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AN *IN VITRO* MODEL OF LIVER INJURY USING PARACETAMOL TREATMENT OF LIVER SLICES AND PREVENTION OF INJURY BY SOME ANTIOXIDANTS

ANDRÉ E. M. MCLEAN and LESLEY NUTTALL

Laboratory of Toxicology and Pharmacokinetics, Department of Clinical Pharmacology, University College Hospital Medical School, University Street, London, WC1 6JJ, U.K.

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Abstract—Rat liver slices exposed to paracetamol for 2 hr show loss of potassium and leakage of isocitrate dehydrogenase (ICD) during the subsequent 4 hr of incubation in paracetamol free Ringer solution. The extent of injury depends on paracetamol dose, and phenobarbital pretreatment of the rats. Treatment of the slices with methionine in this second stage of incubation increases the slices acid soluble SH content, but does not protect against K loss or ICD leak. In contrast, methylene blue, promethazine and +Catechin prevent injury when added after paracetamol exposure has ended. It is inferred that the mechanism of paracetamol injury to cells may involve some reversible process subsequent to covalent binding of paracetamol metabolites to sensitive cell sites.

The sequence of events of cell injury by drugs and chemicals can be observed in whole animals but the underlying mechanisms of injury may be obscure [1]. In order to study the underlying mechanisms, an *in vitro* model system may be useful.

We have used rat liver slices to study liver cell injury. In this system the influence on injury of changes in extracellular fluid can be studied, or effects of addition of drugs can be followed. In the slice system such changes can be made without having to consider the problems of survival of the rest of the animal, or of distribution and metabolism of drugs by other tissues.

Also, using a model system it may be possible to assess the action of potentially toxic compounds on the liver, even if only a few milligrammes of the substance are available.

All such studies are useful in shedding light on toxicity only if the model system has some relevance to the events that take place in the whole animal.

The validation of a model requires that there be some parallel between the events *in vitro* and those found *in vivo*. The present paper describes the validation of a model system using rat liver slices and paracetamol, and describes some results using this system.

In particular, some compounds are shown to protect the slice even when they are added after exposure to the injurious agent has ended.

Paracetamol (*N*-acetyl-para-aminophenol, acetaminophen) in overdosage leads to liver damage in man and many species of animals [2–5]. Recent work on the mechanism of toxicity of paracetamol has produced the view that the drug is mostly metabolised in the liver. Glucuronide and sulphate conjugation are safe pathways of disposal [6], but a small fraction of the paracetamol is oxidised in the cytochrome P450 system, to form a reactive metabolite which combines with glutathione. As glutathione levels fall, further metabolite attacks other, as yet unknown, cell components to cause cell destruction [7, 8]. In the rat the sequence of toxic effects usually takes 8–16 hr from oral dosage to the development of signs of liver injury such as histological cell necrosis, increased liver water content or increased enzyme activity in the plasma.

In rats made susceptible by low protein diets or pretreatment with phenobarbital, and then given doses of paracetamol in the region of the LD_{50}, the animals may become ill and die within 6 hr, and at that time have evidence of severe liver injury [9, 10].

In this respect, paracetamol differs from most hepatotoxins, such as carbon tetrachloride, pyrrolizidine alkaloids or dimethyl nitrosamine, where even lethal doses do not usually kill the animal or cause obvious cell injury before 12 hr or more have passed, and often do not kill until 48 hr have elapsed ([11] and A. E. M. McLean, unpublished observations).

The rapid development of cell injury, and its dependence on the physiological state of the animal as well as the dose of paracetamol, suggested that it might be possible to develop a model system for investigating drug injury to liver cells, *in vitro*. It would be possible to separate the first phase of exposure to paracetamol, activation to the toxic compound and attachment of the metabolite to cell structures, from the second phase of cell injury, during which the cell runs down as a consequence of the primary attack.

We have developed a model where liver slices from rats made sensitive to paracetamol by phenobarbital treatment, are incubated with paracetamol for 2 hr; they are then moved into a fresh Ringer solution without paracetamol, and yet develop evidence of cell injury in the form of loss of potassium and leakage of enzymes, 4–6 hr after first exposure to paracetamol.

Abbreviations: BHT 2.6. di tert Butyl p. cresol (Butylated hydroxy Toluene); BHA Butylated hydroxy anisole; DPPD N.N Diphenyl para phenylene diamine.

Table 1. Effect of phenobarbital pretreatment and paracetamol concentration on leakage of isocitrate dehydrogenase from rat liver slices

Rat diet	ICD leakage per cent Paracetamol concentration in the first 2 hr of incubation 0	4 mM	8 mM	12 mM
Stock pellets	1.9 ± 0.6	2.7 ± 0.2	7.1 ± 2.8	6.6 ± 1.9
Stock pellets + phenobarbital	1.3 ± 0.4	9.2 ± 2.8	33 ± 19	—

Slices were incubated with varying amounts of paracetamol for 2 hr, and then put into fresh Ringer solution for a further 4 hr. At the end of this time ICD leakage into the incubation medium was measured. Each figure gives the mean ± ISD of results from at least 4 flasks from 2 separate animals.

MATERIALS AND METHODS

Animals. Male albino rats of a Wistar strain weighing 120–150 g (Tuck and Sons, Rayleigh, Essex) were fed stock pellets (Diet 41B, Oxoid Limited) and either tap water, or else were given Na phenobarbital 1 mg/ml in distilled water as the sole source of drinking water [12]. Phenobarbital treatment was given for at least 6 days. Vitamin E (5 mg α tocopherol acetate in 2 drops of olive oil) was given by mouth the day before rats were killed.

Rats weighing 130–200 g were killed by exsanguination under ether anaesthesia, the liver rapidly removed, and the liver slices cut by hand on a Stadie–Riggs stage with a long razor blade (A. H. Thomas Co., Philadelphia). Slice thickness was about 0.3 mm (0.3 mg/mm^2), thicker slices were rejected. Slices of about 80 mg weight were distributed into 50 ml Erlenmeyer flasks containing 5 ml of Ringer solution. Two or three slices were weighed into each flask, in order to minimise the variation between flasks.

The slices were put into the Ringer solutions at room temperature and the experiment started by placing the flasks into an incubator bath at 37° under oxygen with shaking (90 strokes/min). After 2 hr the slices were taken out of the first flask and put into another flask containing 5 ml fresh Ringer solution, and the incubation continued for a further 4 hr.

Hepes–Ringer albumin solution. A Ringer solution suitable for measurement of potassium transport and buffered with N2 hydroxy ethyl piperazine ethane sulphonic acid (Hepes, tissue culture grade, Hopkins & Williams, Essex) was made up with the following composition:
Na Cl 125 mM, K Cl 6 mM, Mg SO_4 1.2 mM, Na H_2PO_4 1 mM, Ca Cl_2 1 mM, glucose 10 mM, Hepes buffer pH 7.4 at 37° 15 mM, albumin 5 mg/ml (Sigma, bovine albumin sterile solution).

Chemicals. NADP was bought from Böhringer, and +Catechin was a gift from Zyma, Nyon. Other antioxidants were bought from BDH Ltd, Dorset, or Koch–Light Laboratories, Bucks. Paracetamol was bought from Sigma Chemical Co., London.

Measurement of ICD, K and glutathione and amidopyrine demethylation. Isocitrate dehydrogenase, water and potassium content of livers, slices and Ringer solutions were measured as previously described [13, 14]. Leakage of isocitrate dehydrogenase into the Ringer solution was expressed as a percentage of the amount of enzyme activity originally added to the flask, based on ICD assays on homogenates of liver slices sampled before incubation.

$$\text{Leak } \% = \frac{\text{ICD activity in the 5 ml Ringer solution}}{\text{ICD activity/mg of liver slice} \times \textit{wt} \text{ of slices added to flask}}.$$

Glutathione (as acid soluble SH) was measured by the method of Ellman [15]. Amidopyrine demethylation to 4-amino antipyrine was measured by incubating slices in Ringer solution without added albumin, and with 2 mM amidopyrine. 4-amino-antipyrine was measured in the Ringer solution 30 min later [14].

RESULTS

Table 1 shows that rat liver slices incubated for 2 hr with paracetamol developed a very extensive leak of isocitrate dehydrogenase 4 hr later. This leak was dependent on paracetamol concentration, and was made very much more severe by phenobarbital pretreatment of the rats.

Table 2 shows that slices incubated for 2 hr with 8 mM paracetamol showed not only extensive leakage of isocitrate dehydrogenase but also loss of potassium content by 6 hr. In contrast, slices incubated without paracetamol showed only minimal leakage of enzyme, and maintained a high potassium content. Sodium and water content altered in a reciprocal manner to K content.

Table 3 shows that both control and paracetamol treated slices lost equal amounts (over 20 per cent) of their isocitrate dehydrogenase activity to the incubation medium in the first 2 hr. Essentially all of this loss took place in the first 15 min of incubation, and was associated with a general loss of protein and dry weight of the slices. It was accompanied by visible loss of cells, clumps of cells and cell particles. On histological examination of slices this seemed to be associated with the loss of damaged cells from the surface of the slices.

The potassium content of all the slices fell drastically during the first few min of incubation and recovered over the next hr, as has been shown previously [13].

Paracetamol treated slices maintained their potassium content for 3 hr, but by 4 hr potassium content had fallen significantly and enzyme leakage was marked.

Table 2. Leakage of isocitrate dehydrogenase and loss of potassium from rat liver slices incubated for 4 hr after 2 hr incubation with 8 mM paracetamol

Incubation medium		n	ICD leak % ± ISD	K content μmoles/g dry weight
0–2 hr	2–6 hr			
Ringer paracetamol	Ringer albumin	9	25.1 ± 14.3	124 ± 48
Ringer	Ringer albumin	9	2.9 ± 2.3	230 ± 15

In nine experiments with phenobarbital pretreated rats, slices were cut and flasks were run in triplicate, and ICD leak and K content of slices measured. The standard deviation about the mean within individual experiments averaged 26 and 16 per cent of the mean for ICD leak, and 4 and 14 per cent for K content, in control and paracetamol treated slices. Results are expressed as mean ± ISD for the means of the nine experiments.

In preliminary experiments it was found that 95 per cent or more of the ICD added to the flasks as slices, could be recovered at the end of the experiment, either in the Ringer solution, or the remaining slices. This was true both for control and paracetamol treated slices.

In all the experiments with paracetamol there was a significant correlation between ICD leakage at 6 hr and the original cytochrome P450 content of the liver, (co-efficient of determination = 0.71) in the range of P450 values (85–140 nmoles/g liver) found in the phenobarbital treated rats. When a wider range of body weights was used, (140–280 g) there was a negative correlation of leakage with body weight, smaller rats giving higher paracetamol/control ratios for leakage, but this was largely due to higher P450 levels in the smaller rats.

Exposure to paracetamol for 1 hr was not sufficient to cause leakage, but 90 min or 2 hr seemed to cause similar amounts of damage.

This may well be because the first hr of incubation is occupied in lowering glutathione levels (Fig. 1). At some time after about 90 min, oxidative drug metabolism is severely inhibited (Table 4).

The fall in acid soluble SH compounds in the slices can be reversed by adding methionine or cystine, even after 2 hr incubation with paracetamol. However, this late addition does not prevent ICD leak and K loss, or loss of pyramidon demethylation activity, nor does cysteamine added at this time block injury. The loss of demethylation is not accompanied by any fall in cytochrome P450 levels per g of protein. (When gross water accumulation takes place there is of course a

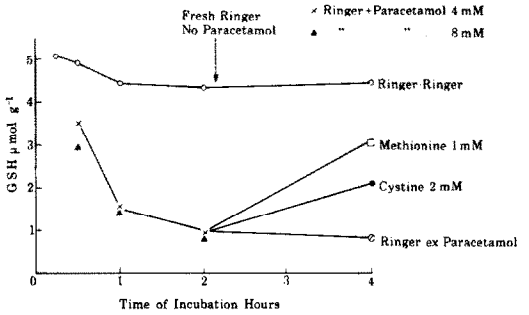

Fig. 1. Glutathione content (μmoles/g wet weight of slice) of liver slices incubated with and without paracetamol (4 and 8 mM) for 2 hr (Phenobarbital treated rat). Subsequent incubation in Ringer solution with and without addition of methionine and cystine. Each point represents the mean value of two slices, and the graph is representative of several similar experiments.

Table 3. Effect of 2 hr incubation with 8 mm paracetamol on potassium content and ICD leakage during 4 hr subsequent incubation in Ringer albumin solution in liver slices from phenobarbital treated rats

Time (hr)	(n)	ICD leakage: ICD found in incubation medium as % of ICD content in original slice R	RP	Potassium content μmoles/g dry weight R	RP
0.25	4	24.5 ± 4.4	24.3 ± 5.2	137 ± 20	108 ± 25
2.0	8	25.5 ± 4.8	25.6 ± 6.0	227 ± 6	213 ± 19
		Medium changed		Medium changed	
3.0	4	1.4 ± 0.5	3.4 ± 0.7	211 ± 18	188 ± 34
4.0	4	1.8 ± 0.4	12.1 ± 1.0	204 ± 16	164 ± 24
5.0	4	1.8 ± 0.4	22.5 ± 3.9	205 ± 16	143 ± 31
6.0	8	2.2 ± 0.2	30.8 ± 6.4	213 ± 9	107 ± 20

Liver slices from two rats were incubated with (RP) or without paracetamol (R) for 2 hr. The slices were then incubated in fresh Ringer albumin solution without paracetamol for a further 4 hr. ICD leak and K content were measured as described in the section on methods. Each value represents the mean ± ISD of the number of separate flasks indicated in column (n). The K content of paracetamol treated slices was significantly reduced from 4 hr onwards ($P < 0.025$). ICD leak was significantly increased from 3 hr onwards ($P = 0.05$).

Table 4. Amidopyrine metabolism by rat liver slices after exposure to paracetamol

Paracetamol concentration mM	Paracetamol exposure time 2 hr	4 hr
	Amidopyrine demethylation μmoles/g liver/hr	
0	2.16 ± 0.23 (3)	2.66 ± 0.41 (6)
2	..	1.8
4	..	1.0
8	1.0	0.45

Slices were incubated in Ringer solution containing various amounts of paracetamol for 2 or 4 hr. They were then taken out and incubated in fresh Ringer containing 2 mM amidopyrine for 30 min, at the end of which time 4 amino antipyrine in the Ringer solution was measured. Stock + phenobarbitone treated rat. Values are means of two slices except for controls where n is given in brackets.

loss of P450 per gram of liver.) When slices unable to demethylate pyramidon are homogenised and re-incubated with pyramidon, with the usual addition of co-factors, pyramidon demethylation is restored. This suggests that a failure of electron transport rather than destruction of drug metabolising enzyme proteins has taken place.

Protective compounds. Compounds that prevent oxidative metabolism of drugs [7], and also methionine [9] prevent liver injury if given before paracetamol treatment or before paracetamol metabolism is completed. A question of both practical and theoretical interest is whether it is possible to prevent injury by any measures taken after the paracetamol has been metabolised Tables 5 and 6 show that a number of compounds do protect the slice from injury even when added at the late stage, after removal of paracetamol. Both K loss and ICD leakage are prevented; Na and water entry are also blocked (A. E. M. McLean, unpublished results).

The effective compounds, methylene blue, (+) catechin, DPPD and promethazine are all antioxidants [16, 17]. But a number of other antioxidants (in particular EDTA, BHT, BHA and Caffeic acid) are not effective.

Table 6. Inhibition of paracetamol induced isocitrate dehydrogenase leak, and K loss by additions after exposure to paracetamol

Addition to Ringer albumin from 2 to 6 hr		% inhibition of ICD leak	% inhibition of K loss
Promethazine	1000 μM	−ve	−ve
	100 μM	>100	>100
	20 μM	>100	>100
	10 μM	94	60
	4 μM	60	53
	1 μM	−ve	−ve
DPPD 4 μM		>100	80
Propyl gallate 470 μM		91	52
Quercetin 330 μM		94	88
(+) Catechin 500 μM		62	52
Ca EDTA 1000 μM		28	20
BHT 455 μM		−ve	−ve
BHA 555 μM		−ve	−ve
Propranolol 340 μM		−ve	−ve
Dexamethasone 250 μM		−ve	−ve

Liver slices from rats treated with phenobarbital were incubated for 2 hr with 8 mM paracetamol in Ringer solution as in Table 1. They were then transferred to Ringer albumin, containing the additions in the concentration tabulated above. At the end of the further 4 hr incubation ICD leakage and K content of slices were measured. Results were expressed as follows:

−ve indicates that leakage was equal to or greater than in the paracetamol treated slice incubated in Ringer albumin alone. >100 indicates that leakage was less than in the control slices incubated without paracetamol in the first 2 hr.

Percentage figures for inhibition of leak were calculated as follows. If A = K level in control slices incubated in Ringer solution throughout, B = K level in paracetamol treated slices, and C = K level in paracetamol treated slices incubated from 2 to 6 hr with the protective compound, then per cent inhibition of K loss

$$100 - \left(\frac{A - C}{A - B}\right) \times 100.$$

Inhibition of ICD leak was calculated in a similar way.

The table summarises results from several experiments, each of which was carried out as set out in Table 5, with control ringer, ringer-paracetamol 2 hr, and ringer-paracetamol 2 hr + ringer-protective compound 2–6 hr as incubation media.

Table 5. Inhibition of paracetamol induced isocitrate dehydrogenase leak and K loss by additions after exposure to paracetamol

0–2 hr	Incubation medium 2–6 hr	ICD leak % of total in slice	% inhibition of ICD leak	K content μmoles/g dry	% inhibition of K loss
Ringer	Ringer albumin	8.2 ± 3.2	(100)	222 ± 14	(100)
Ringer + 8 mM paracetamol	Ringer albumin	44 ± 5	(0)	63 ± 13	(0)
Ringer + 8 mM paracetamol	Ringer albumin methylene blue 2.5 μM	7.7 ± 0.8	101	146 ± 18	52
Ringer + 8 mM paracetamol	Ringer albumin caffeic acid 600 μM	57 ± 8	−38	68 ± 8	3

ICD leak and K content are calculated as described in the section on methods. Results are expressed as mean ± ISD for at least three slices for each value.

All the rats were treated with 5 mg vitamin E 18 hr before they were killed, in order to ensure that cation transport by slices should be adequately protected against the injury that overtakes E deficient slices [13]. However, this did not prevent injury by paracetamol, nor did large doses of vitamin E (10 or 50 mg) protect.

ICD activity in Ringer solution or saline is destroyed during incubation with a number of compounds, especially those such as (+) catechin or cysteine, which can undergo oxidation-reduction reactions. However, the addition of albumin to the incubation medium stabilised the ICD activity so that leakage could be detected. In addition, experiments where ICD was measured in the slice at the end of the experiment confirmed that the protective effects were not artefacts due to destruction of ICD in the incubation medium.

Compounds can be termed protective if both ICD leak and K loss are prevented, since one might postulate that while a compound might appear to protect by destroying ICD as it emerges from the cell, it would be unlikely to do so and also to prevent K loss.

DISCUSSION

In the model system described here the liver slice behaves in a similar way to the liver in the whole animal, in its response to paracetamol. Injury is dependent on dose and phenobarbital pretreatment. It is preceded by a fall in glutathione levels, and takes several hr to develop.

In contrast to the whole animal, the phase of exposure to paracetamol can be brought to an abrupt end. Paracetamol content of the slices falls to undetectably low levels within a few min of change of incubation medium and the phase of paracetamol metabolism can be sharply separated from the phase of K loss and leakage of ICD.

Many molecules are toxic because they are biological alkylating agents. Often it is the activated metabolite that is the electrophilic reactant [18–20]. There is good correlation between alkylation of DNA, especially in the O_6 position of guanine and the carcinogenic effects of alkyl nitrosamines [21, 22], and there is good correlation between carcinogenicity and the ability to damage DNA in a number of systems [19].

However, the sites of attack for compounds that cause acute cell injury are not so well defined. Studies on bromobenzene, carbon tetrachloride, paracetamol and furosamide [7, 23, 24], have revived interest in the suggestion that cell necrosis is the consequence of covalent binding of drug to cell macro-molecules [18]. The present study suggests that cell injury is not a simple question of 'knocking out' some essential part of cell metabolism with an alkylating metabolite, since the addition of protective agents after the end of exposure to the toxic substance would be ineffective in this schema.

It seems more that cell injury is a process which involves a sequence of reactions, only starting with alkylation, and ending much further on in cell death [25].

It seems likely that the loss of control of cell volume and loss of permeability barriers leading to accumulation of water and sodium and leak of K and enzymes, is a terminal stage of injury. While ATP deficiency in ethionine poisoning leads to loss of K and increase of Na and water [26], it does not cause any major leak of enzymes from cells, so one can infer that in paracetamol injury one is dealing not only with a failure of ion transport but a loss of organised membrane structure.

Lipid peroxidation has been postulated as being the major destructive process in liver injury due to carbon tetrachloride [27]. It is accompanied by loss of cytochrome P450 [28]. However, loss of cytochrome P450 is also found in CS_2 poisoning where lipid peroxidation is not involved [29, 30].

The coincidence of antioxidant activity and ability to protect slices after paracetamol exposure might suggest that free radical generation and lipid peroxidation are involved in the injury process. However, autocatalytic lipid peroxidation is well inhibited by the antioxidants BHT, BHA and EDTA or by vitamin E [16]. Since these compounds are not effective as protective agents in the paracetamol model, it is unlikely that autocatalytic lipid peroxidation is of major importance, and catechin and methylene blue probably act at some other point in the cells breakdown. Losowsky and co-workers have shown protective effects by vitamin E and some but not other antioxidants *in vivo* [31]. We have also observed protection by vitamin E but only in rats fed a diet high in unsaturated fats and low in vitamin E (McLean and Greaves, unpublished work). It seems likely that lipid peroxidation can take place in injured tissues and make the lesions worse. However, it is not a necessary part of paracetamol poisoning.

Reutter *et al.* [32] (and also Perrissoud—personal communication) have observed protection by + catechin against galactosamine injury, so it is possible that the protective agents are acting not on the particular attack by one toxic agent, but with a later part of the sequence of events whereby the initial interaction of toxin with cell leads eventually to cell death. The possibility arises that + catechin and other flavones such as quercitin act by preventing the uncoupling of ion dependent ATPases, and so preventing Na influx into injured cells [1, 33].

The daily occurrence of death and liver injury after paracetamol overdose is a challenge. It should be possible by understanding the pathogenesis of cell injury after paracetamol poisoning, to prevent the injury, even when the patient arrives after the paracetamol has been metabolised.

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