The first observation of O_2^- generation at real time in vivo from non-Kupffer sinusoidal cells in perfused rat liver during acute ethanol intoxication

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Abstract Infusion of ethanol or phorbol myristate acetate (PMA) into the perfused rat liver immediately produces O_2^- which was detected directly by infusion of a *Cypridina* luciferin analogue, MCLA as a chemiluminescence reagent. The MCLA photon emission was inhibitable by SOD. Generation of O_2^- in the liver was further verified by nitroblue tetrazolium, formazan precipitate formation. Ethanol-induced O_2^- generation was unaffected by gadolinium chloride ($GdCl_3$), an inhibitor of kupffer cells, while PMA induced O_2^- generation was completely abolished by $GdCl_3$. Since PMA is a known stimulator of phagocytic cells including Kupffer cells, the results indicate, for the first time that ethanol stimulates a non-Kupffer cell population, probably liver sinusoid endothelial cell to produce O_2^-.

Key words: Sinusoidal cell; Superoxide; MCLA-dependent organ luminescence; SOD; $GdCl_3$; Ethanol-intoxication

1. Introduction

Superoxide, O_2^- derived active oxygen species have been implicated in the pathogenesis of ethanol-induced tissue injury [1–3]. Bautista and Spitzer [4] were the first to demonstrate that short-term exposure of the liver to ethanol stimulates O_2^- production as monitored by O_2^--dependent cytochrome *c* reduction. Kupffer cells generate more O_2^- than hepatocytes or endothelial cells. We have previously reported [5] that O_2^- generated by the sinusoidal cells following stimulation by phorbol myristate acetate (PMA) can most conveniently be measured by MCLA, a highly sensitive O_2^- and $^1O_2$ (singlet oxygen) specific chemiluminescence reagent.

Gadolinium (a rare earth metal) chloride, $GdCl_3$ is reported to block selectively phagocytosis by Kupffer cells in vivo [6]. In line with this view, enhanced production of O_2^- by Kupffer cells isolated from sublethal endotoxaemic rats was suppressed by pretreatment with $GdCl_3$ [7]. In the rat liver with ethanol infusion, if organ luminescence is high and is markedly suppressed by pretreatment with $GdCl_3$ then the emitted light is assumed to be originating from the reaction of MCLA with O_2^- produced by Kupffer cells in the hepatic sinusoids. The present study was undertaken to examine the above assumption, by comparing the results obtained with PMA with that from ethanol. Formazan precipitate test was applied as an additional test to ascertain generation of O_2^- in the liver.

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2. Experimental procedures

2.1. Animal investigations

The study protocol was approved by the Committee on Ethics of animal experimentation of the Japan Immunoresearch Laboratories and extra care was taken to avoid animal suffering throughout the study. Male Wistar rats (Charles River, Japan) weighing 250–300 g were fed on standard laboratory animal chow and tap water ad libitum. Before operation, the rats were anaesthetized by i.p. injection of 50 mg/kg sodium pentobarbital and then were given 1000 units/kg sodium heparin by i.v. injection.

2.2. Reagents

MCLA, a *Cypridina* luciferin analogue was obtained from Tokyo Kasei Co. Ltd. It was dissolved in sterile saline (pH 7.4) prior to use. The MCLA concentration was based on $\varepsilon_{430} = 9{,}600\ M^{-1} \cdot cm^{-1}$ [5]. PMA (Sigma) was dissolved in DMSO and then diluted with Krebs-Hensleit bicarbonate buffer (KHB), pH 7.4. $GdCl_3$ (Aldrich) was dissolved in saline (pH 7.4) just before use. Heat inactivated SOD was prepared by treating 50 μM Cu, Zn SOD (Sigma) in an oven at 120°C for 30 min.

2.3. Measurement of light emission from the liver

The photon counting system used in the present study (Fig. 1) was essentially the same as described by Uehara et al. [5]. The rat liver was carefully exposed and cannulated through the portal vein. Perfusion was started with KHB, then the inferior vena cava and the abdominal aorta were immediately cut. The rat was then placed in a light tight box as close as possible to the window of a photomultiplier unit, such that the distance between the photomultiplier window and the liver was about 10 cm. The KHB was efficiently gassed with a mixture of 95% O_2 and 5% CO_2 and was infused at a constant rate of 900 ml/h with a peristaltic pump. After washing out the blood, 100 μM MCLA was continuously infused at a rate of 9 ml/h using a syringe infusion pump A. Under steady state conditions, the concentration of MCLA in the liver sinusoids where O_2^- was to be detected was 1 μM. After 5 min, 20 mg/l PMA in a syringe pump B was infused at a rate of 9 ml/h for 10 min. Ethanol in syringe B was infused at a rate of 2.1 ml/h for 20 min. Under steady state conditions, the concentrations of PMA and ethanol in the sinusoids were 0.3 μM and 40 mM, respectively. In some experiments, 50 μM SOD or the heat inactivated SOD in a syringe pump C was infused at a rate of 9 ml/h for 5 min. The rat was covered with a black sheet with a 1 cm^2 hole over the liver to exclude light emitted from neighbouring organs. The light from the 1 cm^2 hole was detected by a luminescence reader (Tohoku Electoric Inc.). In the $GdCl_3$ experiments, rats were given 5 mg/kg $GdCl_3$ (i.v.) twice, at 48 h and 24 h before perfusion experiments.

2.4. Formazan precipitate formation

The liver was perfused by nitroblue tetrazolium dissolved in KHB (0.5 mg/ml) at a rate of 900 ml/h for 10 min, 10 min after PMA infusion or 20 min after ethanol infusion. The residual NBT in the liver was washed out by 5 min perfusion with KHB. All other conditions were the same as described above except that MCLA was omitted. In certain experiments, SOD was also infused during NBT infusion.

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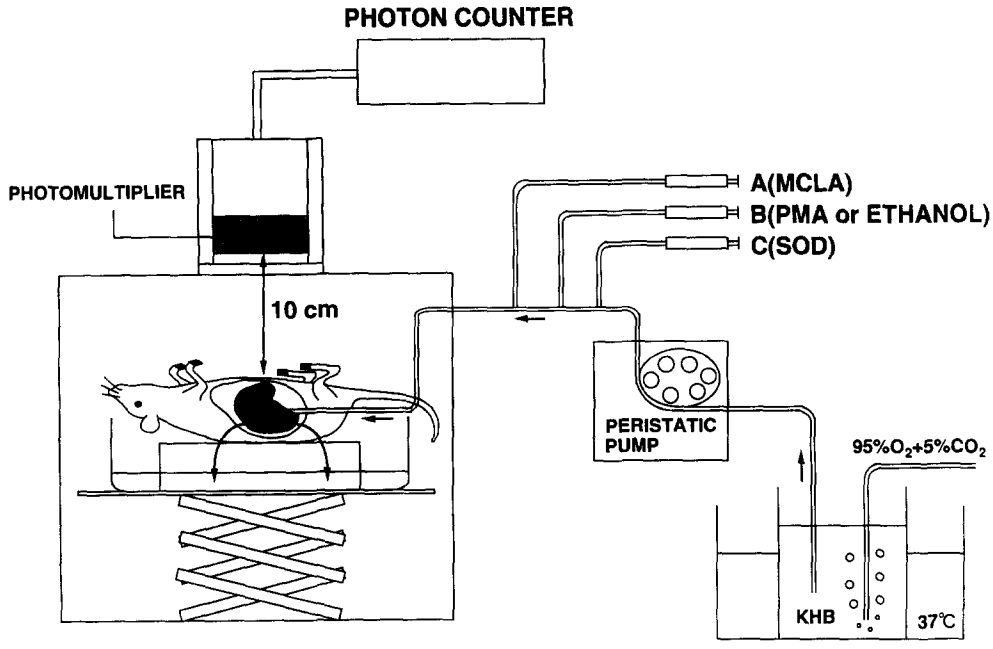


Fig. 1. The perfusion and photon counting system used for the measurement of luminescence from the surface of the rat liver.

3. Results

3.1. O_2^--dependent MCLA photon emission

Fig. 2 shows luminescence of MCLA recorded from the liver surface following infusion of ethanol. As seen a marked rise over control luminescence was observed when ethanol was infused which was unaffected by pretreatment with $GdCl_3$. Infusion of catalytic amounts of SOD for a brief time during increasing organ luminescence produced instaneous suppression of the luminescence to control level and caused a reincreasment of the luminescence thereafter (Fig. 2, inset A). Under the same experimental conditions, save that the heart inactivated SOD (iSOD) was used instead of SOD, little or no suppression of the organ luminescence occured (Fig. 2, inset B). Further more, concomitant infusion of catalytic amounts of SOD with MCLA+ethanol did not elevate the organ luminescence over control level, while that of the heat inactivated SOD with MCLA+ethanol had no effect on the elevated organ luminescence (data not shown). These results support our assertion

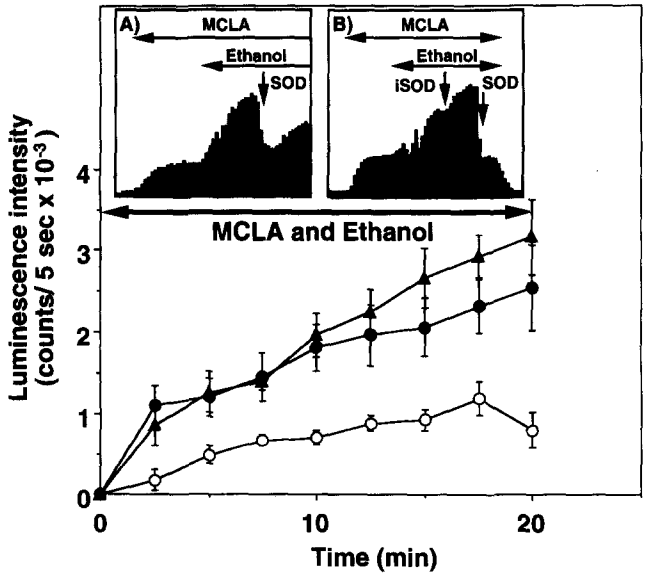


Fig. 2. Time courses of MCLA-dependent luminescence intensities from perfused liver surface after ethanol + MCLA infusion to normal rat liver (●) or $GdCl_3$-pretreated rats (▲) and those after MCLA + saline infusion to normal rat liver, control (○). Data are mean ± S.E.M., n = 5–7. (Insets) Effect of SOD on the ethanol-induced luminescence. (A) SOD was infused during 5 min at 8 min after ethanol + MCLA infusion. (B) Heat inactivated SOD (iSOD) and SOD were infused during 5 min at 6 min and at 12 min, respectively, after ethanol + MCLA infusion.

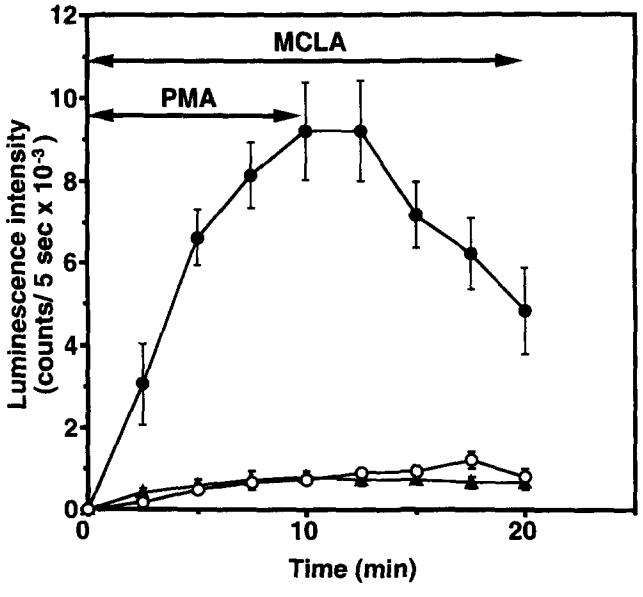
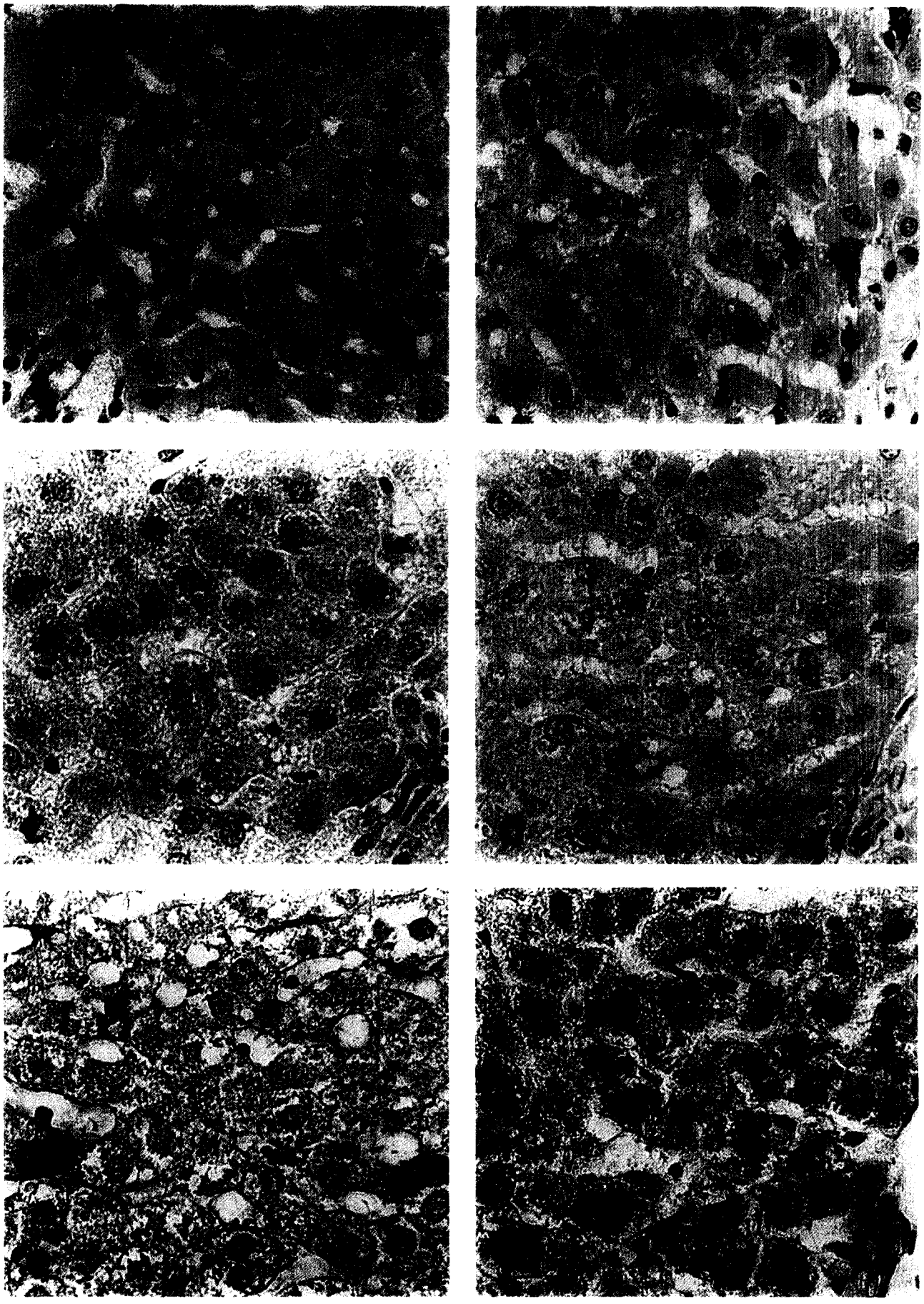


Fig. 3. Time courses of MCLA-dependent luminescence intensities from perfused liver surface after PMA + MCLA infusion to normal rat liver (●) or $GdCl_3$-pretreated rats (▲) and those after PMA+saline infusion, control (○). Data are mean ± S.D., n = 5.



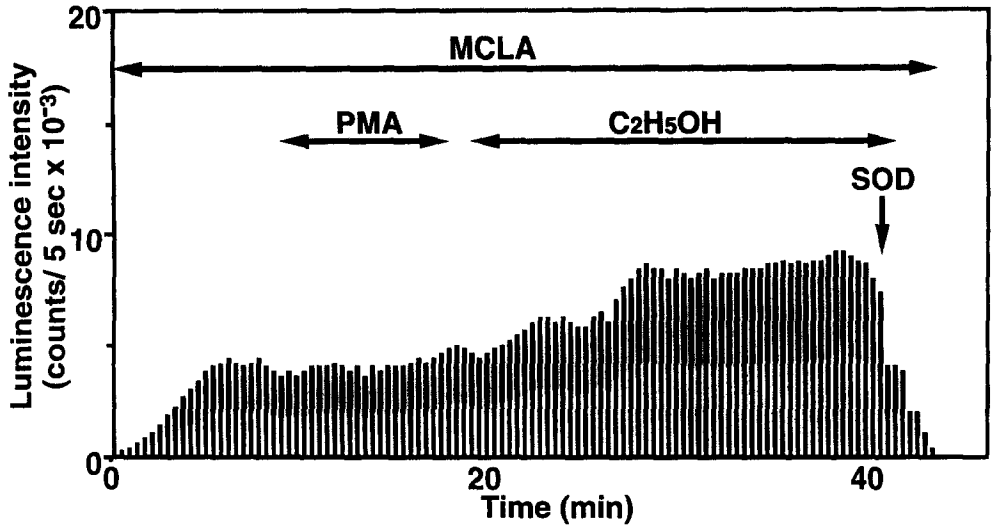


Fig. 4. Time cource of MCLA-dependent luminescence intensity from $GdCl_3$-pretreated rat liver after PMA + MCLA infusion and C_2H_5OH + MCLA infusion. SOD was also infused at the time cited.

hat the emitted light over control level originates for the reaction between O_2^- and MCLA in blood vessels and sinusoids. Luminescence of $GdCl_3$ (a substance known to block phagocytosis by Kupffer cells) treated liver was also sensitive to inhibition by SOD (deta not shown).

Fig. 3 shows the luminescence when PMA (a stimulator of phagocytic cells including Kupffer cells) was infused instead of ethanol. Comparing the luminescence traces in Fig. 2 with those in Fig. 3, luminescence of MCLA in PMA system was stronger than that in ethanol system and also sensitive to inhibition by SOD (deta not shown), but surprisingly, it could be completely abolished by $GdCl_3$ pretreatment. MCLA-dependent luminescence in PMA-infused system in which the rat had been pretreated with $GdCl_3$, which was almost equal to the control level, increased just after ethanol infusion (Fig. 4), indicating that ethanol stimulates cells other than Kupffer cells to generate O_2^-.

3.2. Histological observations

Histological observations providing qualitative evidence for the generation of O_2^- in the liver during infusion of ethanol or PMA are presented in Fig. 5a,b. Formazan deposition was observed on the sinusoidal cells around the portal areas in ethanol (a) or PMA (b) infused liver. Deposition was more extensive on the sinusoidal walls in ethanol infused liver as opposed to PMA-infused liver. No blue formazan precipitate was observed in SOD-treated liver (c and d). In contrast, $GdCl_3$ pretreatment did not affect blue formazan formation on the sinusoidal walls in the ethanol-treated liver but abolished it in PMA-treated liver (e and f).

4. Discussion

In this study, infusion of the rat liver with ethanol or PMA resulted in the generation of O_2^- which could be detected by a highly sensitive chemiluminescence reagent, MCLA. The generation of O_2^- was verified by SOD and the nitroblue tetrazolium test. In line with our previous study [5], O_2^- production in the liver in response to PMA infusion was not unexpected as it is a stimulator of Kupffer cells; however, O_2^- generation in the liver immediately after ethanol infusion was completely unexpected and may have pathological relevance in alcohol abuse. The fact that $GdCl_3$ which is a known inhibitor of phagocytic cells (Kupffer cells) could completely abolish O_2^- generation in response to PMA, but being ineffective on O_2^- generation in response to ethanol, clearly indicates that ethanol stimulates a different cell population to produce O_2^- than PMA, most likely endothelial cells.

Judging from rapid appearance of SOD-inhibitable light emission immediately following ethanol infusion, the sinusoidal endothelial cells could be stimulated directly by ethanol itself and not by its metabolite, acetaldehyde formed in the liver parenchymal cells, enen if the aldehyde is a possible stimulator of the same cell type as is ethanol. Further, although spontaneous production of O_2^- by Kupffer cells isolated from ethanol infused rat liver has been reported [4], this could not have been a direct effect of ethanol on these cells but most likely that Kupffer cells were stimulated by cell debris formed during homogenisation of the liver tissue.

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Fig. 5. Light photographs showing formazan deposition in the liver tissue. (a) Ethanol-infused liver; (b) PMA infused liver; (c) ethanol+SOD infused liver; (d) PMA + SOD infused liver; (e) $GdCl_3$ pretreated and ethanol infused liver; (f) $GdCl_3$ pretreated and PMA infused liver.

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