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Picroliv preconditioning protects the rat liver against ischemia–reperfusion injury

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Abstract

Cell death following ischemia–reperfusion injury is a major concern in clinical issues such as organ transplantation and trauma. The need to identify agents with a potential for preventing such damage has assumed great importance. We have evaluated the efficacy of picroliv, a potent antioxidant derived from the plant *Picrorhiza kurrooa*, in protecting against hepatic ischemia–reperfusion injury in vivo. Picroliv was fed to male Sprague Dawley rats in a dose of 12 mg/kg once daily by oral gavage for 7 days prior to hepatic ischemia. Ischemia was induced by occluding the hepatic pedicel with a microaneurysm clip for 30 min and reperfusion was allowed thereafter for varying period (15–120 min) by releasing the microaneurysm clip. Picroliv pretreatment resulted in better hepatocyte glycogen preservation and reduced apoptosis. Reduction in apoptosis was associated with decreased mRNA expression of caspase-3 and Fas. Oxidant induced cellular damage as measured by tissue malondialdehyde (MDA) levels was significantly less following picroliv pretreatment. Both a reduction in neutrophil infiltration and an increased level of intracellular antioxidant enzyme superoxide dismutase possibly contributed to the reduction in tissue lipid peroxidation. Tissue inflammatory cytokines level of interleukin-1α (IL-1α) and interleukin-1β (IL-1β) was also lower in picroliv group. Furthermore, picroliv pretreatment resulted in enhanced proliferating cell nuclear antigen (PCNA) immunoreactivity. These studies strongly suggest picroliv to be a promising agent for ameliorating injury following ischemia–reperfusion.

Keywords: Ischemia–reperfusion injury; Picroliv; Liver; Antioxidant

1. Introduction

Ischemia causes functional and structural damage to liver cells. A further impairment of the microenvironment occurs in the liver following reperfusion. Mediators released from accumulated polymorphonuclear cells and activated Kupffer cells such as oxygen derived free radicals and inflammatory cytokines are associated with this ischemia–reperfusion injury. Regulation of these mediators has been considered a therapeutic necessity, especially so since interruption of liver blood flow is often necessary as an operative technique in liver surgery (Kurokawa et al., 1996). In spite of recent reports indicating mechanisms leading to the absolute collapse of liver function and finally organ failure during ischemia–reperfusion injury, the exact mechanism(s) involved in such liver impairment remains inconclusive.

Numerous studies have suggested involvement of polymorphonuclear cells in mediating microcirculatory endothelial injury, thereby leading to reperfusion injury (Jaeschke et al., 1990; Suzuki et al., 1993). Peroxidation of the phospholipid fatty acid is generally recognized as one of the most important mechanism through which active oxygen derivatives induce cellular damage (Comporti, 1985; McCord, 1985). It is believed that lipid peroxidation

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inflicts cell damage whenever conditions of increased oxidative stress occur in the cell. Studies from several independent laboratories have shown beneficial effects of free radical scavengers on liver function following ischemia–reperfusion (Marzi et al., 1992).

Picroliv is a natural plant product extracted from the roots and rhizome of *Picrorhiza kurrooa*. Picroliv is basically a mixture of two iridoid glycosides, picroside-1, and kutkoside (1:1.5 w/w) and has been shown to impart significant hepatoprotective activities by modulation of free radical induced lipid peroxidation in in vitro systems (Chander et al., 1992). Picroliv also modulates the expression of hypoxia inducible genes during hypoxia-reoxygenation in different cells (Gaddipati et al., 1999a), and favorably regulates the expression of insulin like growth factor-1 and its receptor in cerebral hypoxia (Gaddipati et al., 1999b).

The present study was designed to assess its potential to be developed as a preconditioning agent against ischemia–reperfusion injury, in an in vivo setting, using a hepatic model in rats. The data reported here indicate that picroliv treatment resulted in lesser neutrophil infiltration, reduced cell death and greater DNA synthesis as compared to untreated control rat. Picroliv pretreatment also reduced the levels of lipid peroxidation, interleukin-1α (IL-1α), interleukin-1β (IL-1β) expression and resulted higher cellular superoxide dismutase levels implying that picroliv protects the liver and enhance its ability to withstand ischemia–reperfusion injury probably through an antioxidant pathway.

2. Material and methods

2.1. Animals

Male Sprague Dawley rats weighing 300–350 g were used in these experiments. All experiments were done in accordance with the US Public Health Service policy and the University Laboratory Animal Review Board. The animals in the test group were pretreated with picroliv prior to procedures. Picroliv was administered by oral gavage as an aqueous solution at a dose of 12 mg/kg once daily for 7 days following the protocols described earlier (Rastogi et al., 1996; Shukla et al., 1992). Control animals were similarly handled and fed an equivalent amount of water.

2.2. Procedures

Sixty rats were divided in four groups. Group 1(control, $n = 6$) and group 2 (picroliv, $n = 6$) were sham controls. The remaining 48 animals were equally divided into group 3 (ischemia–reperfusion control, $n = 24$) and group 4 (ischemia–reperfusion with picroliv, $n = 24$). Animals from groups 3 and 4 were anesthetized with pentothal (50 mg/kg) and laparotomy performed by a right paramedian incision. The hepatic pedicel was exposed by blunt dissection and hepatic ischemia induced by clamping the hepatic artery and portal vein together using a microaneurysm clip. Ischemia was maintained for periods of up to 30 min, and reperfusion was allowed thereafter by releasing the clip. Animals were euthanized at 0, 15, 60 and 120 min after beginning of reperfusion. At each time point of reperfusion, there were at least six rats in control as well as picroliv prefed group. The liver was perfused with isotonic saline and one half of each lobe was collected in 10% buffered neutral formalin for histopathology study. The other halves were stored at −70°C for RNA studies. Small portion from each lobe (1–2 mm) were also fixed in 2.5% glutaraldehyde and processed for electron microscope evaluation. Individuals who scored the staining, analyzed the histo-pathology and studied the gene expressions were blind to the tissues and treatments.

2.3. Histology, myeloperoxidase staining and glycogen content

Tissues collected in formalin were processed routinely and embedded in paraffin. Multiple sections were taken from different area for histology evaluation. Sections were stained with hematoxylin and eosin (H&E). Myeloperoxidase stain was used in other sections for identifying neutrophils. Briefly, paraffin sections were deparaffinized and hydrated. Peroxidase indicator reagent solution (0.05% *p*-Phenylenediamine, 0.1% Catechol in Tris buffer, pH 6.3) was pre-warmed at 37°C for 5 min before adding 0.2 ml of 3% hydrogen peroxide solution. Hydrated liver slides were incubated for 30 min in peroxidase indicator reagent. The sections were then air dried for 15 min, followed by incubation of slides with acid hematoxylin (0.1% hematoxylin, 0.02% sodium iodate, acetic acid) for 10 min. The sections were rinsed with distilled water, air dried, and then mounted with permount. The stained neutrophils were counted under light microscopy (magnification 60 ×) from multiple sections of different fields. The results were rounded off to the nearest number and expressed as mean neutrophil ± S.E.M. Glycogen content in hepatocytes was evaluated by using periodic acid schiff (PAS) method. The PAS stain was performed both with and without pretreatment with diastase. Glycogen stained pink by this method and staining was abolished when sections were pretreated with diastase thereby confirming specificity of staining for glycogen. Quantitation of staining was performed as described earlier (Sidhu et al., 1999). Briefly, The images were scanned, and macros were loaded to quantitate the mean density at color scale in NIH image 1.62 program. The images were magnified and a fixed area was mapped for staining in all the pictures. The process was repeated for several pictures from different areas of the liver tissue. The average was calculated to represent the mean density.

2.4. Scanning electron microscope

The liver pieces obtained after surgery were fixed in 2.5% glutaraldehyde in 0.1 mol/l sodium cacodylate buffer (pH 7.3). The specimens were post-fixed with osmium tetraoxide, dehydrated in graded alcohol and embedded in Epon 812 mixture. Sections were cut on an LKB ultramicrotome, stained with uranyl acetate and lead citrate, and examined in a Philips electron microscope.

2.5. Immunohistochemical studies

Immunostaining for copper–zinc superoxide dismutase (Cu^{2+}/Zn^{2+} SOD) was performed using human polyclonal anti-Cu/Zn SOD (Calbiochem-Novabiochem, San Diego, CA, USA). Immuno-staining was also performed to evaluate proliferating cell nuclear antigen (PCNA) (Oncogene Products, Cambridge, MA, USA) expression using a monoclonal antibody. We used an indirect avidin–biotin–immunoperoxidase technique (Quick Universal Kit, Vector Laboratories, CA, USA) following manufacturer's protocol. PCNA is a stable cell cycle related nuclear protein (37 kDa), which exclusively expressed in late G1 and throughout the S-phase of the cell cycle (Mathews et al., 1984). Briefly, tissue sections on slides were deparaffinized, hydrated, and treated with 3% hydrogen peroxide in methanol for 10 min at room temperature to inactivate endogenous peroxidase. Blocking serum, provided in the kit, was used

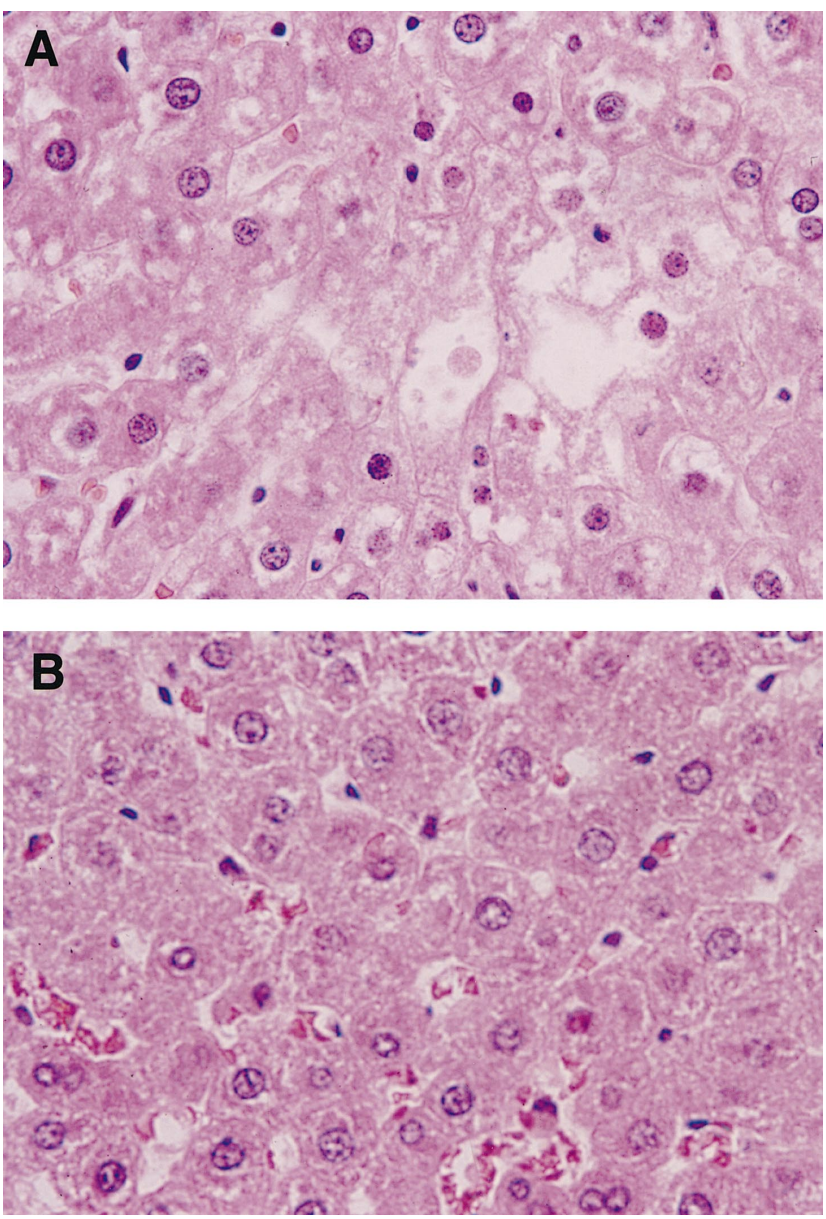

Fig. 1. Representative H&E sections from liver of control rats (A) show edema, hydropic hepatocytes, and condensation of some hepatocyte nuclei as compared to picroliv fed rats (B) after 30 min ischemia and 120 min reperfusion (H&E, 60 ×).

to block non-specific staining. Sections were then incubated with respective antibody for 1 h at room temperature in a humidified chamber. Slides were washed with phosphate buffered saline (PBS) after each incubation with antibody/kit reagents. Respective biotinylated secondary antibody was added on to sections for 15 min at room temperature, followed by avidin–biotin–peroxidase complex for 15 min. Diaminobenzidine was used as a substrate for peroxidase and slides were incubated in dark for 5–8 min and sections were counter stained with Gill's hematoxylin (Vector Laboratories). Specificity of test antibody was ascertained by incubating sections from each set with blocking serum separately. Quantitation of staining was performed as described earlier.

2.6. In situ cell death detection

The extent of apoptotic cell death was determined by a terminal deoxynucleotidyl transferase (TdT) mediated D-Uridine Tri Phosphate nick end labeling (TUNEL) technique using Apoptag kit (Oncor Lab, Gaithersburg, MD) as per the manufacturer's instructions. This method is based on the specific binding of TdT to 3′-OH end of DNA and ensuring synthesis of a polydeoxynucleotide polymer. Briefly, sections were digested using proteinase K and the endogenous peroxidase activity was blocked using 2% hydrogen peroxide in PBS. These slides were then placed in equilibration buffer and incubated with working strength of TdT enzyme. The reaction was terminated after 30 min using stop/wash buffer, provided with the kit. The apoptotic nuclei were stained by direct immunoperoxidase detection of digoxigenin-labeled DNA in test sections.

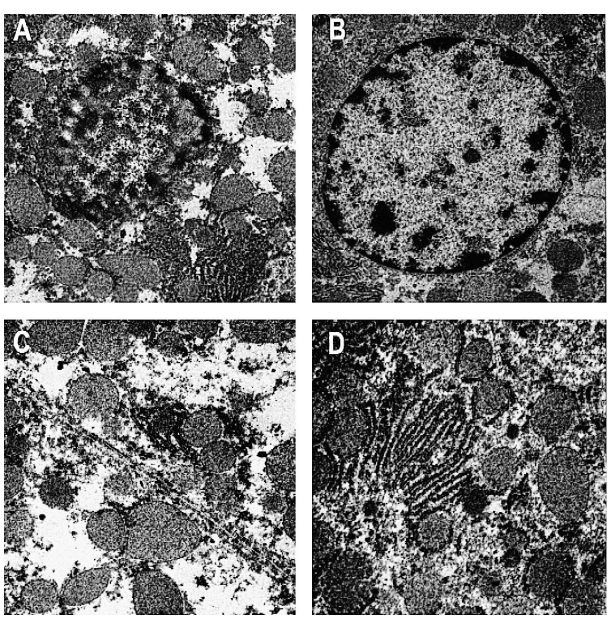

Fig. 2. Representative electron micrographs of rat liver from (A) untreated group after 30 min ischemia and 120 min reperfusion showing chromatin clumping and early loss of integrity of nuclear membranes. Whereas in the picroliv treated rats (B) hepatocyte nuclei showed normal ultrastructural morphology. (magnification A and B: 8700x) (C) Control animals (i30r120 min) showing early swelling and break down of the endoplasmic reticulum as compared to in the livers of picroliv fed animals (i30r120 min) (D).(magnification C and D: 12,500x)

Table 1
Myleoperoxidase staining of liver section after 30 min ischemia followed by 15 and 60 min reperfusion

Groups	Number of infiltrating neutrophil	
	15 min reperfusion	60 min reperfusion
Control	27 ± 2[a]	14 ± 1
Picroliv	14 ± 1[*]	9 ± 3

[a] Values are mean ± S.E.M. of average number of neutrophil, $n = 6$.
[*] $P < 0.001$ compared with control rats.

2.7. Assessment of free radical mediated lipid peroxidation

Liver samples stored at −70°C were homogenized in ice cold 20 mM Tris–HCl buffer (pH = 7.4) in order to prepare a 10% w/v homogenate. Cell debris was removed from sample preparations by centrifuging the homogenates at $3000 \times g$ for 10 min, at 4°C. Free radical mediated lipid peroxidation was estimated in tissue homogenates in terms of μM malondialdehyde (MDA)/mg tissues protein, using a calorimetric biochemical kit (Calbiochem-Novabiochem), following manufacture's protocol. MDA contents were calculated using standard MDA curve plotted using the standard provided with the kit.

2.8. Ribonuclease protection assay (RPA) for IL-1 and apoptotic genes

Total RNA was isolated from frozen liver tissues using Trizol (Life Technologies, Gaithersburg, MD) and was quantitated and equalized. Equalized RNA samples (20 μg) were then hybridized at 56°C for 12–14 h with a ^{32}P alpha-UTP labeled probe which was prepared using the human apoptosis template (Pharmingen, San Diego, CA). Hybridized samples were later subjected to RNAs digestion for 45 min at 30°C to remove unhybridized RNAs. Templates for IL-1α, IL-1β apoptotic, and anti-apoptotic genes like *bclX*$_L$, *Fas*, *Fas-L*, *caspases*-1,2,3, *bax*, and *bcl*$_2$ were used. GAPDH template was used for the analysis of house keeping gene transcript. The ribonuclease-protected bands were then resolved on denaturing urea-poly acrylamide gel electrophoresis gels, followed by autoradiography.

2.9. Statistical analysis

Unless otherwise indicated, data are presented as mean ± S.E.M. of 5–6 animals. Comparisons of means were performed using Student's *t*-test, which was contained within the StatView program II (Abacus Concepts, Berkley,

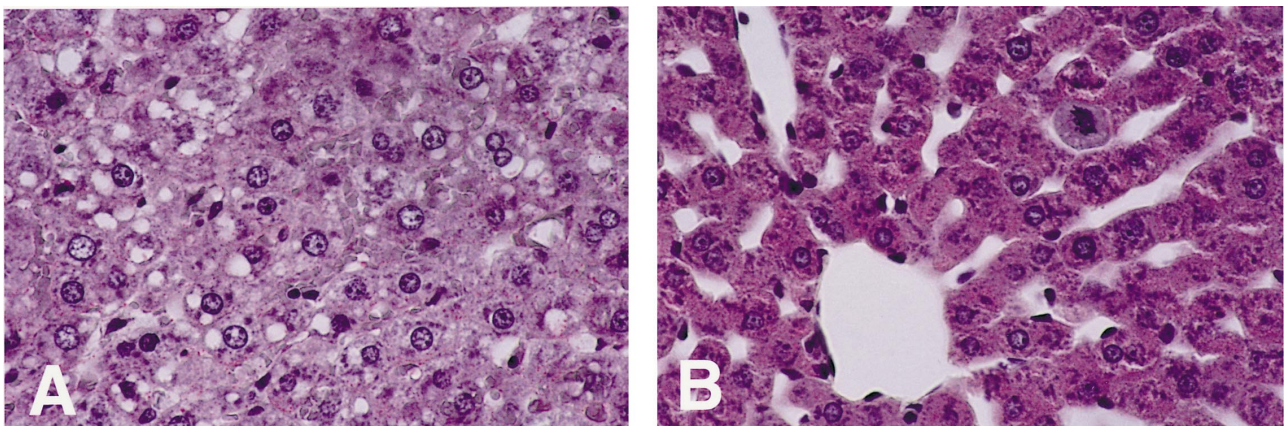

Fig. 3. PAS staining was used to evaluate glycogen content of the livers. Glycogen stains pink by this method. Control rats (A) showed significant glycogen depletion as compared to picroliv pre-fed rats following 30 min ischemia and 120 min reperfusion (PAS, 60×).

CA). A ''*P* value'' less than 0.05 was accepted as statistically significant.

3. Results

3.1. Histopathology, myeloperoxidase staining and glycogen content

Histopathological analysis of hematoxylin-eosin stained sections from multiple area of liver of control and picroliv pre-treated animals were compared. There was little or no significant difference following 30 min ischemia and early periods of reperfusion. However, with continuing reperfusion, hepatocyte damage was noted in control animals in the form of tissue edema, hydropic changes, nuclear pyknosis, and Kuffer cell hyperplasia. These changes were more pronounced in the periportal area of the hepatic lobule than in the centrilobular region (Fig. 1A). This data suggests that the mediators of injury were brought in by the in-flowing blood from the portal vein and hepatic artery, rather than caused by ischemia. Picroliv pretreated animals did not show such loss of viability till 120 min reperfusion (Fig. 1B). These results were consistent and reproducible in the control and picroliv prefed animals of multiple sections from different animals.

Table 2
Quantiatation of glycogen content and superoxide dismutase expression as seen with PAS and immunohistochemistry staining, respectively, in the liver tissues after 30 min ischemia followed by 120 min reperfusion

Groups	Superoxide dismutase	Glycogen content
	120 min reperfusion	120 min reperfusion
Control	4007 ± 727[a]	11,156 ± 3431
Picroliv	10,316 ± 298*	28,939 ± 2905*

[a] Values are mean ± S.E.M. of average density, $n = 6$.
* $P < 0.05$ compared with control rats.

Although nuclear condensation and fragmentation of the type associated with apoptotic cell death was focally identified in hepatocytes on H&E sections, evaluation of the full morphological attributes was better achieved by electron microscope. Histologically viable areas were selected for electron microscopy studies. In the picroliv treated rats, hepatocyte nuclei showed normal ultrastructural morphology, the control animals showed chromatin clumping and early loss of integrity of nuclear membranes (Fig. 2A and B). Furthermore, mitochondrial swelling and break down of the endoplasmic reticulum were also more advanced in the livers of control (Fig. 2C) versus picroliv-treated animals (Fig. 2D).

A large number of studies have already demonstrated the deleterious effect of accumulated neutrophils that are marginated in hepatic micro-vessel and cause liver failure. Myeloperoxidase stains revealed neutrophil exudation mainly in the periportal regions, as would be expected in a

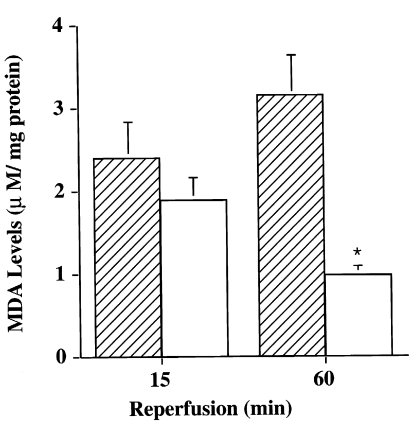

Fig. 4. Alterations in lipid peroxidation levels by picroliv following hepatic ischemia reperfusion injury. MDA levels were measured in 10% homogenates of liver samples from rats subjected to 30 min ischemia and varying times of reperfusion. Hatched bars represent mean MDA levels of control rat liver tissues and the hollow bars are that of picroliv pre-treated rats. * $P < 0.02$ as compared to untreated rat hepatic sample.

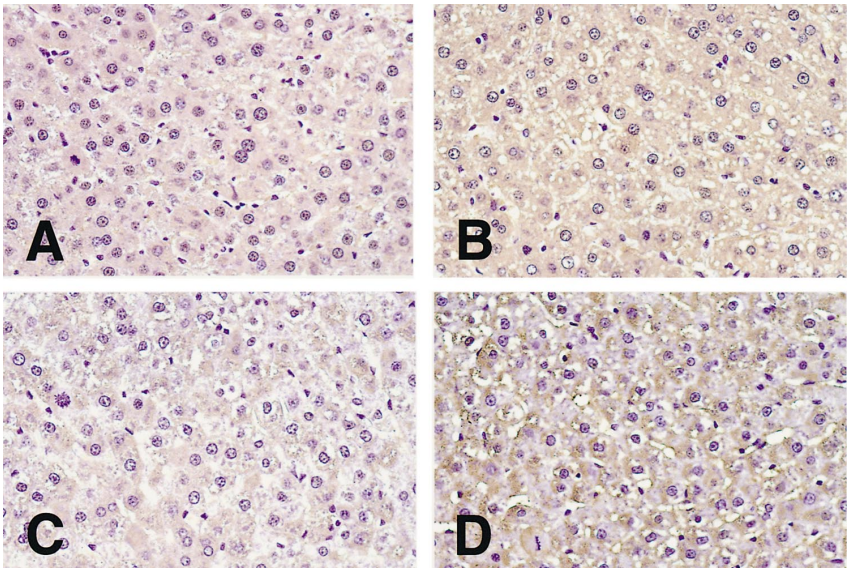

Fig. 5. Superoxide dismutase expression in liver sections. Immuno-histochemistry was performed on paraffin sections of livers from treated and untreated groups using Quick Universal Kit (Vector Laboratories) and polyclonal anti-Cu/Zn SOD (Calbiochem-Novabiochem). (A) Control animal (i30r15 min). (B) Picroliv fed control animals (i30r15min). (C and D) Control animals (i30r120 min), picroliv fed animal section (i30r120 min).

reperfusion-induced inflammatory response, since blood inflow during hepatic reperfusion would be from the portal vein and hepatic arteries, both being vessels in the portal triad. The degree of such infiltration was lower in the livers of picroliv treated rats as compared to its respective control (Table 1).

Glycogen depletion was also more pronounced in control animals (Fig. 3), suggesting that picroliv pretreatment allowed for better hepatocyte vitality and an enhanced capacity to withstand reperfusion mediated injury (Table 2). The pink staining seen in liver sections of the same and different rats in control and picroliv treated groups were reproducible.

3.2. Picroliv modulates lipid peroxidation levels and superoxide dismutase expression

MDA levels are widely used as marker of free radical mediated lipid peroxidation injury. We measured MDA levels as μM/mg protein of tissue homogenates. Picroliv treatment, by itself, had no effect on the constitutive MDA levels (data not shown). However, in control animals' tissue, MDA levels progressively increased with increasing duration of reperfusion, suggesting an ongoing lipid peroxidation because of reintroduction of oxygen in to these tissues. In picroliv pre-treated rats, MDA levels were always lower than in control rats at the same time point. It

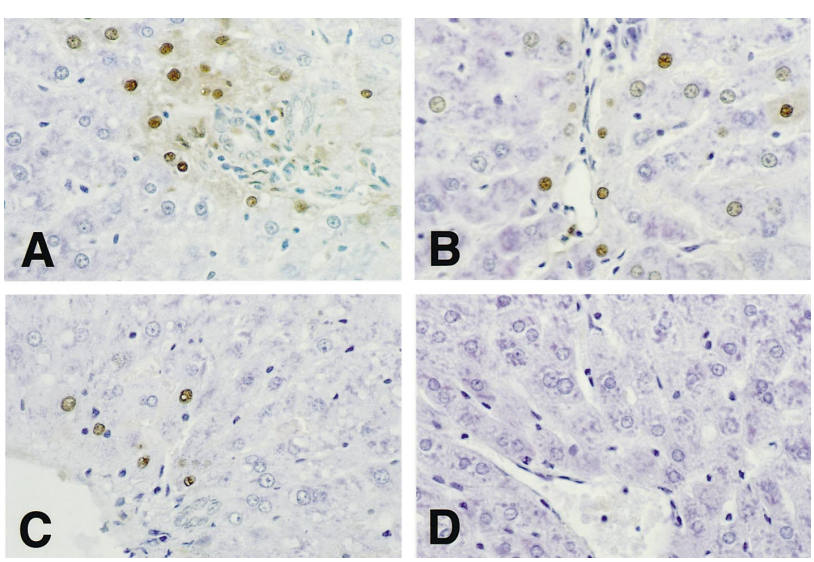

Fig. 6. In situ TUNEL staining of liver sections revealed that 30 min ischemia followed by 60 and 120 min reperfusion resulted in increased cell death in untreated control as compared to picroliv pretreated animal sections (magnification 60×). (A) Control animal (i30r60 min). (C) Picroliv fed control animals (i30r60 min). (B and D) Control animals (i30r120 min); picroliv fed animal section (i30r120 min), respectively.

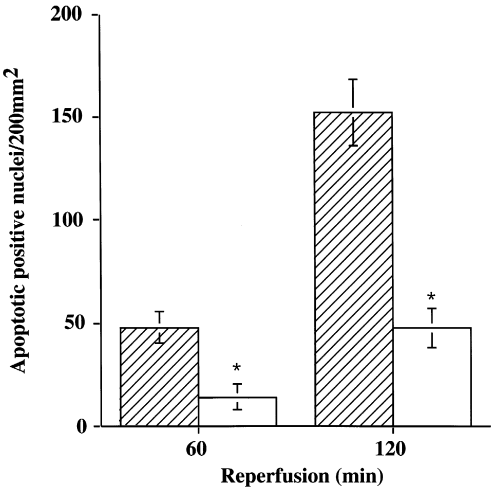

Fig. 7. The mean ± S.E.M. number of apoptotic positive nuclei per 200 mm^2 are shown after 30 min ischemia followed by varying period of reperfusion. The degree of liver damage was significantly lowered the picroliv treated animals ($P < 0.01$). Hatched bars represent values obtained from vehicle treated rats while hollow bars represent values from picroliv prefed rats. $^{*}P < 0.01$ as compared to corresponding vehicle treated group, at 30 min ischemia followed by 60 and 120 min reperfusion.

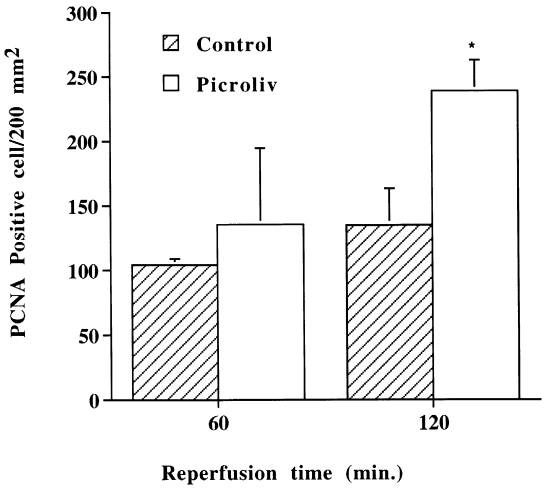

Fig. 9. The number of PCNA positive cells was quantitated and expressed as the mean ± S.E.M. number of nuclei per 200 mm^2 are shown after 30 min ischemia followed by varying period of reperfusion. Hatched bars represent values obtained from vehicle treated rats while hollow bars represent values from picroliv prefed rats following ishemia–reperfusion. $^{*}P < 0.05$ as compared to corresponding vehicle treated group, at 30 min ischemia followed by 60 and 120 min reperfusion.

is notable that MDA levels continued to increase in control livers after 15 min reperfusion but in picroliv pretreated rats, there was actually a reduction ($P < 0.02$) in MDA level at 60 min. This suggests that the free radicals being released were simultaneously being more effectively scavenged in the liver following picroliv treatment resulting in a better ability of the cellular and subcellular membranes to withstand oxidant damage (Fig. 4).

We studied the effect of Picroliv on superoxide dismutase protein expression using immuno histochemical staining on multiple liver sections with reproducible results both in the control and picroliv pre-treated rats. Data demonstrated that Picroliv pretreatment had no significant effect alone, until 15 min reperfusion. However, superoxide dismutase expression was markedly increased by 120 min reperfusion in picroliv prefed animals (Fig. 5; Table 2). This suggests that picroliv treatment resulted in enhanced antioxidant status.

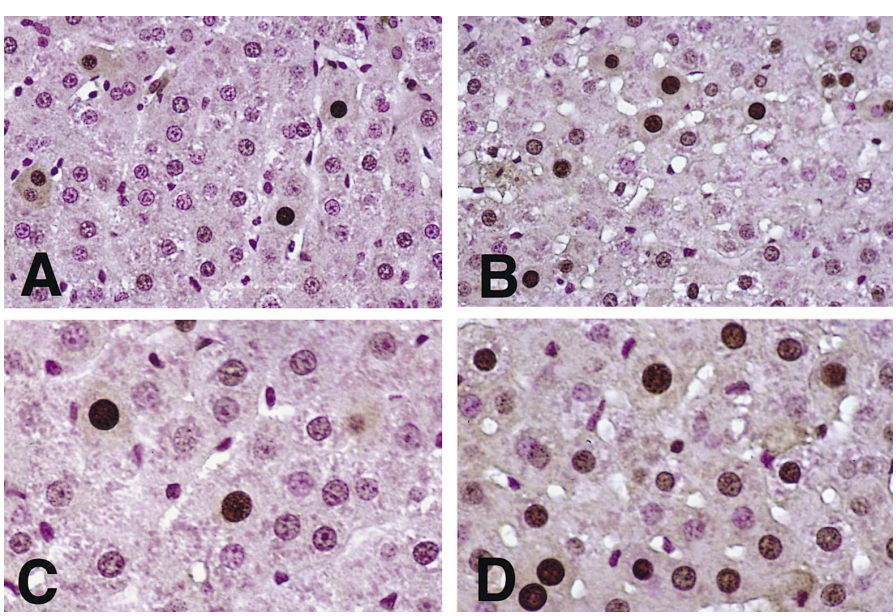

Fig. 8. (A) PCNA immunoexpression following ischemia–reperfusion. PCNA expression was analyzed by immunohistochemistry. Photomicrographs shows the level of PCNA immnoreactivity after 30 min ischemia followed by 120 min reperfusion. Lower magnification 10 ×: (A) control, (B) picroliv prefed rats; and at higher magnification 60 ×: (C) control, (D) picroliv prefed rats.

3.3. Modulation of apoptosis by picroliv following ischemia–reperfusion

We have assessed the extent and cellular localization pattern of apoptosis in liver using in situ end labeling technique that labels the large number of DNA ends in oligonucleosomes generated within the apoptotic nuclei. This technique was used on hepatic sections obtained from animals fed with or without picroliv during ischemia–reperfusion injury. Apoptotic responses began by 60 min post reperfusion predominantly in the periportal region and maximal by 120 min post reperfusion (Fig. 6). There was an increase in apoptotic injury to hepatocytes with increasing time of reperfusion. In contrast, picroliv fed animals showed a significant lesser number of apoptotic positive nuclei as compared to untreated control and hence picroliv significantly protected the cell death ($P < 0.05$) during reperfusion (Fig. 7).

3.4. Hepatocellular regeneration following ischemia–reperfusion injury

The extent of hepatocellular regeneration following ischemia–reperfusion was assessed by immunohistochemical localization of PCNA. The result demonstrated a dramatic onset of PCNA immunoreactivity at 60 min reperfusion injury, which was further increased after 120 min of reperfusion. Livers of picroliv prefed animals showed higher staining in a time dependent manner as compared to their respective control (Fig. 8). This increase in nuclear staining was found statistically significant ($P < 0.05$) after 120 min reperfusion (Fig. 9).

3.5. Effect of picroliv on cytokine IL-1α, IL-1β and apoptotic genes

IL-1α and IL-1β mRNA levels were studied by RPA. IL-1α and IL-1β mRNA levels increased upon 15 min. reperfusion in the control animals (fig. 10, lane 3; 3.0- and 3.5-fold, respectively) as compared to control rats undergoing 30 min ischemia only. (fig. 10, lane 1) Expression of these genes, however, remained much less in the picroliv fed animal and never increased significantly upon reperfusion (Fig. 10). This data suggests that a lower degree of inflammatory response consistent with the lesser degree of cellular damage and thus better hepatocyte vitality.

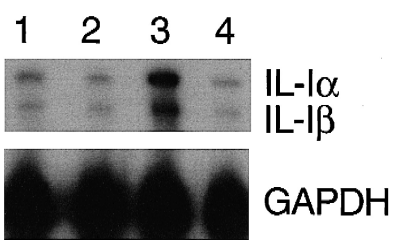

Fig. 10. IL-1α and IL-1β gene expression. Differential regulation of IL-1α and IL-1β mRNA levels in (1) control i30 min only, (2) picroliv i30 min only, (3) control i30r15 min, and (4) picroliv i30r15 min. RPA was carried out by the RiboQuant Multiprobe RPA kit (Pharmingen). GAPDH was used as internal control to equalize the mRNA levels in different groups.

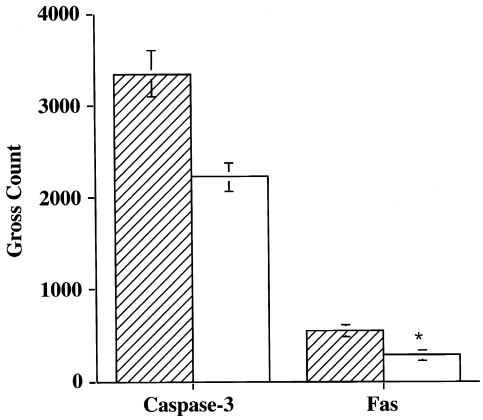

Fig. 11. Expression of *Fas* and *caspase*-3 genes. Differential regulation of *Fas* and *caspase*-3 mRNA levels in liver tissues after 30 min ischemia followed 120 min of reperfusion. RPA was carried out by the RiboQuant Multiprobe RPA kit (Pharmingen). GAPDH was used as internal control to equalize the mRNA levels in different groups. mRNA expression were visualized by autoradiography and quantitated by Phosphoimager (Beckton Dickinson) analysis using Imagequant software and were expressed as mean gross count (counts per min) ± S.E.M. The hatched bar represents gene expression of control rats where as hollow bar are the picroliv prefed rats. $^{*}P < 0.05$ as compared to corresponding vehicle treated group, at 30 min ischemia followed by 120 min reperfusion.

In order to elucidate the mechanism of cell death during ischemia–reperfusion, we performed RPA to study the regulation of various genes involved in apoptosis. Phospho-imager data obtained from the autoradiogram indicated no significant changes in bcl_2, *bax*, $bclX_L/s$, *caspases*-1, and *caspase*-2 gene expression from control to picroliv prefed rats after 30 min ischemia followed by 120 min reperfusion. However, there was nearly 1.6-fold inhibition in *caspase*-3 and 1.9-fold reduction in *Fas* expression in picroliv fed group (Fig. 11). This inhibition of *caspase*-3 and *Fas* mRNA expression may be partly responsible for reduced cell death observed during reperfusion in picroliv prefed animals.

4. Discussion

Prolonged ischemia has been known to result in tissue and organ damage. The concept of reperfusion-induced tissue injury, defined as tissue damage occurring as a direct consequence of revascularization, is relatively recent. Reperfusion injury may increase the morbidity and mortality of patients undergoing vascular reconstruction, trauma surgery, and transplantation, and protocols that reduce its impact need to be developed (Homer-Vanniasinkam et al., 1997; Jaeschke, 1998). In this regard, we have investigated the effect of Picroliv in ameliorating

hepatic ischemia–reperfusion injury. The results presented in this report strongly suggest that picroliv preconditioning has an overall protective effect against hepatic ischemia–reperfusion injury in a rat model. We chose to evaluate the utility of a preconditioning agent as opposed to a therapeutic agent since hepatic surgery is more often elective in nature and reperfusion injury is known to cause morbidity in such a clinical setting (Kurokawa et al., 1996).

Nutritional status greatly modulates ischemia–reperfusion injury in normal livers. Depletion of energy storage of liver transplant recipients, as measured by liver glycogen content, has been proposed to be involved in the deterioration of peri-operative energy metabolism and the exaggerated post-operative cytokine response (Miki et al., 1999). Glycogenolysis, as measured by glucose release, is significantly increased during hypoxia, and does not return to normal levels following reoxygenation (Villalobos-Molina et al., 1998) and glycogen-depleted grafts produced more ketone bodies until 24 h after reperfusion (Miki et al., 1997). Rats with elevated liver glycogen have been shown to undergo significantly reduced hepatocellular injury in isolated perfused livers after 40 h cold storage (Lindell et al., 1996). Repletion of hepatic glycogen content has, therefore, been proposed as a treatment strategy to reduce reperfusion injury (Caraceni et al., 1999). In our study, animals prefed picroliv showed higher hepatocyte glycogen content whereas control animals had glycogen depleted livers at comparable time periods of reperfusion.

Several independent research laboratories have shown the importance of activated neutrophils during reperfusion injury following hepatic ischemia and various avenues are being explored to reduce neutrophil activation in such settings (Tomizawa et al., 1999; Okuhama et al., 1998). In fact, activation of neutrophils and macrophages and of the complement cascade with the formation of biologically active substances may be one explanation for the circulatory instability often seen in patients undergoing orthotopic liver transplantation (Tomasdottir et al., 1996; Terashima et al., 1996). Neutrophils are involved in organ damage induced by an excessive acute inflammatory response after ischemia–reperfusion. In addition to causing vascular injury, neutrophils can transmigrate and attack parenchymal cells by way of releasing proteases and reactive oxygen species. The development of therapeutic strategies to attenuate these excessive acute inflammatory responses without compromising essential host defense mechanisms has been considered critical (Jaeschke and Smith, 1997). Picroliv pretreatment resulted in reduction in numbers of infiltrating neutrophils in our experiments. Whether this involves downregulation or modification of adhesion molecules remains to be further explored (Toledo-Pereyra and Suzuki, 1994). We have indeed seen a downregulation of intracellular adhesion molecule-1 (ICAM-1) in kidneys following picroliv treatment (Seth et al., 2000).

Neutrophil derived reactive oxygen species are responsible for an intracellular oxidant stress in hepatocytes. Even after short periods of hepatic ischemia (20–30 min) with only moderate reperfusion injury, phagocytes are 'primed' for enhanced formation of such reactive oxygen species. Therefore, secondary stimuli such as those generated during sepsis can cause a potentiation of post-ischemic oxidant stress and liver injury leading eventually to acute liver failure (Jaeschke, 1994). Peroxides from neutrophils diffuse into hepatocytes leading to cell death. Thus, strengthening defense mechanisms against reactive oxygen species in target cells can attenuate excessive inflammatory injury without affecting host defense reactions (Jaeschke et al., 1999). Tissue MDA levels have been widely studied in the past as indicators of such oxidant damage (Chen et al., 1998; Tetik et al., 1999). We have also determined MDA levels in tissue homogenates as an indicator of membrane lipid peroxidation in order to compare our test and control groups. In the control group, as was expected, MDA levels rose with increasing duration of reperfusion indicating increasing degree of oxidant damage with time. In the picroliv treated group, not only were levels significantly lower, but they also did not rise appreciably with increasing reperfusion time, bearing out an anti-oxidant tissue preservation role for picroliv. These findings were consistent with increased superoxide dismutase expression in picroliv pre-fed rats which is known to scavenge free radicals and provides first line of defense against oxidative injury in tissues (Mizuta et al., 1989). This better tissue preservation was also confirmed on ultrastructural examination of livers from the test and control groups.

Although necrosis and apoptosis are distinct, but non-exclusive mechanisms of cell death and their modulation play a pivotal role in warm ischemia–reperfusion injury of the liver. Cell death by apoptosis is involved in various pathophysiological situations involving the liver. It is believed to be an important source of hepatocyte morbidity as related to ischemia–reperfusion injury, a circumstance which physicians often face in the field of the liver surgery (Miyoshi and Gores, 1998). Given the distinct nature of apoptosis and the highly regulated and conserved pathway for its initiation, inhibition of apoptosis may serve to decrease allograft reperfusion injury after orthotopic liver transplantation (Kuo et al., 1998). In fact, hepatocyte apoptosis is activated during early phase of reperfusion after liver transplantation (Sasaki et al., 1997a) and in-situ staining of liver biopsy specimens using TUNEL has shown significant apoptosis after reperfusion. (Kohli et al., 1999). In another report, it has been demonstrated that apoptotic bodies were maximal after 180 min reperfusion and subsequently the number of apoptotic bodies decreased as the length of reperfusion period increased following warm ischemia (Sasaki et al., 1997b). Pretreatment with picroliv was able to substantially reduce hepatocyte apoptosis following ischemia–reperfusion and this reduced apoptosis was associated with decreased expression of *Fas* and *caspase*-3. Earlier studies have indicated a possible

role of *Fas* expression in enhanced apoptosis of hepatocytes during allograft rejection (Tannapfel et al., 1999; Afford et al., 1999). Likewise, *caspase*-3 inhibitors have also been shown to prevent liver damage after ischemia–reperfusion injury (Cursio et al., 1999). If we couple the extent of apoptosis with PCNA responses, our results are even more significant. Not only was apoptosis reduced in treated animals but concomitantly an immediate hepatocellular response of PCNA expression was seen following reperfusion, which was significantly enhanced in Picroliv prefed animals indicating greater DNA synthesis in this group. Earlier IL-6 has been indicated to promote hepatocyte proliferation against warm ischemia–reperfusion injury (Camargo et al., 1997). The exact mechanism of enhanced tissue regeneration in picroliv prefed animals appears to be different than its effects in reducing the ischemia–reperfusion induced injury. Presently, studies are in progress to delineate the molecular mechanism for the enhanced mitotic activity in picroliv prefed animals.

Cytokines mediate inflammatory injury and studies have shown that cytokines may mediate binding of leukocytes to hepatocytes and cause upregulation of adhesion molecules (Sano et al., 1999; Matsushita et al., 1998). Overproduction of cytokines in the post-operative period is therefore deleterious to the host. We found a reduction in the expression of IL-1α and IL-1β following hepatic ischemia–reperfusion in animals prefed with picroliv. Interestingly, depletion of energy stores has been proposed to be a causative mechanism in the exaggerated post-operative cytokine response following liver transplantation (Miki et al., 1999). This is in concordance with our results, wherein the untreated control animals have shown higher tissue IL-1 levels as well as depleted glycogen stores.

In summary, we have shown that picroliv, a novel pharmacological agent with antioxidant properties, can attenuate warm hepatic ischemia–reperfusion injury in an in-vivo rat model. Pretreatment of rats with oral picroliv prior to induction of ischemia led to a reduced hepatocyte damage and preserved hepatocyte vitality. Picroliv could thus be evaluated as a pre-clinical agent with ability to attenuate reperfusion-induced hepatic injury.

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