*Arch Pharm Res Vol 23, No 6, 620-625, 2000*



# Role of Kupffer Cells in Cold/Warm Ischemia-Reperfusion Injury of Rat Liver

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*(Received August 11, 2000)*

The mechanisms of liver injury from cold storage and reperfusion are not completely understood. The aim of the present study was to investigate whether the inactivation of Kupffer cells (KCs) by gadolinium chloride ($GdCl_3$) modulates ischemia-reperfusion injury in the rat liver. Hepatic function was assessed using an isolated perfused rat liver model. In livers subjected to cold storage at 4°C in University of Wisconsin solution for 24 hrs and to 20 min rewarm-ing ischemia, oxygen uptake was markedly decreased, Kupffer cell phagocytosis was stimulated, releases of purine nucleoside phosphorylase and lactate dehydrogenase were increased as compared with control livers. Pretreatment of rats with $GdCl_3$, a selective KC toxicant, suppressed Kupffer cell activity, and reduced the grade of hepatic injury induced by ischemia-reperfusion. While the initial mixed function oxidation of 7-ethoxycoumarin was not different from that found in the control livers, the subsequent conjugation of its meta-bolite to sulfate and glucuronide esters was suppressed by ischemia-reperfusion. $GdCl_3$ restored sulfation and glucuronidation capacities to the level of the control liver. Our findings suggest that Kupffer cells could play an important role in cold/warm ischemia-reperfusion hepatic injury.

**Key words:** Kupffer cells, Ischemia-reperfusion, Cell viability, Drug metabolism.

## INTRODUCTION

Primary nonfunction and dysfunction occur in 5 to 30 % of liver transplantation cases, resulting in either a requirement for retransplantation or the death of the recipient (Ploeg *et al.*, 1993). Because liver transplantation is the therapy of choice in an increasing number of liver diseases (Pichlmayr *et al.*, 1987) and the organ pool is limited, there is an urgent need to understand the under-lying mechanisms responsible for graft failure.

Multiple mechanisms, including the formation of oxygen free radicals (Marzi *et al.*, 1989), the activation and migration of leukocytes (Takei *et al.*, 1991), the injury of endothelial cells (Caldwell-Kenkel *et al.*, 1989), and the disturbance of hepatic microcirculation (Thurman *et al.*, 1988) have been identified as accounting for cold/warm ischemia-reperfusion injury in the liver. Previous studies have also suggested that Kupffer cells (KCs), resident macrophages of the liver, are activated during cold storage (Carles *et al.*, 1994), on reperfusion of the isolated liver (Caldwell-Kenkel *et al.*, 1991), as well as upon organ transplantation (Marzi *et al.*, 1991). Direct evidence, however, of the involvement of KCs in such type of injury is still lacking in the literature.

In patients with chronic liver disease, the elimination of drugs metabolized by the liver is often impaired (Huet and Villenueve, 1983). Indeed, our recent studies have suggested that abnormalities in microsomal mixed function oxidation associated with lipid peroxidation occur during hepatic ischemia and reperfusion *in vivo* (Lee *et al.*, 2000). However, the subsequent conjugation of oxidized metabolites has not been investigated yet.

Therefore, the objective of the current investigation was to test the hypothesis that KC activation is a contributing factor to cold/warm ischemia-reperfusion injury in the liver. In order to elucidate this hypothesis, rats were pretreated with gadolinium chloride ($GdCl_3$), a selective Kupffer cell toxicant, and the liver viability and functional parameters were studied.

## MATERIALS AND METHODS

### Chemicals

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Gadolinium chloride ($GdCl_3 \cdot 6H_2O$), 7-ethoxycoumarin, glucuronidase, sulfatase, xanthine oxidase, uric acid, inosine and LDH kit were supplied by the Sigma Chemical Co. All other chemicals used in this study were of reagent grade and were locally and commercially available.

### Animals

Male Sprague-Dawley rats weighing 280 ± 20 g were obtained from Jeil Animal Breeding Company of Korea and were acclimatized to laboratory conditions at Sung kyunkwan University for at least one week. During this period, food (Samyang Co., Korea) and tap water were supplied *ad libitum*. The experimental animals were kept in a temperature and humidity controlled room (25 ± 1 °C, 55 ± 5%, respectively) with 12 hrs of light-dark cycle and were fasted for 18 hrs before the experiment.

### Hepatectomy and perfusion

The rats were anesthetized by the intraperitoneal injection of pentobarbital sodium (40 mg/kg). A midline incision was made to the abdomen, and the portal vein was then cannulated with PE-190 and flushed with Krebs-Henseleit bicarbonate buffer (KHBB) containing (in mmol/L) NaCl, 118; KCl, 4.6; $CaCl_2$, 2.5; $KH_2PO_4$, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; and glucose 5 (pH 7.4, 37°C). The flow rate was 4 ml/min/g liver and the perfusate was saturated with 95% $O_2$-5% $CO_2$ gas mixture. After flushing, the inferior vena cava was ligated above the right renal vein and cut distally. During flushing, the liver was dissected free from the rat and moved to the perfusion apparatus. The liver was flushed with 30 ml of Ringers lactate solution (4°C) and stored in University of Wisconsin solution (UW, Du Pont Pharmaceuticals, DE, U.S.A.) for 24 hrs at 4°C. Livers were then immersed in oxygenated Ringers lactate solution at 37°C for 20 min. Following sequential cold and warm ischemia, UW was flushed from the liver with 10 ml of Ringers lactate solution at 37°C and the liver was reperfused with KHBB for 80 min. The livers were perfused at rates of ~4 ml/min/g liver, essentially as previously described (Lee and Clemens, 1992). Samples of perfusate were taken at 5, 20, 40, 60 and 80 min of reperfusion. After 80 min of reperfusion the liver was weighed.

### Administration of $GdCl_3$ and experimental groups

$GdCl_3$, dissolved in distilled saline (vehicle) solution, was administered by intraperitoneal injection at a dose of 10 mg/kg of body weight 48 hr prior to hepatectomy. In the vehicle-treated rats, distilled saline solution was injected in the same volume and manner as $GdCl_3$. Three experimental groups were studied: (a) control group, (b) vehicle-treated cold/warm ischemia-reperfusion group and (c) $GdCl_3$-treated cold/warm ischemia-reperfusion group.

### Kupffer cell activity and oxygen consumption

Kupffer cell activity was measured according to the method of Cowper *et al.* (1990). Briefly, rates of colloidal carbon uptake (mg/g liver/hr) were calculated from the influent minus effluent carbon concentration differences measured spectrophotometrically at 623 nm and at the perfusion flow rate. Oxygen concentration in the effluent perfusate was monitored continuously with a Clark-type oxygen electrode. The $O_2$ uptake rates of the whole livers were calculated from influent minus effluent $O_2$ concentration differences at constant flow rates and were normalized for the wet weight of the liver (Lee and Clemens, 1992)

### Analytical procedures

Lactate dehydrogenase (LDH) activity, a marker of hepatocyte injury, was determined by spectrophotometric procedures using Sigma kit #228-50 (Sigma Chemical Co.). Purine nucleoside phosphorylase (PNP) activity, a marker of endothelial cells, was measured according to the method previously described by Hoffe *et al.* (1978). The rates of hepatic oxidation of 7-ethoxy-coumarin and of the subsequent conjugation of the 7-hydroxycoumarin to sulfate and glucuronide esters were assayed according to Cha *et al.* (1987).

### Statistical analysis

Data is presented as mean ± SE (standard error of the mean). The statistical significance was determined using a two-tailed unpaired Student's *t*-test. A p-value $<0.05$ was deemed to be significant.

## RESULTS

### Kupffer cell activity and oxygen consumption

The mean activity of Kupffer cells in isolated perfused livers was approximately 62.3 ± 1.5 mg/g liver/hr. In the vehicle-treated ischemic livers, the activity of Kupffer cell was significantly increased as compared to the control livers at 5, 20, 40 and 60 min of reperfusion. However, this increase was suppressed by pretreatment with $GdCl_3$ (Fig. 1). The rate of oxygen uptake averaged 141.0 ± 5.6 μmol/g liver/hr in isolated perfused livers. In the vehicle-treated ischemic livers, the rate of oxygen uptake was significantly decreased during reperfusion. $GdCl_3$ treatment did not prevent a decrease in the rate of oxygen consumption (Fig. 2).

### LDH and PNP

The mean release of LDH in isolated perfused livers was found to be approximately 66.9 ± 13.5 U/l. In the vehicle-treated ischemic-livers, the release of LDH was significantly increased as compared to the control livers

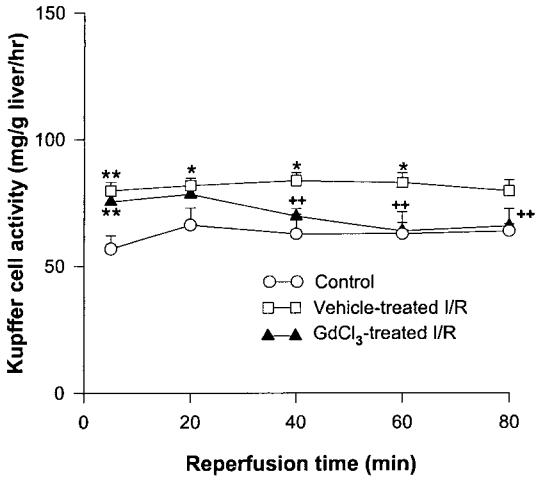


**Fig. 1.** Effect of $GdCl_3$ on the activity of Kupffer cells following cold/warm ischemia-reperfusion. Values represent means ± S.E.M. for 7 to 10 rats per group. *,**=Significantly different ($P<0.05$, $P<0.01$) from the control group. $^{++}$=Significantly different ($P<0.01$) from the vehicle-treated I/R group. $GdCl_3$, gadolinium chloride; I/R, ischemia-reperfusion.

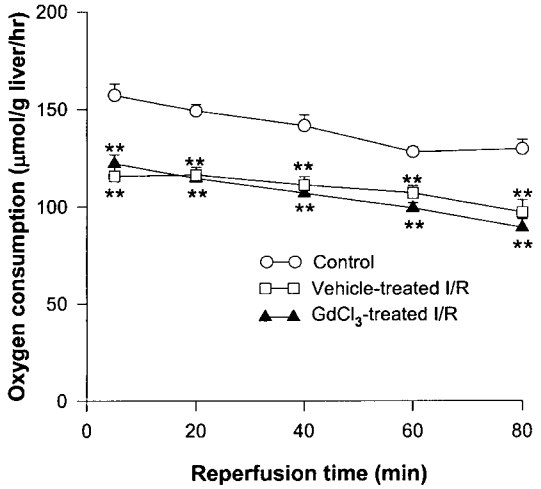


**Fig. 2.** Effect of $GdCl_3$ on the oxygen uptake following cold/warm ischemia-reperfusion. Values represent means ± S.E.M. for 7 to 10 rats per group. **=Significantly different ($P<0.01$) from the control group. $GdCl_3$, gadolinium chloride; I/R, ischemia-reperfusion.

during reperfusion. However, this increase was suppressed by pretreatment with $GdCl_3$ (Fig. 3). The release of PNP averaged 1.35 ± 0.18 U/l in isolated perfused liver. In the vehicle-treated ischemic livers, the release of PNP was significantly increased during reperfusion as compared to the controls. However, this increase was suppressed by treatment with $GdCl_3$ (Fig. 4).

## Drug metabolism

The rates of hepatic oxidation of 7-ethoxycoumarin

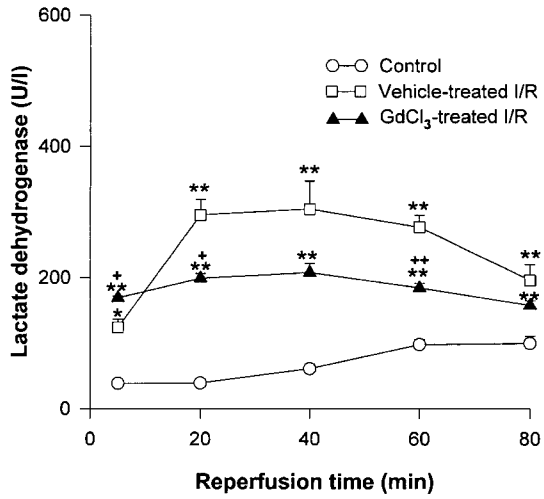


**Fig. 3.** Effect of $GdCl_3$ on the release of lactate dehydrogenase following cold/warm ischemia-reperfusion. Values represent means ± S.E.M. for 7 to 10 rats per group. *,**=Significantly different ($P<0.05$, $P<0.01$) from the control group. $^{+}$,$^{++}$= Significantly different ($P<0.05$, $P<0.01$) from the vehicle-treated I/R group. $GdCl_3$, gadolinium chloride; I/R, ischemia-reperfusion.

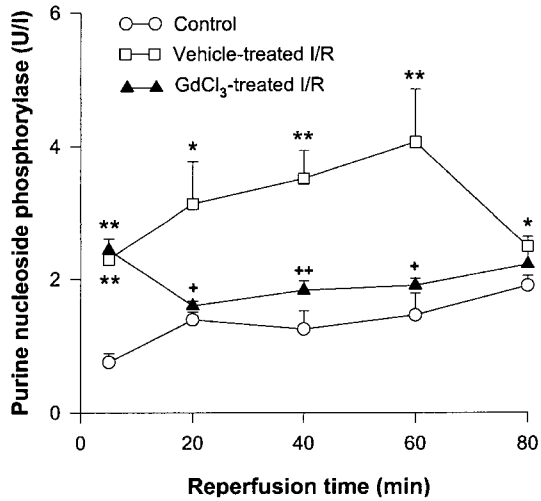


**Fig. 4.** Effect of $GdCl_3$ on the release of purine nucleoside phosphorylase following cold/warm ischemia-reperfusion. Values represent means ± S.E.M. for 7 to 10 rats per group. *,**= Significantly different ($P<0.05$, $P<0.01$) from the control group. $^{+}$,$^{++}$=Significantly different ($P<0.05$, $P<0.01$) from the vehicle-treated I/R group. $GdCl_3$, gadolinium chloride; I/R, ischemia-reperfusion.

and of the subsequent conjugation of the 7-hydroxycoumarin to sulfate and glucuronide esters are shown in Table I. The mean rate of hepatic oxidation of 7-ethoxycoumarin in isolated perfused livers was found to be approximately 0.54 ± 0.08 nmol HC/g liver/min. In the vehicle-treated ischemic livers, the rate of hepatic oxidation of 7-ethoxycoumarin did not change significantly as compared to the control livers. However, in the $GdCl_3$-treated ischemic livers, there was a significant increase in

**Table I.** Effect of $GdCl_3$ on hepatic drug metabolism after cold/warm ischemia-reperfusion

| | Reperfusion time | | | | |
|---|---|---|---|---|---|
| | *5 min* | *20 min* | *40 min* | *60 min* | *80 min* |
| Oxidation (nmol HC/g liver/min) | | | | | |
| Control | 0.26 ± 0.02 | 0.49 ± 0.02 | 0.68 ± 0.06 | 0.64 ± 0.03 | 0.64 ± 0.03 |
| Vehicle-treated I/R | 0.70 ± 0.15 | 0.54 ± 0.06 | 0.62 ± 0.08 | 0.62 ± 0.06 | 0.57 ± 0.06 |
| $GdCl_3$-treated I/R | 0.74 ± 0.17 | 0.76 ± 0.07$^{**\#}$ | 0.82 ± 0.08 | 0.73 ± 0.07 | 0.71 ± 0.06 |
| Glucuronidation (nmol HC/g liver/min) | | | | | |
| Control | 0.66 ± 0.10 | 1.03 ± 0.13 | 1.23 ± 0.23 | 1.31 ± 0.24 | 1.26 ± 0.23 |
| Vehicle-treated I/R | 0.07 ± 0.01$^{*}$ | 0.34 ± 0.06$^{**}$ | 0.50 ± 0.08$^{**}$ | 0.65 ± 0.10$^{*}$ | 0.64 ± 0.11$^{*}$ |
| $GdCl_3$-treated I/R | 0.06 ± 0.00$^{*}$ | 0.52 ± 0.11$^{*}$ | 1.02 ± 0.17$^{\#\#}$ | 1.16 ± 0.18$^{\#}$ | 0.95 ± 0.13$^{\#}$ |
| Sulfation (nmol HC/g liver/min) | | | | | |
| Control | 9.24 ± 0.68 | 11.50 ± 0.67 | 12.41 ± 0.67 | 13.31 ± 0.88 | 12.51 ± 0.89 |
| Vehicle-treated I/R | 5.52 ± 0.92$^{*}$ | 7.86 ± 0.84$^{**}$ | 8.60 ± 0.93$^{**}$ | 8.73 ± 1.10$^{**}$ | 8.70 ± 0.97$^{*}$ |
| $GdCl_3$ treated I/R | 6.06 ± 0.82 | 11.69 ± 0.93$^{\#\#}$ | 12.09 ± 1.01$^{\#}$ | 12.41 ± 1.12$^{\#}$ | 12.02 ± 0.95$^{\#}$ |

Each value represent the mean ± S.E.M. for 7 to 10 rats per group.
*,**=Significantly different ($P<0.05$, $P<0.01$) from the controls.
#,##=Significantly different ($P<0.05$, $P<0.01$) from the vehicle-treated I/R group.
HC, hydroxycoumarin; $GdCl_3$, gadolinium chloride; I/R, ischemia-reperfusion.

oxidation at 20 min of reperfusion as compared to the control livers. In isolated perfused livers, the rate of hepatic glucuronidation, in the subsequent conjugation of 7-hydroxycoumarin, averaged 1.10 ± 0.12 nmol HC/g liver/min. In the vehicle-treated ischemic livers, the rate of hepatic glucuronidation was significantly decreased during reperfusion as compared to the control livers. However, this decrease was recovered by treatment with $GdCl_3$. The mean rate of hepatic sulfation, in the subsequent conjugation of 7-hydroxycoumarin, was approximately 11.8 ± 0.7 nmol HC/g liver/min. In the vehicle-treated ischemic livers, the rate of hepatic sulfation was significantly decreased during reperfusion as compared to the control livers. However, this decrease was restored by $GdCl_3$ treatment.

## DISCUSSION

Reperfusion injury to liver graft occurs *in vivo* shortly after orthotopic transplant (Thurman *et al.*, 1988). Ischemia-reperfusion-induced hepatic injury is one of the major problems following transplantation of the liver (Thurman *et al.*, 1988). There is evidence that tumor necrosis factor and reactive oxygen species released from neutrophils and/or monocytes are found to be involved in the ischemia-reperfusion-induced tissue injury such as injury to the heart, lung, and intestine (Kennedy *et al.*, 1989). Recent studies revealed that Kupffer cells are activated after reperfusion following cold storage (Rao *et al.*, 1991). The objective of the present study was to investigate whether Kupffer cell activation is a contributing factor in the mechanisms of cold/warm ischemia-reperfusion injury of the rat liver. The potential injury to drug metabolism after cold storage was also elucidated.

Data on PNP release presented here suggested that the stimulation of Kupffer cells may damage the integrity of endothelial cells during cold/warm ischemia and reperfusion. Although there is some controversy about the use of PNP as an indicator of endothelial injury (Brass and Mody, 1995), the enzyme is believed to originate primarily from damaged endothelial cells (Rao *et al.*, 1990).

A widely accepted hypothesis proposes that reductions in microvascular perfusion would be the first sign of liver injury and, as a result, predispose the parenchyma to injury during ischemia and reperfusion (Chun *et al.*, 1994). Kupffer cells, when stimulated or activated, have been found to contribute to hepatic injury in several experimental models (Shiratori *et al.*, 1994). Kupffer cells are known to produce a number of biologically active metabolites, the role of oxygen radical intermediates by activated macrophages in the liver and tumor necrosis factor has been found to contribute to experimental hepatic injury (Nakagawa *et al.*, 1990). In the present study, treatment of $GdCl_3$, which suppressed the phagocytic functions of Kupffer cell, reduced the LDH release in perfusate. The increased perfusate level of LDH may account for the greater metabolic activity in damaged hepatocytes or the shedding of cytosolic components in blebs formed during ischemia and reperfusion (Lemasters and Thurman, 1991). Thus, these results emphasize the importance of Kupffer cell activation in postischemic hepatic cell damage. Similary, Jaeske and Farhood (1991) showed that Kupffer cells take part in the initial phase of warm ischemia-reperfusion injury in the *in vivo* model.

Under normal conditions, most of the infused 7-ethoxycoumarin is oxidized to 7-hydroxycoumarin and forms sulfate (major) and glucuronide (minor) esters (Yang and Carlson, 1991). In our data, sulfation and glucuronidation of 7-hydroxycoumarin significantly decreased. The diminished ability of liver to form the sulfate ester conjugate was prevented by pretreatment with $GdCl_3$. While the exact mechanism by which Kupffer cells inhibit sulfate conjugation remains unclear, activation of Kupffer cells may inhibit energy production or decrease the adenylate pool size and again limit the synthesis of 3-phosphoadenosine-5-phosphosulfate, a cofactor required for sulfate conjugation, and thereby suppress the sulfate conjugation. A decrease in the cellular level of ATP is common to all ischemic tissues and has been confirmed by many researchers (Marubayashi *et al.*, 1980).

$GdCl_3$ also inhibited the decreased glucuronidation of 7-hydroxycoumarin. This inhibition may have been caused by the Kupffer cells dependent suppression of the synthesis of UDP-glucuronic acid, a cofactor required for glucuronidaion. Griffeth *et al.* (1987) have shown that hind-limb ischemia resulted in a decrease in hepatic UDP-glucuronic acid content.

In summary, we have demonstrated that Kupffer cells contribute in part to abnormalities in the drug-metabolizing function during cold/warm ischemia-reperfusion. Our findings suggest that Kupffer cell activation is prime causative factor in the pathogenesis of liver graft failure.

## ACKNOWLEDGEMENTS

This work was supported by grant 981-0714-110-2 from the Basic Research Program of the Korea Science & Engineering Foundation.

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