), CYP1A2 (Kimura *et al.* 1984), and CYP2E1 (McLaughlin *et al.* 1992), 2. *Related to cell division:* c-fos (Curran *et al.* 1983) and histone 3 (His3) (Taylor *et al.* 1986). Specificity of the probes was

ascertained by autoradiography of northern blots, showing signals from hybridised mRNA at the expected position in relation to ribosomal RNA 18S and 28S. This also ensured that RNA was not degraded.

Restriction enzymes were from Promega and Boehringer. DNA fragments (inserts) were separated by agarose gel electrophoresis. Determination of mRNA levels by slot blot analysis was performed according to the previously described protocol (Tygstrup *et al.* 1997).

The membranes were prehybridised for 30 min. at 68° and hybridised at 68° for 1 hr. Filters were washed twice for 15 min. at room temperature by 2×SSC and 0.1% SDS, and for 30 min. at 60° by 0.1×SSC and 0.1% SDS.

Autoradiography was made on an imaging plate BASIII (under lead shield) and the hybridisation signal analysed in a FUJIX i-maging analyser system BAS 2000 (FUJI Photo Film Co). Afterwards filters were immersed in boiling SDS 0.1% and glycerol 1%, left to cool and washed in sterile water, and then re-hybridised with cDNA for 18S rRNA, used to compensate for variations in RNA loading, the median variation being 9%.

**Statistics:** Data are expressed as per cent of the mean of 6 control rats receiving vehicle and no acetaminophen. Comparison between groups before a toxic dose was given has been analysed by t-statistics (table 1). Comparisons of changes in time between the groups was analysed by two-way analysis of variance ('group' is difference between groups irrespective of time, 'time' is differences between points in time irrespective of group, and 'interaction' is difference in change over time between the groups (table 2). P values <0.05 were considered statistically significant.

### Results

Comparable toxicity of acetaminophen (7.5 mg/kg b.wt. given to pre-treated rats and 4.3 mg/kg b.wt. to naïve rats) is demonstrated by similar (33%) mortality rates in both groups (fig. 1).

Table 1.

Differences between pretreated rats and naïve rats before administration of the toxic dose of acetaminophen.

Variable		Pre-treated (n=5)	naïve (n=6)
PCNA	Score	3.4±0.4*	0±0
Necrosis	Score	2.1±0.3*	0±0
ALT activity	U/L	124±8*	54±2
Glutathione contents	µmol/g	3.9±0.1*	6.7±0.3
Cysteine contents	µmol/g	0.17±0.03*	0.25±0.01
GST activity	µmol/min./g	42.8±2.2*	25.8±0.4
GST mRNA	Arb. units	1260±451*	100±8
GCSlc mRNA	Arb. units	379±96*	100±9
GCShc mRNA	Arb. units	266±79*	100±17
CYP1A2 mRNA	Arb. units	44±12*	100±18
Histone 3 mRNA	Arb. units	144±12*	100±13

Mean values and standard error (S.E.M.) of observations with significant difference between rats pretreated with acetaminophen in sublethal doses for 3 weeks (pretreated rats, N=4-6) and naïve rats (N=6). (ALT=alanine aminotransferase, GST=glutathione-S-transferase π subunit 7, GCS=γ-glutamylcysteine synthetase, lc=light chain subunit, hc=heavy chain subunit). \*P<0.05.

Abbreviations: PCNA: proliferating cell nuclear antigen, NAPQI: N-acetylbenzoquinoneimine, GST: glutathione-S-transferase π subunit 7, GCSlc: γ-glutamylcysteine synthetase, light chain subunit, GCShc: γ-glutamylcysteine synthetase, heavy chain subunit, His3: histone 3, ALT: alanine aminotransferase

Table 2.

Statistical analysis (two-way analysis of variance) of differences between groups of pretreated rats receiving 7.5 g/kg b.wt. of acetaminophen and naïve rats receiving 4.3 g/kg b.wt. of acetaminophen.

Variable	Group	Time	Interaction
PCNA	<0.001	<0.001	0.002
Necrosis	<0.001	NS	<0.001
ALT	0.009	NS	NS
Acetaminophen	NS	<0.001	<0.001
Glutathione	0.002	0.027	0.008
Cysteine	NS	0.032	0.011
GST	<0.001	NS	0.011
GCSlc	NS	0.015	0.002
GCShc	<0.001	<0.001	<0.001
CYP1A2	0.03	0.003	0.018
CYP2E1	NS	0.003	NS
c-fos	NS	0.003	0.001
Histone 3	NS	<0.001	0.008

Data are shown in fig. 2 to 5. 'Group' relates to the probability that data from pretreated rats and naïve rats are different, irrespective of time, 'Time' relates to the probability that data during the experimental period are different, irrespective of group, and 'Interaction' relates to the probability that data from pretreated rats and naïve rats vary differently during the experimental period. NS signifies  $P \geq 0.05$ . For abbreviation of variable names see fig. 2 to 5.

Before administration of the toxic dose the level of mRNA of glutathione-S-transferase and  $\gamma$ -glutamylcysteine synthetase (both the light and heavy chain subunits) in the liver and the activity of alanine aminotransferase in plasma were significantly higher in pretreated than in naïve rats. The concentration of cysteine and glutathione, the activity of glutathione-S-transferase, and mRNA of CYP1A2 in the

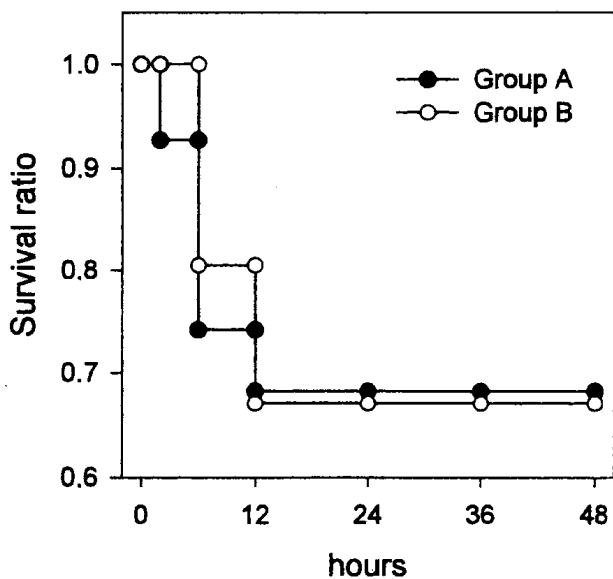


Fig. 1. Survival curves (timetable method) of rats pretreated with acetaminophen following a toxic dose of 7.5 g/kg b.wt. of acetaminophen (●) and of naïve rats after a toxic dose of 4.3 g/kg b.wt. of acetaminophen (○). Dots show means, error bars indicate standard error of the mean.

liver were significantly lower in pretreated than in naïve rats (table 1).

During the experimental period, as shown in fig. 3A and B, the concentration of glutathione in the liver was rising in pretreated rats and fell initially in naïve rats, and similar, less pronounced changes were seen for the concentration of cysteine. Acetaminophen was found in low concentration in the serum in pretreated rats and stayed at that level throughout the experiment (fig. 3C). In naïve rats high values were seen at 12 hr only. Alanine aminotransferase activity in serum was increased in pre-treated rats and rose in naïve rats (fig. 3D). The necrosis score (fig. 2B) was elevated in pre-treated rats before the toxic dose was given, rose to a maximum after 24 hr and then declined. In naïve rats the levels were lower the first 24 hr and later rose to levels similar to pretreated rats. The correlation coefficient between alanine aminotransferase level and necrosis score was +0.62.

The mRNA level of  $\gamma$ -glutamylcysteine synthetase light and heavy chain subunit and glutathione-S-transferase was

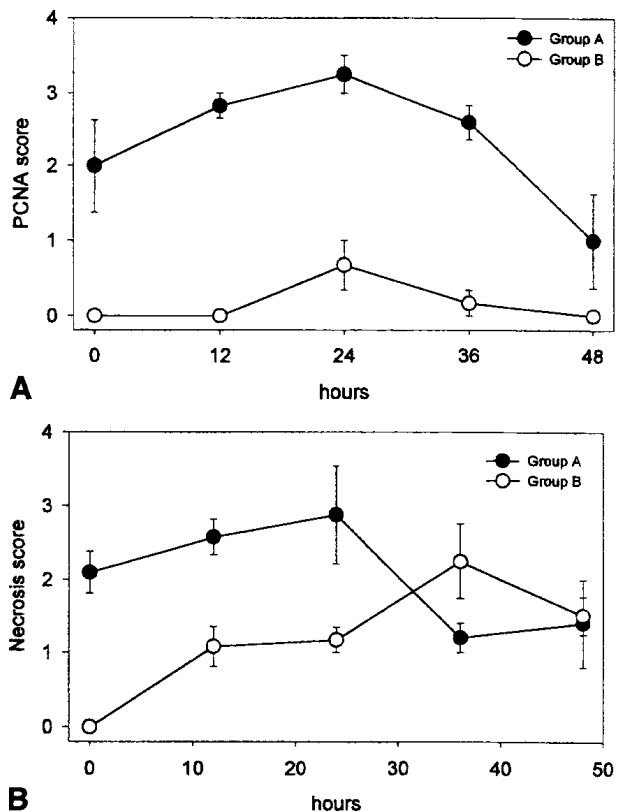


Fig. 2. Hepatocytes stained for proliferating cell nuclear antigen (panel A) before and after a toxic dose of 7.5 g/kg b.wt. of acetaminophen to rats pretreated with acetaminophen (●), and before and after a toxic dose of 4.3 g/kg b.wt. of acetaminophen to naïve rats (○). Histological liver cell necrosis (panel B) before and after a toxic dose of 7.5 g/kg b.wt. of acetaminophen to rats pretreated with acetaminophen (●), and before and after a toxic dose of 4.3 g/kg b.wt. of acetaminophen to naïve rats (○). Dots show means, error bars indicate standard error of the mean. Definition of scores is given in Materials and Methods. Statistics: see table 1 and 2.

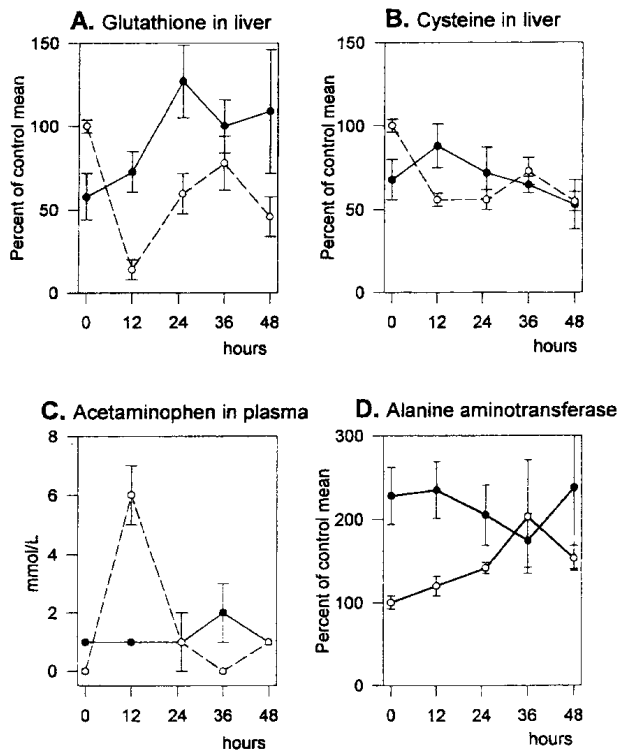


Fig. 3. The concentration of glutathione (panel A) and cysteine (panel B) in the liver, the concentration of acetaminophen (panel C) and alanine aminotransferase activity (panel D) in plasma before and after a toxic dose of 7.5 g/kg b.wt. of acetaminophen to rats pretreated with acetaminophen (●), and before and after a toxic dose of 4.3 g/kg b.wt. of acetaminophen to naive rats (○). Dots show means, error bars indicate standard error of the mean. Statistics: see table 1 and 2.

decreased from elevated levels of pretreated rats (fig. 4). In naive rats the mRNA level of  $\gamma$ -glutamylcysteine synthetase heavy chain subunit increased seven times above the control after 12 hr. The mRNA level of CYP1A2 (fig. 5A) fell to low values at 24 hr in both groups and was partially restored at 48 hr. Changes were much smaller for CYP2E1 mRNA (fig. 5B).

The mRNA levels of the cell-division related proteins Fos and Histone 3 were sharply increased at 12 hr in naive rats, both with highly significant interaction (fig. 5C and D). The mean proliferating cell nuclear antigen score (fig. 2A) was high before administration of the toxic dose in pretreated rats and increased further to a maximum after 24 hr and then declined, in naive rats, proliferating cell nuclear antigen positive cells were only found after 24 hr, and none after 48 hr.  $^3\text{H}$ -Thymidine incorporation in DNA was significantly increased in only two naive rats at 24 hr (data not shown).

### Discussion

This study compared hepatic responses to acetaminophen in naive and pretreated rats suffering a similar degree of acetaminophen toxicity. Using mortality as a measure of

toxicity, similar survival (fig. 1) of naive rats receiving 4.3 g/kg of acetaminophen and of pretreated rats receiving twice that dose indicates a redoubled increase in tolerance from pretreatment.

Liver damage is the most conspicuous but not the only toxic effect of an acetaminophen overdose. Although it is not known whether liver damage was the immediate cause of mortality, the increased tolerance resulting from pretreatment is likely to be related to changes in the response of the liver to the toxin.

Acetaminophen toxicity is mostly attributed to the reactive metabolite of acetaminophen NAPQI, which forms adducts with and inactivates proteins necessary for the function and survival of liver cells (Nelson 1990). Thus likely

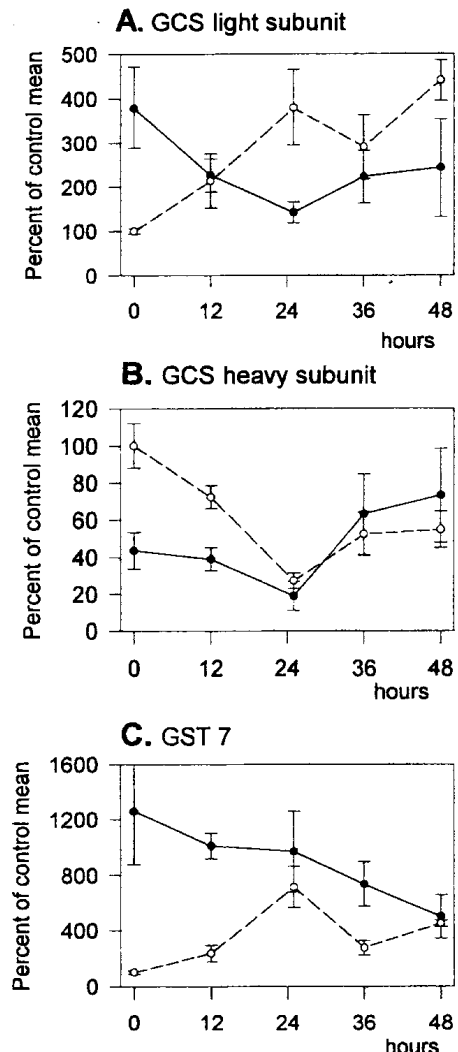


Fig. 4. Messenger RNA steady-state level in the liver of  $\gamma$ -glutamylcysteine synthetase light chain subunit (GCSlc) (panel A), heavy chain subunit (GCShc) (panel B), and glutathione-S-transferase (GST  $\pi$  subunit 7) (panel C) before and after a toxic dose of 7.5 g/kg b.wt. of acetaminophen to rats pre-treated with acetaminophen (●), and before and after a toxic dose of 4.3 g/kg b.wt. of acetaminophen to naive rats (○). Dots show means, error bars indicate standard error of the mean. Statistics: see table 1 and 2.

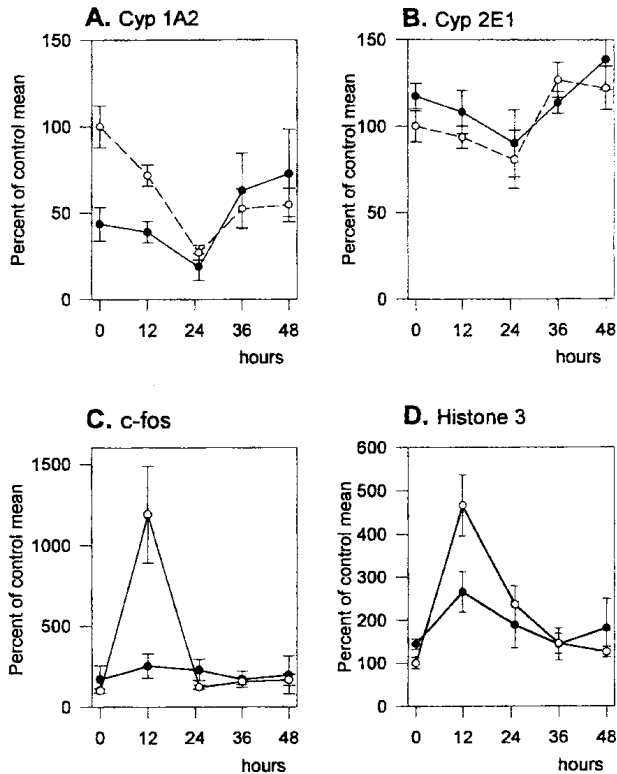


Fig. 5. Messenger RNA steady-state level in the liver of CYP1A2 (panel A), CYP2E1 (panel B), c-fos (panel C), and histone 3 (panel D) before and after a toxic dose of 7.5 g/kg b.wt. of acetaminophen to rats pretreated with acetaminophen (●), and before and after a toxic dose of 4.3 g/kg b.wt. of acetaminophen to naïve rats (○). Dots show means, error bars indicate standard error of the mean. Statistics: see table 1 and 2.

causes for an increased tolerance are relatively reduced formation of NAPQI (i.e. ‘bioactivation’ mediated by the microsomal CYP enzymes) and/or enhanced detoxification of NAPQI (i.e. ‘protection’ by conjugation with glutathione), or replacement of inactivated proteins.

Data concerning the role of bioactivation in tolerance are inconclusive. In the present study the expression of CYP1A2 was reduced in pretreated rats, whereas that of CYP2E1 was practically unchanged (fig. 5B). CYP2E1 is considered to be the most active enzyme in this respect (Morgan *et al.* 1983). In one study CYP1A2 knockout mice showed increased tolerance to acetaminophen (Sinclair *et al.* 1998), while another study did not confirm this (Tonge *et al.* 1998). There is therefore little evidence that reduced bioactivation contributes significantly to the increased tolerance to acetaminophen toxicity in pretreated rats.

Acetaminophen was more rapidly eliminated from the blood in pretreated rats (fig. 3C), possibly leading to higher NAPQI production. Chronic administration has been found to enhance the elimination rate of acetaminophen and to increase the excretion of the mercapturate metabolite (Poulsen & Thomsen 1988), an indicator of NAPQI formation. Furthermore, in mice pretreatment with acetaminophen did not reduce the formation of the 3 (cysteine-S-yl) acetaminophen-protein adduct (Shayiq *et al.* 1999).

Increased elimination of NAPQI, the alternative explanation for ‘autoprotection’, assumes an increased availability of glutathione. Before the toxic dose was given, the glutathione content in the liver of pretreated rats was reduced to 60% of that of the controls (fig. 3A), indicating depletion of the cytosolic (extranuclear and -mitochondrial) pool (Bellomo *et al.* 1992; Meredith & Reed 1982). This may be the result of either low synthesis rate or high consumption rate of glutathione. The finding of significantly increased expression of both subunits of  $\gamma$ -glutamylcysteine synthetase in pretreated rats (fig. 4A and B) is more consistent with increased synthesis of glutathione, and this, together with the increased activity (table 1) and expression of glutathione-S-transferase (fig. 4C), strongly suggests that the turn-over rate of glutathione was accelerated in the pretreated rats. In these pretreated rats the hepatic glutathione content was restored to control levels 24 hr after the toxic dose, in naïve rats recovery lagged behind, awaiting stimulated transcription of the  $\gamma$ -glutamylcysteine synthetase gene (fig. 4A and B). Our interpretation is that the liver of pretreated rats has been programmed to increase capacity to synthesise glutathione, possibly by feedback regulation from the reduced GSH

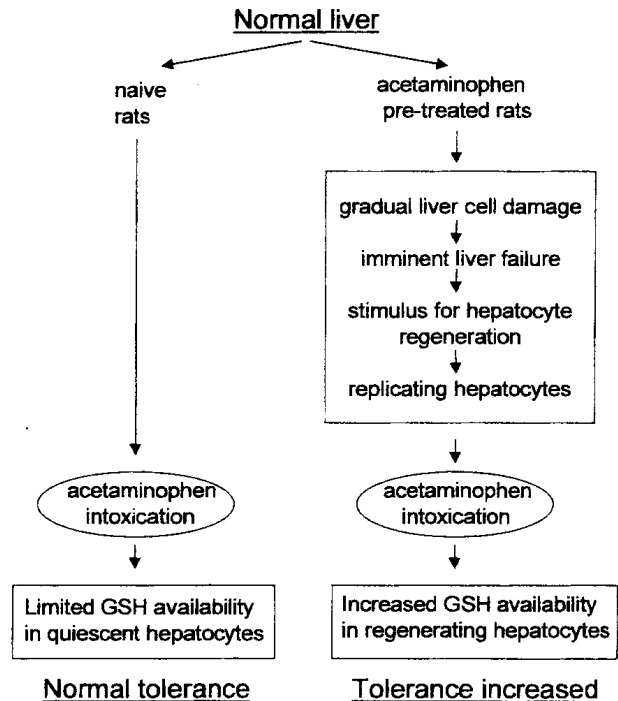


Fig. 6. Hypothetical effect of treatment with increasing doses of acetaminophen on the tolerance to a sublethally toxic dose of acetaminophen. In naïve rats the tolerance is determined by the availability of GSH in quiescent hepatocytes for detoxification of reactive metabolites. In the acetaminophen pretreated rats the drug has caused sufficient liver damage to induce hepatocyte regeneration. Regenerated hepatocytes have greater capacity for GSH production and thus for detoxification of reactive metabolites so that greater toxic doses are tolerated. Also quiescent hepatocytes may benefit due to a “by-stander effect”. In naïve rats regeneration is initiated, but too late to rescue the liver.

level (Meister & Anderson 1983), thereby increasing the tolerance to acetaminophen toxicity.

It is noteworthy, however, that this redoubled increase in tolerance in pretreated rats over that of the naïve rats is achieved by a liver which is obviously damaged by the pretreatment, as shown by the serum levels of alanine aminotransferase (fig. 3D) and histological hepatocellular necroses (fig. 2B). Besides, the livers of the pretreated rats were engaged in replication, apparently attempting to replace damaged hepatocytes by regeneration, revealed by numerous proliferating cell nuclear antigen positive hepatocytes (Assy *et al.* 1998) (fig. 2A). A regenerative response was also elicited in native rats after the toxic dose, shown by increased expression of c-fos and histone 3 (fig. 5C and D), but few proliferating cell nuclear antigen positive hepatocytes were found (fig. 2A). In mice, protection against high doses by pretreatment was blocked if colchicine was given in antimetabolic doses (Mangipudy *et al.* 1996). Resistance to toxicity in dividing hepatocytes has been demonstrated, using thioacetamide as inducer of replication and CCl<sub>4</sub> as toxin (Manautou *et al.* 1996). Our data indicate that acetaminophen may act as inducer as well as toxin.

Replicating hepatocytes may contribute to increased tolerance against toxins simply by replacing damaged hepatocytes at a rate that keeps the functional liver mass above the limit needed for survival. This process will be enhanced if regenerating hepatocytes are less sensitive to toxins, as suggested by Roberts *et al.* (1983) and supported by demonstration of reduced concentration of several CYP proteins (Favre *et al.* 1998) and their mRNA levels (Tygstrup *et al.* 1996) in the liver regenerating after partial hepatectomy. What may be more important, however, is the substantial increase in  $\gamma$ -glutamyl cysteine synthetase activity in the hepatectomy model (Huang *et al.* 1998), since this, if it applies to regenerating hepatocytes in the present model, may increase the supply of glutathione by increasing the capacity to synthesise it in endangered, not replicating hepatocytes (Mitchell *et al.* 1973). Further evidence of higher resistance to acetaminophen in dividing hepatocytes is found in the observation that glutathione conjugation is several times higher in weanling than in adult rats (Allameh *et al.* 1997), and perhaps also in the protective effect of treatment with peroxisome proliferator (Manautou *et al.* 1996).

Finally the possibility of selection bias must be considered, i.e. that the pretreatment eliminated the more sensitive rats from the trial. However, since only 4 rats died during the pretreatment, this is not likely to be the case.

We conclude that our data are consistent with the view that the increased tolerance to acetaminophen in pretreated rats is due to increased glutathione availability from regenerating hepatocytes (fig. 6).

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