Curative Effect of Picroliv on Primary Cultured Rat Hepatocytes Against Different Hepatotoxins: An in Vitro Study

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Picroliv, the standardized active principle from the plant *Picrorhiza kurrooa* showed significant curative activity in vitro in primary cultured rat hepatocytes against toxicity induced by thioacetamide (200 mg/mL), galactosamine (400 mg/mL), and carbon tetrachloride (3 ml/mL). Activity was assessed by determining the change in hepatocyte viability and rate of oxygen uptake and other biochemical parameters (GOT, GPT, and AP). The toxic agents alone produced a 40–62% inhibition of cell viability and a reduction of biochemical parameters after 24 h of incubation at 37° C which (on removal of the toxic agents) was reversed after further incubation for 48 h. Incubation of damaged hepatocytes with picroliv exhibited a concentration- (1–100 mg/mL) dependent curative effect in restoring altered viability parameters. The results warrant the use of this in vitro system as an alternative for in vivo assessment of hepatoprotective activity of new agents. © 1999 Elsevier Science Inc.

Key Words: Curative; Picroliv; Hepatocytes; Hepatotoxins

Introduction

The present study was undertaken to standardize an in vitro test system using primary cultured rat hepatocytes to detect the curative effect of new agents against chemical toxin-induced cellular damage. In vitro models can offer a more detailed approach to understanding the mechanism of toxic actions (Roberfroid, 1991), thus permitting a better analysis of the hepatoprotective action of new drugs being developed. Several studies have demonstrated a hepatoprotective activity with picroliv, the active principle from the plant *Picrorhiza kurrooa,* against a variety of hepatotoxins in animals (Dhawan, 1995) but few data are available on its curative effect. In the present study, the effect of picroliv was examined against the hepatotoxins galactosamine, thioacetamide, and carbon tetrachloride, using primary cultured rat hepatocytes. Changes in cell viability and rate of oxygen uptake together with alterations in GOT (glutamic oxaloacetic transaminase), GPT (glutamic pyruvate transaminase), and AP (alkaline phosphatase) levels were used as criteria for toxicity and drug efficacy.

Materials and Methods

Animals

Albino rats (Druckery strain) weighing 200–225 g and of either sex were employed. The animals were obtained from the Division of Laboratory Animals of the Central Drug Research Institute (CDRI), Lucknow, and maintained under environmentally controlled conditions.

Plant Materials

Picroliv was kindly prepared by Dr. D.K. Kulshreshtha of the Division of Medicinal Chemistry of the CDRI. The test material was used in concentrations of 1, 10, and 100 mg/mL.

Chemicals and Enzymes

Galactosamine hydrochloride, HEPES buffer, trypan blue, RPMI-1640 (Rosewell Park Memorial Institute), calf serum, gentamycin, trypsin, and collagenase enzymes were procured from Sigma Chemical Co., USA. Carbon tetrachloride, thioacetamide, sodium chloride,

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and potassium chloride were purchased from Sisco Research Laboratory, Bombay, India.

Hepatotoxins

Galactosamine hydrochloride (400 mg/mL), thioacetamide (200 mL/mL) and carbon tetrachloride (3 mL/mL) were used to produce submaximal toxicity in isolated rat hepatocytes.

Isolation of Rat Hepatocytes

Rat hepatocytes were isolated by the method of Seglen (1975), with slight modifications (Visen et al., 1991a,b). Perfusion of the liver was carried out in situ with collagenase type IV. Immediately after sacrifice, a buffer solution containing NaCl 0.142 M, KCl 0.0067 M, HEPES 0.01 M, bubbled with O_2 for 10 min, (pH 7.4) was perfused at a flow rate of 2.5–3 mL/min/g of liver for 12–15 min to remove blood. As soon as the liver became greyish brown in color, a second buffer solution containing collagenase (1.6×10^{29} g/mL, 630 units/*mg* solid, 37° C, pH 7.6) was perfused at the same flow rate for 7–8 min. Hepatocytes were obtained after mechanical disruption of the collapsed liver, filtration, and cold centrifugation (4° C, 200 rpm/min for 1 min, three times) and suspended in buffer I. The viability of hepatocytes was assessed by trypan blue (0.2%) exclusion and oxygen consumption.

Primary Cultures of Rat Hepatocytes

The method of Tingstrom and Obrink (1989), for the short-term culture of hepatocytes was used with slight modification. The freshly isolated viable hepatocytes were suspended in culture medium RPMI-1640 supplemented with calf serum (10%) and gentamycin (1 mg/mL). These cells (approximately 1×10^6) were then seeded into tissue culture dishes (35 mm diameter) and maintained in an atmosphere of 5% CO_2 + 95% O_2 in a humidified incubator at 37° C. The adherence of cells to the culture dish plate occurred between 30 and 60 min later, so that within 24 h the hepatocytes had formed a monolayer. The newly attached cells were round and most appeared as individual cells. These cells were 95–96% viable as confirmed by trypan blue exclusion.

In vitro Drug Testing

Twenty-four hours after the establishment of the monolayers, the medium was decanted, and the culture washed and finally covered with fresh medium. Two batches of cells were made: batch 1 had a normal hepatocyte monolayer with control medium; and batch 2 contained normal cells plus medium to which had been added one of the toxins in appropriate concentration. Both batches of cells were then further incubated for 24 h at 37° C by which time toxicity had become established in the toxin-treated cells. Fresh toxin-free medium was now introduced after decanting the old medium from petri dishes. The effect of picroliv added to the fresh medium at different concentrations of 1, 10, and 100 mg/mL was studied on normal as well as hepatotoxin-damaged cells. The cells of all the groups were incubated at 37° C for 48 h after adding picroliv.

At the end of the incubation period, the cells were loosened by 2% trypsin. The separated cells were washed three times with medium, each time decanting the medium after cold and low-speed centrifugation (4° C; 200 rpm for 1–2 min) before examination for viability and enzyme levels. The viability of cells was assessed by trypan blue exclusion and oxygen uptake (the trypan blue dye stained the nonviable hepatocytes). The percentage of viable cells was calculated accordingly. The rate of oxygen consumption, determined by a Gilson Oxygraph (Estabrook, 1967), was calculated as mL O_2 utilized/h/mg protein at atmospheric pressure. The reaction mixture contained 1.7 mL of HEPES buffer and 0.1 mL of hepatocyte suspension (1–1.2 million cells).

The method of Reitman and Frankel (1957) was followed for the assessment of activity of glutamic oxaloacetic transaminase and glutamic pyruvic transaminase in the cells. The level of alkaline phosphatase was determined by the method of Kind and King (1954).

Assessment of Curative Activity

Picroliv curative activity was determined by measuring an increase in the percent viable cells and the improvement in the rate of oxygen uptake in that group of cells incubated with picroliv, compared with the normal and damaged groups. Reversal of toxin-induced reductions in the levels of enzymes was also considered to be an important criterion of curative activity.

Statistical Analysis

Analysis of variance (ANOVA) was employed for judging the significance of variations followed by individual comparison between the groups by the Students *t* test. A value of $p < 0.05$ was considered significant.

Results

After 24 h, significant toxicity (40–62%) was observed following incubation with the chemical toxins galactosamine, thioacetamide, and carbon tetrachloride as assessed by trypan blue exclusion and oxygen up-

Table 1. Curative effect of picroliv on the viability of galactosamine (400 mg/mL) treated rat hepatocytes

Parameter	Normal (group I)	Galactosamine only (group II)	Concentration of picroliv (mg/mL)	Picroliv 1 galactosamine (group III)
Trypan blue exclusion (% viable cells remaining)	93.6 6 2.7	45.9 6 1.9	1	63.8 6 3.2 (37% restoration of viability)
			10	75.3 6 5.1 (62%)
			100	96.6 6 1.6 (100%)
Oxygen uptake (mL/h/mg protein)	4.6 6 0.2	1.4 6 , 0.1	1	2.9 6 0.2 (46% restoration of oxygen uptake)
			10	3.2 6 0.4 (56%)
			100	4.7 6 0.4 (100%)

Note: All values are mean 6 SD of six samples. Group II compared with group I (p , 0.001 for both values). Group III compared with group II (p , 0.001 for all values). Values in parentheses indicate percent reversal.

take—these levels were maintained at the same level after further incubation for 48 h. Picroliv showed significant activity when incubated with damaged cells for 48 h.

Effect on Toxin-Induced Changes in Viability

Effect against galactosamine. Hepatocytes incubated with galactosamine (400 mg/mL) showed a significant reduction in viability, as shown by the trypan blue exclusion test (50%) and oxygen consumption rate (68%) after incubation for 48 h. Picroliv showed a concentration-dependent (1–100 mg/mL) curative effect (37–100%) by restoring the viability of hepatocytes as shown by trypan blue exclusion and O_2 uptake tests. Results are given in Table 1.

Effect against thioacetamide. Thioacetamide- (200 mg/mL) incubated cells exhibited a 50–61% reduction in viability as evidenced by trypan blue exclusion and O_2 uptake tests. Picroliv reversed these viability parameters (38–100%) in a concentration- (1–100 mg/mL) dependent manner (Table 2).

Table 2. Curative effect of picroliv on viability of thioacetamide- (200 mg/mL) treated rat hepatocytes

Parameter	Normal (group I)	Thioacetomide only (group II)	Concentration of picroliv (mg/mL)	Picroliv 1 galactosamine (group III)
Trypan blue exclusion (% viable cells remaining)	93.4 6 4.2	36.4 6 2.3	1	58.2 6 3.4 (38% restoration of viability)
			10	65.8 6 3.2 (51%)
			100	93.1 6 4.2 (99%)
Oxygen uptake (mL/h/mg protein)	4.46 6 0.4	2.2 6 0.5	1	2.1 6 0.4 (39% restoration of oxygen uptake)
			10	3.6 6 0.1 (61%)
			100	4.5 6 0.4 (100%)

Note: All values are mean 6 SD of six samples. Group II compared with Group I (p , 0.001 for both the values). Group III compared with Group II (p , 0.001 for all values). Values in parentheses indicate percent reversal.

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Table 3. Curative effect of picroliv on viability of carbon tetrachloride- (3 mL/mL) treated rat hepatocytes

Parameter	Normal (group I)	Carbon tetrachloride-treated (group II)	Concentration of picroliv (mg/mL)	Picroliv 1 carbon tetrachloride (group III)
Trypan blue exclusion (% viable cells remaining)	91.9 6 1.8	36.3 6 1.2	1	36.2 6 8.3 (inactive)
			10	56.4 6 9.2* (36% restoration of viability)
			100	83.3 6 1.4* (84%)
Oxygen uptake (mL/h/mg protein)	4.1 6 , 0.1	2.0 6 0.3	1	2.6 6 0.2 (inactive)
			10	3.2 6 0.1* (57% restoration of oxygen uptake)
			100	3.9 6 0.4* (90%)

Note: All values are mean 6 SD of six samples. Group II compared with Group I (p , 0.001 for both the values). Group III compared with Group II (*p , 0.001). Values in parentheses indicate percent reversal.

Effect against carbon tetrachloride. Carbon tetrachloride at a concentration of 3 ml/mL decreased the viability of cells by 50–60% in both the tests. Picroliv-treated cells exhibited 84–90% curative effect at 100 mg/mL concentration. A weak effect (36–57%) was observed at 10 mg/mL. No activity was found at a lower concentration of 1 mg/mL (Table 3).

Effect on Toxin-induced Changes in Marker Enzymes

Galactosamine model. The level of marker enzymes in the cells (GOT, GPT, and AP) was found to be decreased to 55–61% of control values after incubation with galactosamine. Picroliv treatment showed concen-

Table 4. Curative effect of picroliv on marker enzymes in galactosamine- (400 mg/mL) treated rat hepatocytes

Parameter (U/mg protein)	Normal (group I)	Galactosamine only (group II)	Concentration of picroliv (mg/mL)	Picroliv 1 galactosamine (group III)
GOT	18.6 6 1.1	7.2 6 0.4	1	11.5 6 2.3 (38% restoration of protein)
			10	14.9 6 2.8 (67%)
			100	17.5 6 4.9 (90%)
GPT	21.5 6 5.1	9.6 6 3.8 (55)	1	14.8 6 6.4 (43% restoration of protein)
			10	17.4 6 7.8 (65%)
			100	20.6 6 2.7 (92%)
AP	15.3 6 0.2	6.4 6 0.4	1	9.8 6 0.7 (38% restoration of protein)
			10	12.5 6 0.6 (68%)
			100	14.0 6 0.4 (85%)

Note: All values are mean 6 SD of six samples. Group II compared with group I (p , 0.001 for both values). Group III compared with group II (p , 0.001 for all values). Values in parentheses indicate percent reversal.

Table 5. Curative effect of picroliv on marker enzyme in thioacetamide- (200 mg/mL) treated rat hepatocytes

Parameter (U/mg protein)	Normal (group I)	Thioacetamide only (group II)	Picroliv concentration (mg/mL)	Picroliv 1 thioacetamide (group III)
GOT	19.8 6 2.3	9.6 6 3.5	1	14.4 6 2.7 (47% restoration of protein)
			10	16.9 6 5.8 (71%)
			100	19.8 6 6.2 (100%)
GPT	22.7 6 6.9	13.4 6 8.9	1	17.6 6 6.2 (45% restoration of protein)
			10	19.6 6 3.4 (67%)
			100	22.6 6 8.2 (99%)
AP	13.5 6 0.1	5.1 6 0.4	1	8.7 6 0.2 (43% restoration of protein)
			10	11.7 6 0.5 (78%)
			100	13.6 6 0.4 (100%)

Note: All values are mean 6 SD of six samples. Group II compared with group I (p , 0.001 for both values). Group III compared with group II (p , 0.001 for all values). Values in parentheses indicate percent reversal.

tration-dependent (1–100 mg/mL) restoration (38–92%) of all the above-mentioned marker enzymes (Table 4).

Thioacetamide model. Thioacetamide-incubated cells showed 40–62% reduction in the marker enzymes. Picroliv (1–100 mg/mL) treatment had a significant curative effect (43–100%) as evidenced by the restoration of altered levels of marker enzymes (Table 5). The effect of picroliv on each enzyme was concentration dependent.

Carbon tetrachloride model. Incubation of hepatocytes with carbon tetrachloride significantly depleted marker enzymes (50–60%). Picroliv restored the altered marker enzymes by 72–82% at a concentration of 100 mg/mL and by 41–44% at lower concentration of 10 mg/mL. No effect was seen at 1 mg/mL concentration of picroliv (Table 6).

Discussion

Isolated hepatocytes have become a useful model for pharmacological, toxicological, metabolic, and transport studies of xenobiotics since the development of techniques for high-yield isolation of rat hepatocytes (Skett, 1994). For xenobiotic studies, freshly isolated hepatocytes and their short-term cultures are currently being used (Blaauboer et al., 1994; Rogiers and Vercruysse, 1993).

Various hepatotoxins viz. galactosamine, thioacetamide, and carbon tetrachloride have been shown to result in the reduction of viability of hepatocytes, rate of oxygen uptake, and leakage of enzymes which are considered to be the markers of cellular injury (Belinsky et al., 1984; Zimmerman and Mao, 1965). Similar changes in the present study confirm these changes and also indicate satisfactory standardization of our isolation and culture procedures. Galactosamine-induced cell damage resembles that seen in acute human viral hepatitis (Reutter et al., 1975), hence, it has been widely used in experimental studies (Keppler et al., 1968; Wang and Wendel, 1990; Visen et al., 1993) to evaluate potential hepato-protective agents. Galactosamine induces liver injury by depletion of uracil nucleotides. This alters the molecular structure of cell membranes and results in permeability changes leading to hepatocyte necrosis (Hussain-Abdul and Mehendale, 1992). Thioacetamide induces hepatoxicity by its S-oxide metabolite (Castro et al., 1974) which interferes with the movement of RNA from the nucleus to the cytoplasm, resulting in structural and functional cellular deformation leading to membrane injury (Zimmerman, 1978). Carbon tetrachloride causes liver injury through its free radical metabolite (CCl_3), which impares the endoplasmic reticulum and lipids of cell membranes which in fact lead to membrane failure (Zimmerman and Mao, 1965).

In the present study, all the three toxic agents employed reduced cell viability possibly due to injury of plasma membranes resulting in the leakage of cellular enzymes. These observations are in agreement with

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Table 6. Curative effect of picroliv on marker enzymes in carbon tetrachloride- (3 ml/mL) treated rat hepatocytes

Parameter (U/mg protein)	Normal (group I)	Carbon tetrachloride only (group II)	Picroliv concentration (mg/mL)	Picroliv 1 carbon tetrachloride (group III)
GOT	21.5 6 1.3	10.8 6 1.9	1	9.8 6 1.9 (0 5 inactive)
			10	15.5 6 1.4* (44% restoration of protein)
			100	19.6 6 1.3* (82%)
GPT	17.7 6 1.2	8.5 6 1.2	1	8.4 6 1.2 (0 5 inactive)
			10	12.3 6 1.4* (41% restoration of protein)
			100	15.7 6 2.3* (78%)
AP	13.7 6 0.2	5.5 6 0.2	1	4.7 6 0.3 (0 5 inactive)
			10	8.9 6 0.6* (41% restoration of protein)
			100	11.4 6 0.1* (71%)

Note: All values are mean 6 SD of six samples. Group II compared with group I (p , 0.001 for all values). Group III compared with group II (*p , 0.001). Values in parentheses indicate percent reversal.

earlier reported findings (Visen et al., 1996; Zimmerman and Mao, 1965). Incubation of the damaged hepatocytes with picroliv significantly restored their viability as well as the reduced biochemical parameters. This suggests a possible stabilizing action of picroliv on plasma membranes. Results of some earlier studies have also suggested a membrane stabilizing action of picroliv on hepatocytes (Singh et al., 1992).

In our study, we have observed significant but submaximal toxicity after 24 h of the incubation with certain toxic agents. These observations were consistent with the work of Olinga et al., (1997), who reported severe cellular damage after 24 h of incubation in a series of systematic comparisons of various incubation systems. Picroliv produces its curative effect by possibly reinstituting the normal cell membrane structures after a 48-h post incubation period.

The results of the present in vitro study are in agreement with our earlier published reports of in vivo activity of picroliv against galactosamine (Dwivedi et al., 1993), thioacetamide (Visen et al., 1991b) and carbon tetrachloride (Dwivedi et al., 1990). The in vivo studies required a large number of animals (six to 10 per group), needed 7 days of drug administration for a significant effect to be produced and thus required large quantities of drugs. The present model on the other hand is more rapid and requires smaller quantities of test compounds and fewer animals. It may be particularly useful in studies on natural products. In such studies, a large number of plant fractions or individual compounds need to be examined which are generally only available in small amounts. This system may also be employed for Q-SAR studies with synthetic compounds.

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