

Glutathione, glutathione-dependent enzymes and antioxidant status in erythrocytes from children treated with high-dose paracetamol

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Aim To investigate glutathione and antioxidant status changes in erythrocytes from febrile children receiving repeated suprathreshold paracetamol doses.

Methods Fifty-one children aged 2 months to 10 years participated in the study. Three groups were studied: group 1 ($n = 24$) included afebrile children who did not receive paracetamol; and groups 2 ($n = 13$) and 3 ($n = 14$) included children who had fever above 38.5°C for more than 72 h. Patients in group 2 received paracetamol at a dose of 50 ± 15 (30–75) mg kg⁻¹ day⁻¹ and those in group 3 received paracetamol above the recommended therapeutic dose, ie 107 ± 28 (80–180) mg kg⁻¹ day⁻¹. A blood sample was taken for the measurement of liver transaminases, gamma-glutamyl transferase (GGT), reduced glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPX), glutathione S-transferase (GST), superoxide dismutase (SOD) and antioxidant status.

Results Aspartate aminotransferase activity in group 3 was higher than in the other groups ($P = 0.027$). GSH, SOD and antioxidant status were significantly lower in group 3 compared with groups 1 and 2 (mean differences: for GSH 3.41 $\mu\text{mol gHb}^{-1}$, 95% confidence interval (CI) 2.10–4.72, and 2.15 $\mu\text{mol gHb}^{-1}$, 95% CI 0.65–3.65, respectively; for SOD 856 U min⁻¹ gHb⁻¹, 95% CI 397–1316, and 556 U min⁻¹ gHb⁻¹, 95% CI 30–1082, respectively; and for antioxidant status 0.83 mmol l⁻¹ plasma, 95% CI 0.30–1.36, and 0.63 mmol l⁻¹ plasma, 95% CI 0.02–1.24, respectively). GR activity was significantly lower in groups 3 and 2 in comparison with group 1 (mean differences 3.44 U min⁻¹ gHb⁻¹, 95% CI 0.63–6.25, and 5.64 U min⁻¹ gHb⁻¹, 95% CI 2.90–8.38, respectively). Using multiple regression analysis, paracetamol dose was found to be the only independent variable affecting GR, GST and SOD activities ($P = 0.007$, 0.003 and 0.008, respectively).

Conclusions In febrile children, treatment with repeated suprathreshold doses of paracetamol is associated with reduced antioxidant status and erythrocyte glutathione concentrations. These significant changes may indicate an increased risk for hepatotoxicity and liver damage.

Keywords: antioxidant status, febrile children, glutathione changes, glutathione-dependent enzymes, repeated paracetamol

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Introduction

Paracetamol is the most frequently used over-the-counter medication in children in the USA [1]. Paracetamol toxicity is rare when it is given in recommended doses. However, repeated suprathreshold doses of paracetamol given for therapeutic reasons may cause hepatotoxicity in adults and children [2–5]. Heubi *et al.* [5] described a

series of 47 children who developed severe hepatotoxicity after receiving multiple therapeutic doses of paracetamol. Twenty-four (55%) died and three survived after liver transplantation. In a case series from a paediatric liver transplant centre in Australia 11 out of 18 cases of liver failure were due to chronic paracetamol exposure [6].

The major metabolic pathways of paracetamol are glucuronidation and sulphation. A minor pathway, catalysed by cytochrome P450, produces a highly toxic metabolite, N-acetyl-*p*-benzoquinoneimine (NAPQI), which is detoxified in the hepatocytes by the glutathione system.

The aim of this study was to compare prospectively glutathione, glutathione-dependent enzymes and antioxidant status between febrile children taking repeated doses of paracetamol (normal or supratherapeutic) and afebrile children who had not received paracetamol.

Patients and methods

The patients comprised a prospective cohort of children 2 months to 10 years of age who presented to the paediatric emergency department and paediatric ambulatory care unit.

The study was approved by the institutional review board for human subject research at the University of Tel-Aviv. Informed written consent was obtained from parents before enrolment.

Three groups of children were studied. Group 1 consisted of afebrile children who did not receive paracetamol and were examined for noninfectious causes. Group 2 consisted of children who had fever above 38.5°C for more than 72 h and had been receiving repeated doses of paracetamol up to 75 mg kg⁻¹ body weight per day during this period. Group 3 consisted of children who had fever above 38.5°C for more than 72 h and received repeated doses of paracetamol exceeding a total daily dose of 75 mg kg⁻¹ body weight.

Patients were excluded from the study if they had known chronic liver disease, suspected viral hepatitis, or treatment with a potentially hepatotoxic drug or with drugs that induce cytochrome P450.

In every child presenting to the paediatric emergency department, a careful history regarding paracetamol administration was taken from the parent or carer. If the reported total daily dose of paracetamol administered was more than 30 mg kg⁻¹ per day for more than 72 h, a questionnaire was filled in by the paediatrician after interviewing the family. The questionnaire included information on the duration of fever, paracetamol doses, frequency and route of administration, and other drugs administered to the child. The total daily dose was verified by asking the parents to recall how many packages of paracetamol were used during the current illness.

Patients for the control group (no fever and no paracetamol treatment) were recruited from the emergency department and from the ambulatory paediatric unit.

Blood was taken from each child, and analysed for aspartate aminotransferase (AST), alanine aminotransferase (ALT), gammaglutamyl transferase (GGT), alkaline phosphatase (ALP), urea, creatinine and uric acid. Reduced glutathione (GSH), glutathione reductase (GR), glutathione peroxidase (GPX), glutathione S-transferase (GST) and superoxide dismutase (SOD) were measured in erythrocytes. Antioxidant status was determined in plasma.

Biochemical analysis

Peripheral blood was collected into heparin vacutainer tubes and immediately transferred on ice to the laboratory. The erythrocyte lysate was prepared according to the method of Beutler *et al.* [7] and rapidly frozen to -70°C.

All activity assays were performed on a Cobas Mira Random Access Chemistry Analyser (F. Hoffmann LaRoche, Ltd. Diagnostic Division, Basel, Switzerland). GPX activity was measured at 340 nm as NADPH oxidized using a commercial kit with cumene hydroperoxide as substrate (Randox Laboratories Ltd, San Francisco, CA, USA). One activity unit was defined as 1 µmol NADPH oxidized min⁻¹ l⁻¹ haemolysate. The lower detection limit was 2.9 µmol l⁻¹ min⁻¹, and the coefficient of variation for the assay was 1.85% at 1048.71 U l⁻¹. GR activity was determined at 340 nm using a commercial kit (Glutathione-reductase; R&D Systems, Oxis International Inc., Portland, OR, USA). One activity unit was defined as 1 µmol NADP (NADPH) formed min⁻¹ l⁻¹ haemolysate. The lower detection limit was 0.14 µmol l⁻¹, and the coefficient of variation was 3% at 2 µmol l⁻¹. GSH content was determined with dithionitrobenzoic acid using the method described by Beutler *et al.* [8] and was expressed in µmol GSH l⁻¹ haemolysate. The lower detection limit was 5 µmol l⁻¹, and the coefficient of variation was 1% at 20 µmol l⁻¹. SOD was determined using a commercial kit (Randox Laboratories, Ltd) at 500 nm as the inhibitor of xanthine oxidation by xanthine oxidase. Activity was defined as SOD U l⁻¹ haemolysate according to the standard provided in the kit. The lower detection limit was 0.2 U l⁻¹, and the coefficient of variation was 3.8% at 236 U l⁻¹. GST was determined at 340 nm using 1-chloro-2,4-dinitrobenzene as a substrate according to the method described by Habig *et al.* [9]. One activity unit was defined as 1 µmol complex formed min⁻¹ l⁻¹ haemolysate. The lower detection limit was 7 µmol min⁻¹ l⁻¹ haemolysate, and the coefficient of variation was 5–11% at 7 U l⁻¹. Antioxidant status was measured in

plasma using the method described by Miller *et al.* [10]. AST and ALT were measured in the serum using a chemistry analyser (ILAB, International Laboratories, Milan, Italy).

Statistical analysis

Groups were compared by one-way analysis of variance with *post hoc* Tukey test for normally distributed variables and by Kruskal–Wallis one-way analysis of variance on ranks with Dunn's method for non-normally distributed variables. Multiple linear regression was used to determine the effect of independent variables on the outcomes. Results are presented as mean \pm SD or median as appropriate. *P*-value of <0.05 was considered significant.

Results

Fifty-one children participated in the study. Group 1 included 24 children aged 6 ± 3 years who were afebrile and did not receive paracetamol. They presented to the hospital for inguinal hernia repair ($n = 12$), abdominal pain ($n = 8$), and cervical lymphadenopathy ($n = 4$). Group 2 included 13 children aged 3.5 ± 2.6 years with fever above 38.5°C who received paracetamol within the recommended dose [50.3 ± 15 (30–75) mg kg⁻¹ body weight per day] for more than 72 h [4.1 ± 1.8 (3–10) days]. Group 3 included 14 children aged 2.2 ± 1.8 years with fever above 38.5°C who received paracetamol above the recommended therapeutic dose [107 ± 28 (80–180) mg kg⁻¹ body weight per day] for more than 72 h [3.9 ± 1.7 (3–9) days]. All the children in this series received paediatric preparations of paracetamol. The majority of the children in group 3 received high dose of the drug because it was given at intervals shorter than recommended or because parents gave more than the recommended dose in an attempt to control the fever.

The ages, weight, sex, duration of fever and time from the last dose of paracetamol for the different groups are presented in Table 1.

There was no significant difference in the duration of

fever or time from the last dose of paracetamol ($P = 0.7$ and $P = 0.94$, respectively), or admission rates between the groups.

Patients in the control group were older in comparison with patients in groups 2 and 3 ($P = 0.042$ and $P < 0.001$, respectively). The difference in ages between groups 2 and 3 was not statistically significant ($P = 0.38$).

There were no cases of severe hepatotoxicity. The median AST activity in patients treated with high-dose paracetamol was 55 IU compared with 29 IU and 28 IU in groups 1 and 2, respectively ($P = 0.027$). There were no significant differences between the groups with respect to urea, creatinine, uric acid, ALT, GGT or ALP.

GSH, GST, GR, GPX and SOD levels in erythrocytes are presented in Table 2.

Erythrocyte GSH content (Figure 1) was significantly lower in group 3 compared with groups 1 and 2 (mean differences $3.41 \mu\text{mol gHb}^{-1}$, 95% CI 2.10–4.72, and $2.15 \mu\text{mol gHb}^{-1}$, 95% CI 0.65–3.65, respectively; $P < 0.001$ and $P = 0.003$, respectively). Although patients in group 2 had lower GSH content than patients in the control group, this difference was not significant (mean difference $1.26 \mu\text{mol gHb}^{-1}$, 95% CI 0.09–2.10, $P = 0.071$).

GR activity was significantly lower in febrile patients exposed to recommended and supratherapeutic doses of paracetamol in comparison with controls (mean differences $3.44 \mu\text{mol min}^{-1} \text{gHb}^{-1}$, 95% CI 0.63–6.25, and $5.64 \mu\text{mol min}^{-1} \text{gHb}^{-1}$, 95% CI 2.90–8.38, respectively; $P = 0.013$ and $P < 0.001$, respectively).

Erythrocyte GST activity (Figure 2) was significantly

Table 1 Demographic features of study groups.

Group	n	Sex M/F	Age (years) \pm s.d.	Duration of fever (days)	Time from last dose of paracetamol (h)
1	24	12/12	6 ± 3	–	–
2	13	7/6	3.5 ± 2.6	4.1 ± 1.8 (3–10)	4.9 ± 6.9 (1–24)
3	14	6/8	2.2 ± 1.8	3.9 ± 1.7 (3–9)	4.8 ± 6 (1–24)

Table 2 Glutathione and glutathione-related enzyme content in erythrocytes of patients treated with paracetamol and in controls.

	GSH ($\mu\text{mol gHb}^{-1}$) mean \pm SD	GPX ($\text{U min}^{-1} \text{gHb}^{-1}$) median*	GST ($\text{U min}^{-1} \text{gHb}^{-1}$) median*	GR ($\text{U min}^{-1} \text{gHb}^{-1}$) median*	SOD ($\text{U min}^{-1} \text{gHb}^{-1}$) mean \pm SD
Group 1	$5.68 \pm 1.88^\dagger$	44.98 [§]	2.18 [¶]	$10.18 \pm 3.92^\dagger$	$2353.5 \pm 461.8^{††}$
Group 2	$4.43 \pm 1.54^\ddagger$	38.16 [§]	3.17	$6.74 \pm 3.18^{††}$	$2053.2 \pm 697.8^{**}$
Group 3	$2.27 \pm 1.06^{††}$	35.76	4.58 [¶]	$4.54 \pm 2.35^{††}$	$1497 \pm 592.7^{**†}$

*Distribution of the data was not normal. [†] $P < 0.001$; [‡] $P = 0.003$; [§] $P = 0.045$; [¶] $P = 0.04$; ^{**} $P = 0.036$; ^{††} $P = 0.013$.

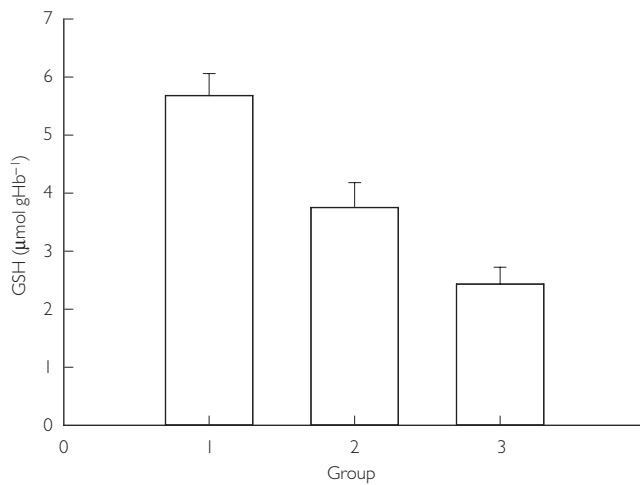


Figure 1 Glutathione (GSH) content in erythrocytes in children treated with paracetamol and in controls. Data presented as mean and standard error ($P < 0.001$ and $P = 0.003$ for the difference between group 3 and groups 1 and 2 by ANOVA).

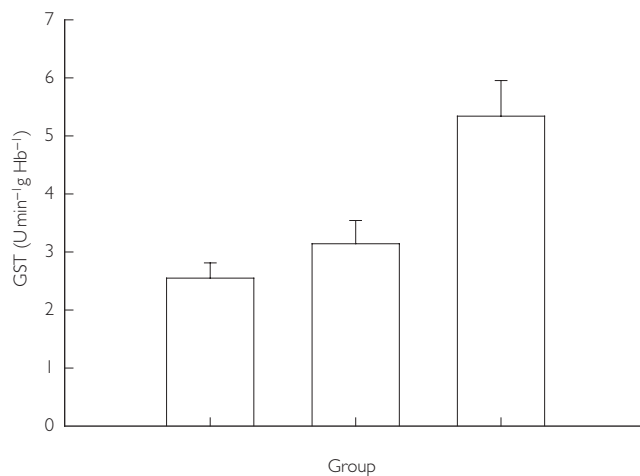


Figure 2 Glutathione S-transferase (GST) activity in erythrocytes in children treated with paracetamol and in controls. Data are presented as group medians and standard deviation ($P = 0.04$ for differences between groups 3 and 1 by ANOVA).

higher in patients treated with high-dose paracetamol compared with controls (mean difference $1.91 \mu\text{mol min}^{-1} \text{gHb}^{-1}$, 95% CI 0.57–3.24, $P = 0.04$). There was no statistically significant difference in GST activity between patients in groups 2 and 3 or between patients in groups 1 and 2.

Erythrocyte GPX activity was lower in patients from group 2 than in patients from group 1 (mean difference $11.5 \mu\text{mol min}^{-1} \text{gHb}^{-1}$, 95% CI 1.1–21.9, $P = 0.045$) (Table 2). SOD activity was significantly lower in patients from group 3 compared with groups 1 and 2 (mean differences $856 \text{ U min}^{-1} \text{gHb}^{-1}$, 95% CI 397–1316, and

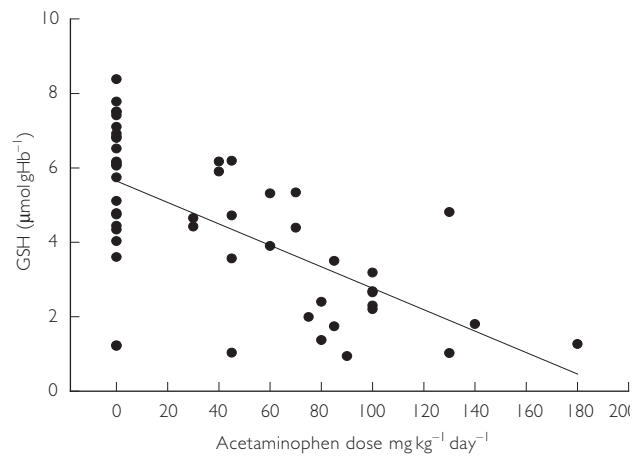


Figure 3 Glutathione (GSH) content in erythrocytes of febrile children treated with paracetamol and controls in relation to dose ($R = 0.65$).

$556 \text{ U min}^{-1} \text{gHb}^{-1}$, 95% CI 30–1082, respectively; $P < 0.001$ and $P = 0.036$) (Table 2).

Mean antioxidant status was 2.066 ± 0.66 , 1.86 ± 0.86 , and $1.23 \pm 0.33 \text{ mmol l}^{-1}$ plasma in groups 1, 2, and 3, respectively. This was significantly lower in group 3 compared with group 1 (mean differences 0.83 mmol l^{-1} plasma, 95% CI 0.30–1.36, and 0.63 mmol l^{-1} plasma, 95% CI 0.02–1.24, respectively; $P = 0.001$ and $P = 0.039$, respectively).

As there was a significant difference in ages between the groups, we used multiple regression analysis to determine the effects of age and daily paracetamol dose on GR, GST, GPX, GSH and SOD.

Paracetamol dose was the only independent variable affecting GR, GST and SOD levels, with P -values of 0.007, 0.003 and 0.008, respectively.

GSH content was affected by paracetamol dose and age ($P < 0.001$ and $P = 0.021$, respectively). The correlation between daily paracetamol dose and GSH is presented in Figure 3.

Discussion

Our data show for the first time that in febrile children, treatment with repeated supratherapeutic doses of paracetamol is associated with reduced erythrocyte glutathione content and antioxidant status.

The major metabolic pathways of paracetamol are glucuronidation and sulphation. Under normal conditions about 5% of paracetamol is metabolized through the cytochrome P450 system, leading to the formation of the highly reactive intermediate NAPQI [11, 12]. NAPQI is conjugated with glutathione and eliminated until glutathione and available liver sulphur stores become critically low. If NAPQI is not removed, it binds to critical

intracellular molecules and eventually leads to toxicity and cell death.

Glutathione, in its reduced (GSH) and oxidized (GSSG) forms, is the main intracellular thiol redox system in erythrocytes [13]. One of its major functions is the detoxification of reactive electrophiles and toxic oxygen metabolites generated during the metabolism of endogenous and exogenous substance [14]. Intermediates such as hydrogen peroxide and hydroperoxides are reduced by glutathione peroxidase at the expense of GSH. Oxidized glutathione (GSSG) is reduced by glutathione reductase (GR) in a NADPH-dependent reaction. The normal functioning of this closed system (redox cycle) maintains a high intracellular GSH:GSSG ratio and protects cells against oxidative damage. Many different endogenous and exogenous electrophiles are also detoxified via conjugation with GSH catalysed by glutathione transferase (GST) [15–17].

Drugs that lead to the depletion of GSH or influence the activity of any of these GSH-dependent enzymes may result in toxic responses. Glutathione complexes with NAPQI in the liver [18], depletion of the former increases the risk of hepatotoxicity [18, 19]. There is evidence that glutathione depletion in erythrocytes reflects glutathione depletion in other tissues including liver cells [20]. Although erythrocytes synthesize glutathione, there is an interorgan glutathione cycle, where liver cells transport glutathione into the plasma and erythrocytes [14, 21, 22]. Based on animal studies, it was postulated that an interorgan glutathione redox cycle may be occurring where the liver is central to the reduction of GSSG [23]. It was also shown that other drugs, such as methanol and azathioprine, can cause depletion of both liver and erythrocyte GSH [24–26].

Some animal studies showed that acute paracetamol overdose can deplete erythrocyte glutathione [27, 28], whereas other studies failed to show such an effect [29]. However, repeated paracetamol dosing in rats produced significant changes in glutathione, glutathione-dependent enzymes and antioxidant status [30]. Although the mechanism for the lower glutathione content in our patients is not clear, it may be evidence of depleted intrahepatic glutathione. This hypothesis should be further tested in future studies. A previous study did not demonstrate glutathione depletion in the erythrocytes of children treated with paracetamol [31]. However, unlike our patients, the children in that study were not treated with high doses of paracetamol.

The enzymatic assay [8] used to detect GSH is not totally specific and other nonprotein thiols in the erythrocytes could affect the GSH results. However, GSH is the most abundant nonprotein thiol in the red blood cell, and GSH content measured by HPLC is comparable to that obtained by the enzymatic assay [32, 33]. As the

enzymatic assay also used a correction for GSSG, it is reasonable to assume that the reactive thiols are mainly GSH.

The lower SOD activity found in the children treated with repeated supratherapeutic doses of paracetamol is another indication of a reduced antioxidant status. Similar changes have been described in hepatocytes of rats exposed to repeated doses of paracetamol.

In a study by O'Brien *et al.* [30], rats treated with paracetamol had higher GR activity than the naive animals. It was suggested that this higher GR activity may have a protective effect against paracetamol toxicity. In contrast to these findings, we found lower GR activity in children exposed to repeated high doses of paracetamol, suggesting a lack of this protective mechanism in erythrocytes.

GST content was greater in patients treated with high-dose paracetamol compared with patients receiving therapeutic doses and with controls. Hepatic GST is a sensitive indicator of hepatocellular injury [34]. Compared with naive rats, those exposed to repeated doses of paracetamol have been shown to have higher GST activity in hepatocytes [35]. However, erythrocyte GST differs from hepatic GST [36] and the increase in GST in hepatocytes after exposure to drugs, which involves a higher mRNA expression [35], does not occur in erythrocytes.

The physiological functions of GST in erythrocytes are not fully understood. One role is to catalyse the binding of glutathione to xenobiotics, including paracetamol [14]. Furthermore, higher GST activity in erythrocytes has been described after exposure to oxidative stress [37]. The mechanism for the high GST activity we found in children treated with high-dose paracetamol is not clear. Possible explanations are increased expression of the protein in erythrocytes, as previously described in patients with uraemia [38], or kinetic modulation of the enzyme.

The role of GST in the treatment of chronic supratherapeutic paracetamol toxicity requires further validation and the development of rapid, simple analytical tests [39]. There are other possible explanations for the reduced antioxidative status found in our patients. Decreased calorie intake has been found to be associated with a lowered erythrocyte glutathione content in both animals and human studies [40, 41]. Nonetheless, in humans, only severe malnutrition associated with oedema was associated with low GSH content in erythrocytes [41]. As none of our patients had signs of malnutrition or was oedematous, it is more likely that at least part of the difference in GSH is due to the effect of paracetamol.

Sepsis and inflammation can cause an increased oxidative stress [42]. However, none of our patients had sepsis, there were no differences in the duration of fever between the groups, and patients treated with high-dose

paracetamol were not considered to be more ill than patients treated with recommended doses.

The febrile patients were younger than the controls. However, there was no significant age difference between patients treated with high-dose paracetamol and those treated with the recommended doses. Multiple linear regression analysis identified paracetamol dose to be an independent variable affecting GR, GST, GSH and SOD.

Our study has some limitations. First, the dose of paracetamol given to the children was calculated based upon the parent/carer's report, which depended on recall and was prone to reporting bias. We tried to minimize this problem by verifying paracetamol dosage in two different ways. However, the possibility of over- or under-estimation of paracetamol dose cannot be excluded. The methodology used in this study cannot prove a causative effect of paracetamol in decreasing glutathione content as other variables that we did not control may have affected the outcome.

Rivera-Penera *et al.* [3] suggested that in sick children who receive multiple, supratherapeutic doses of the drug, the therapeutic index for paracetamol may be much lower than that associated with intermittent, therapeutic administration of the drug. It is important to note that the studies [2–6] reporting liver failure after repeated doses of paracetamol in children are case reports or case series. It is possible that in some of these reports the infectious disease itself caused the liver disease, not exposure to paracetamol. Given that millions of children are treated with paracetamol worldwide, and the few cases of hepatotoxicity reported to date, liver damage is probably a rare phenomenon. Our findings may suggest an explanation for this observation. Further studies are needed to assess the role of repeated supratherapeutic doses of paracetamol in depleting intrahepatic glutathione, and to evaluate whether changes in GSH and glutathione-related enzymes in erythrocytes, following paracetamol treatment, are related to an increased risk of liver damage.

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