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# MICROCIRCULATORY ABERRATIONS IN THE ISOLATED PERFUSED RAT LIVER INDUCED BY SODIUM CYANIDE, ANOXIA OR ACETAMINOPHEN

Kihwa Jung

*Department of Pharmacy, College of Pharmacy,*
*Duksung Women's University, Seoul 132-714, Korea*

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***ABSTRACT:*** *When acetaminophen (25 mM) was introduced into the perfused rat liver, the hepatic $O_2$ uptake was rapidly inhibited first and then later slow-down. The rapid inhibition was found to be due to mitochondrial blockade, whereas the so-called slow inhibition" was associated with microcirculatory aberrations as evidenced by inhomogeneous staining of the liver tissue by trypan blue infusion (0.1%).NaCN (0.5 mM) also caused rapid and slow respiratory inhibitions, giving heterogeneous trypan blue staining. Similarly inhomogeneous trypan blue staining was noted when the liver was perfused with anoxic medium. Accompanying the slow inhibition and heterogeneous trypan blue staining appeared to be a release of lactate dehydrogenase from the liver into the effluent.*

***Key words:*** *Microcirculatory aberration, Acetaminophen hepatotoxicity, Perfused liver, Hepatic respiration, Local ischemia*

## INTRODUCTION

Acetaminophen (N-acetyl-p-aminophenol, paracetamol, Tylenol$^{R}$) is one of the most widely used over-the-counter non-narcotic analgesics and antipyretics. This drug is safe when administered in pharmacological dosages, but can cause often fatal liver injuries when taken in large quantities (Prescott *et al.*, 1971; Rumack *et al.*, 1981; Black, 1984). In general a single dose of 15 grams or more will produce hepatotoxicity in most individuals (Zimmerman, 1981).

The mechanism of acetaminophen-induced liver injury has been intensify investigated since the classical studies performed by Mitchell, Jollow, Gillette and their coworkers in the early 1970's (Mitchell *et al.*, 1973; Jollow *et al.*, 1973; Davis *et al.*, 1974; Jollow *et al.*, 1974; Potter *et al.*, 1973). The major conclusions derived from these investigations are 1) that acetaminophen itself is not hepatotoxic but must be first metabolized by the cytochrome P-450-mediated monooxygenase system to produce a reactive intermediate, later identified to be N-acetyl-p-benzoquinoneimine (NAPQI)

(Dahlin *et al.*, 1984), and 2) that hepatocytes are equipped with various detoxifying mechanisms including reduced glutathione, GSH, with which to remove potentially cytotoxic electrophiles such as NAPQI.

The experimental results that have been obtained by S. Ji and his coworkers during the past five years indicate that the mechanism of acetaminophen hepatotoxicity is much more complex than previously thought. For example, they have demonstrated that acetaminophen itself, prior to any metabolism, can be cytotoxic since it inhibits mitochondrial respiration by blocking Complex I (NADH-ubiquinone oxidoreductase) (Cheng *et al.*, 1984; Esterline, 1989), leading to a rapid depletion of the hepatic ATP content (Ji *et al.*, 1984).

The purpose of the present series of experiments was to delineate the possible cellular and physiological mechanisms underlying the phenomenon of inhibition of hepatic respiration. The experimental evidence obtained supports that hypothesis that a part of the inhibition of hepatic respiration induced by acetaminophen given to the Perfused liver from acutely alcohol-pretreated rats is due to regional collapse of sinusoids in the liver, leading to the development of ischemia (*i.e.*, lock of microcirculation. We also used NaCN and anoxic medium to similar results obtain.

## MATERIALS AND METHODS

### Chemicals and Enzymes

Acetaminophen and the lactate dehydrogenase (EC. 1.1.1.27) assay kit (LD No. 228-UV) were purchased from Sigma Co. (St. Louis, MO). All other chemicals were reagent grade from commercial sources.

### Animals

Female Sprague-Dawley (180-250g) and female Fischer 344 rats (200-250 g) were purchased from Taconic Laboratory Animals and Services (Germantown, N.Y.). All animals were subjected to a 12 hour day/night cycle with lights on at 6:00 a.m. and off at 6:00 p.m., with a free access to food (Purina Rodent Mouse Chow, Fisher Distributor, Bound Brrok, N.J.) and tap water. Rats were acclimated to the animal facility for at least one week prior to experiments.

### Liver Perfusion

Livers were removed from rats under sodium pentobarbital anesthesia (50 mg/kg body weight) and perfused with the Krebs-Henseleit bicarbonate buffer essentially as described in the literature (Scholz *et al.*, 1969; Sies, 1978), except that the liver was placed in a cup-shaped plastic holder in an upside-down position to improve microcirculation. The liver perfusion apparatus used is schematically shown in Figure 1. The perfusate was equilibrated with 95% $O_2$ and 5% $CO_2$ in a constant temperature water bath kept at 45-47°C and pumped through the liver via a polyethylene cannula inserted into the portal vein. The oxygen tension of the inflow perfusate was Kept at $670 \pm 20$ torr. The temperature of the perfusate arriving at the liver was continuously monitored with a thermocouple and maintained at 36-38°C. Any air bubbles formed

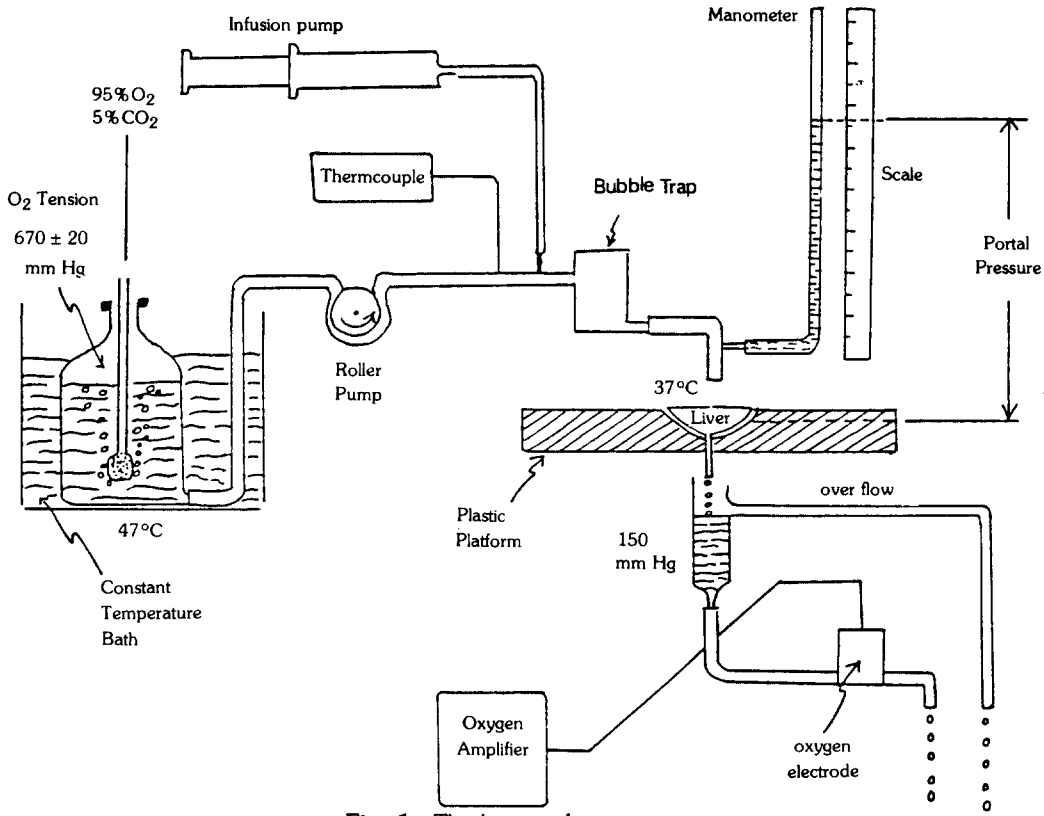


**Fig. 1.** The liver perfusion apparatus.

in the tubings were removed by a bubble trap located just prior to the liver. The effluent dripped into a funnel connected to a Clark-type oxygen electrode (Bachofer laboratory Equipment, Inc., Reutlingen, West Germany) which continuously measured the oxygen tension of the effluent. The perfusion flow rate was kept constant at 4-6 ml/ming liver and the effluent was not recirculated. Addition of acetaminophen was carried out by switching the perfusate bottle from control to that containing acetaminophen dissolved in the Krebs-Henseleit bicarbonate buffer at a desired concentration.

## Rate of Hepatic Oxygen Uptake

The oxygen electrode was calibrated before and after each experiment in order to ascertain that there was no undue drift of the sensitivity of the electrode during a given experiment. The rate of hepatic oxygen uptake was calculated from the inflow-outflow oxygen concentration differences, flow rate, and liver wet weight.

## Rate of Lactate Dehydrogenase (LDH) Leakage from the Perfused Liver

At 15 and 25 minutes following the beginning of the liver perfusion, two samples (1-3 ml) of the effluent was taken and stored on ice. Immediately thereafter, the control perfusate was switched to one containing 25 mM acetaminophen. Four more samples were taken at every 20 minutes. Each effluent sample was analyzed for LDH activity

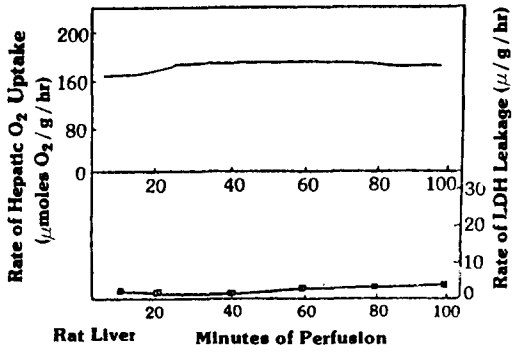


**Fig. 2.** The stability of the isolated perfused rat liver with respect to respiration and hepatocyte viability as measured by LDH leakage.

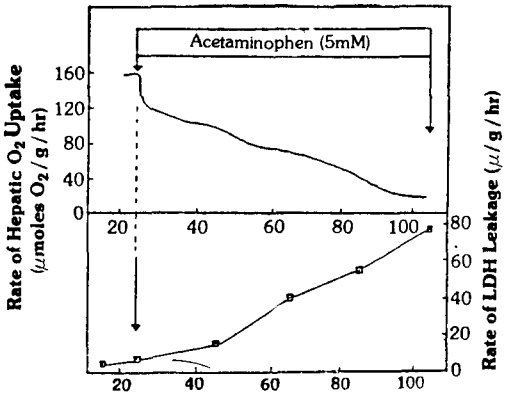


**Fig. 3.** Acetaminophen-induced inhibition of hepatic respiration and LDH leakage into the effluent of the isolated perfused rat liver.

according to the Sigma Diagnostics kit for LDH, Procedure No. 228-UV, except that the absorbance at 340 nm was continuously recorded using a strip chart recorder, and the slope of the initial portion of the recording was used to calculated the LDH activity. The rate of LDH leakage from the perfused liver in Unit/g liver/hr was calculated from the effluent concentration of LDH (L) in Unit/liter measured at a given time point, flow rate (F) in liter/hr, and liver weight (W) in *g*, according to the formula, LF/W.

### Hepatic Microcirculation Viewing by Trypan Blue Staining

Trypan blue infusion was previously utilized to visualize regional microcirculatory blockades induced by the electrical stimulation of the hepatic nerves in the isolated perfused rat liver; microregions, 1-3 mm in diameter, with collapsed capillaries could be detected as unstained areas upon infusion of trypan blue through the portal vein (Ji *et al.*, 1984). The same technique was employed to determine the status of microcirculation in the perfused liver exhibiting inhibition of hepatic respiration. A 0.1% trypan blue solution was prepared by diluting the 0.5% membrane-filtered trypan blue solution (Hazelton Research Products, Inc., St. Lenexa, KS) with the Krebs-Henseleit bicarbonate buffer. The pH of the 0.1% trypan blue solution was adjusted to 7.4 and equilibrated with 95% $O_2$ and 5% $CO_2$ at 47°C for at least 30 min. before use. At the end of a perfusion experiment (typically about 90 minutes after perfusion), the perfusate was switched to the 0.1% trypan blue solution. At 0, 5, 10, 20, and 40 seconds after the arrival of trypan blue at the portal vein, the liver surface was photographed with a Nikon F3 camera equipped with a 55 mm Micro-NIKKOR lens and a motor drive. Ektachrome 200 (Kodak) was used at a film speed setting of 300 in order to remove the yellowish tint in the final prints.

## RESULTS

### Rapid and Slow Inhibitions of Hepatic Respiration

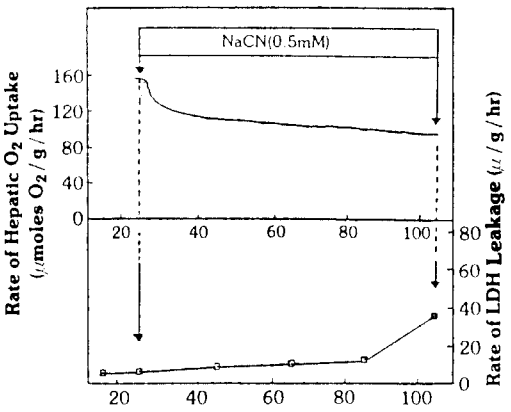


**Fig. 4.** NaCN-induced inhibition of hepatic respiration and LDH leakage into effluent of the isolated perfused rat liver.

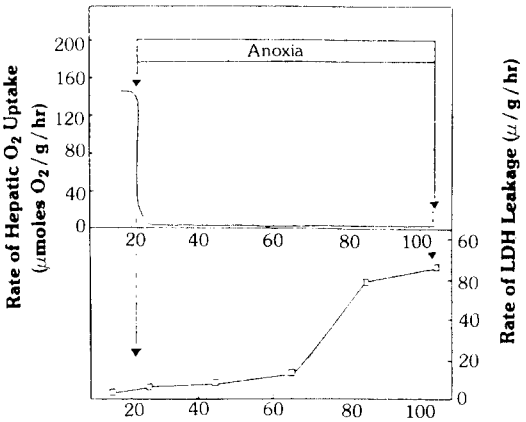


**Fig. 5.** Anoxia-induced inhibition of hepatic respiration and LDH leakage into the effluent of the isolated perfused rat liver.

The control rate of hepatic respiration measured under our experimental conditions remain fairly constant over a time period of 1-2 hours as shown in Fig. 2. The viability of the liver is well maintained as indicated by a low rate of LDH leakage.

In contrast, an infusion of 25 mM acetaminophen causes two types of respiratory inhibitions-one called "rapid inhibition" (19 ± 1.3% of control rate inhibited within 2.2 ± 0.4 min; mean SEM, n = 5) (Ray *et al.*, 1988) and the other termed "slow inhibition". The latter can be detected at varying time points following acetaminophen infusion, ranging from a few minutes to 1/2 hr, and proceeds with a rate of 50-100 % of the control rate inhibited per hr, depending on the physiological state of the perfused liver. A typical experiment showing these two types of respiratory inhibitions is depiced in Fig. 3. From a series of similar experiments, it has been found that 25 mM acetaminophen inhibited hepatic respiration by 33.4 ± 4.3% through the mechanism of a rapid inhibition and by about 42% (i.e., 75.4%-33.4%) through the mechanism of a slow inhibition. Sodium cyanide (0.5 mM) also caused rapid and slow inhibition of hepatic respiration (Fig. 4). When anoxic medium is infused (Fig. 5), the hepatic respiration rapidly reduces to zero.

## LDH Leakage Accompanying the Slow Inhibition of Hepatic Respiration

Table 1 shows leakage of LDH in the effluent as determined by the procedure described in methods.

**Table 1.** The Rate of LDH leakage in the isolated perfused rat liver

| Times (min) / Parameters | 15 | 25 | 45 | 65 | 85 | 105 |
|---|---|---|---|---|---|---|
| Acetaminophen | 3.89 ± 0.32 | 5.76 ± 1.16 | 14.92 ± 3.87 | 41.62 ± 15.26 | 53.37 ± 9.77 | 76.73 ± 12.24 |
| NaCN | 2.38 ± 0.50 | 3.18 ± 0.57 | 3.97 ± 0.55 | 4.48 ± 0.63 | 5.86 ± 0.62 | 17.66 ± 5.53 |
| Anoxic medium | 4.47 ± 1.16 | 5.13 ± 1.03 | 6.33 ± 1.12 | 10.27 ± 2.15 | 59.85 ± 23.71 | 65.84 ± 16.48 |

Mean ± S.E.

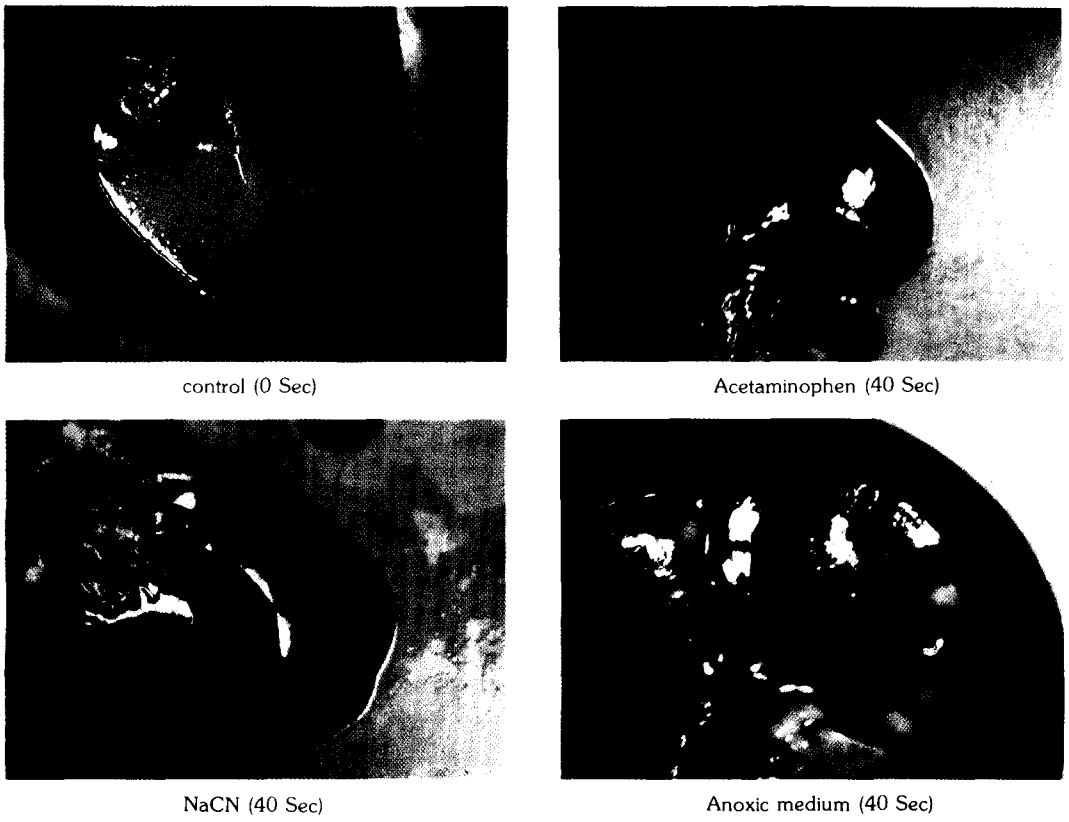


**Fig. 6.** Heterogeneous trypan blue staining of the isolated perfused rat liver. Acetaminophen (25 mM), NaCN and Anoxic medium were infused through the portal vein for 100 minutes and the trypan blue solution (0.1%) was then introduced into the liver for 40 seconds. Other details are given in methods.

### Microcirculatory Aberrations as Visualized by the Pattern of Trypan Blue Staining

The trypan blue staining technique described in methods was employed to visualize local ischemia. As shown in Figure 6, the infusion of 0.1% trypan blue into the portal vein for 40 seconds stains the liver tissue completely and homogeneously. However, if the same dye infusion is carried for 40 seconds following an 80 minute exposure of the liver to 25 mM acetaminophen, 0.5 mM NaCN or anoxic medium, the trypan blue staining is incomplete and heterogeneous, indicating the presence of microregions of ischemia in the liver.

## DISCUSSION

The experimental data presented in this paper strongly indicate that the phenomenon of the "slow inhibition" of hepatic respiration is mechanistically connected to a regional collapse of sinusoids in the perfused liver, leading to the development of local ischemia (Fig. 6). The postulated capillary collapse is intimately associated with liver cell injury, since LDH is found to be released into the effluent in parallel with the pro-

**Table 2.** Two distinct mchanisms of the inhibition of liver functions

| Mechanisms | Component Processes or Factors |
|---|---|
| Metabolic | 1) inhibition of respiratory enzymes<br>2) substrate insufficiency (e.g., anoxia)<br>3) cell death (loss of enzymes) |
| Microcirculatoly | 1) capillary collapse (local ischemia)<br>2) micro-flow redistribution (e.g., microcirculatory shunting) |

gression of slow inhibition of hepatic respiration (Fig. 3,4 and 5).

The questions as to how acetaminophen, NaCN or anoxia can cause sinusoidal collapse cannot be clearly answered at present. However, an intriguing possibility is suggested by the recent developments in the field of endothelium-derived relaxing factors (EDRF) (Palmer *et al.*, 1987; Gryglewski *et al.*, 1986; Moncada *et al.*, 1986), according (or NaCN or $N_2$)-induced sinusoidal collapse is due to an inhibition of the production of EDRF from hepatic endothelium; Acetaminophen (or NaCN or $N_2$) — Injury to hepatic endothelium — Inhibition of EDRF production — Vassoconstriction — Slow inhibition of hepatic respiratory

The causal relationship between slow inhibition of hepatic respiratiory and LDH release cannot be clearly defined on the basis of the experimental data presented herein. In principle, LDH release can precede capillary collapse; or a capillary collapse can lead to cell injuries downstream leading to LDH release. In some experiments the slow inhibition preceded LDH release, and in others the reverse order was found, indicating that either one of the respiratory inhibition and LDH leakage (ie, cell death) these processes can act as the cause of the other. Further experiment are in progress in Ji's laboratories to clarify this point.

The most important conclusion that can be derived from the experimental data presented in this paper is that there exist two fundamentally different mechanisms by which hepatic respiration can be inhibited the metabolic and microcirculatory mechanisms (Table 2). The metabolic and microcirculatory mechanisms can be further broken down into component processes and factors as detailed in Table 2. The microcirculatory mechanisms uniquely available to intact organs can be viewed as an emergent property intrinsic to the more complex biological structure of organs in comparison to suborgan systems of cells, subcellular organelles and enzymes. The availability of the extra degree of freedom in intact organs may make it difficult to directly extrapolate the experimental data obtained from simpler systems of cellsand subcellular fractions to whole organs and organisms. Some of the toxicological effects traditionally attributed to metabolic mechanisms may in fact implicate primarily microcirculatory mechanisms or both metabolic and microcirculatory mechanisms.

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