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Kupffer cells promote lead nitrate-induced hepatocyte apoptosis via oxidative stress

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Abstract

Background: Apoptosis and its modulation are crucial factors for the maintenance of liver health, allowing hepatocytes to die without provoking a potential harmful inflammatory response through a tightly controlled and regulated process. Since Kupffer cells play a key role in the maintenance of liver function, the aim of this study was to verify whether Kupffer cells are involved in the induction of liver apoptosis after i.v. injection of $\text{Pb}(\text{NO}_3)_2$ likely by secretion mechanisms.

Results: The *in vivo* hepatic apoptosis, induced by $\text{Pb}(\text{NO}_3)_2$ was prevented by a pre-treatment with gadolinium chloride (GdCl_3), a Kupffer cells toxicant, that suppresses Kupffer cell activity and reduces to a half the apoptotic rate. In addition, *in vivo* $\text{Pb}(\text{NO}_3)_2$ administration deprives hepatocytes of reduced glutathione, whereas the loss of this important oxidation-preventing agent is considerably mitigated or abolished by pre-treatment with GdCl_3 . However, incubation of isolated hepatocytes and Kupffer cells and HepG2 cells with $\text{Pb}(\text{NO}_3)_2$ for 24 hours induced necrotic but not apoptotic cells. Apoptosis of hepatocytes and HepG2 cells was observed only after the addition of conditioned medium obtained from Kupffer cells cultured for 24 hours with $\text{Pb}(\text{NO}_3)_2$, thus indicating the secretion of soluble mediators of apoptosis by Kupffer cells. Apoptosis in the HepG2 cells was observed upon 24-hours incubation of HepG2 cells with 1 mM buthionine sulfoximine, a glutathione depleting agent, thus showing that there is an oxidative apoptogenic pathway in HepG2 cells.

Conclusion: $\text{Pb}(\text{NO}_3)_2$ has, at most, a direct necrotic (but not apoptogenic) effect on hepatocytes and HepG2 cells, giving a clue about the regulatory role of Kupffer cells in the induction of liver apoptosis after a single $\text{Pb}(\text{NO}_3)_2$ injection without pre-treatment with GdCl_3 , probably via secreting soluble factors that trigger oxidative stress in target cells.

Background

Every cell contains the making of its own demise, and apoptosis is the genetically programmed housekeeping mechanism by which the organism maintains health and

homeostasis – ridding itself of aging, infected, damaged, mutated or excessive cells. Apoptosis is a normal physiological response to a lack of survival signals or to specific "suicidal signals" from the cytoplasm or from the

intercellular environment, always leading to programmed pathways with well-defined biochemical and morphological features [1–4]. The cytoplasm of a cell undergoing apoptosis shrinks without membrane rupture, plasma and nuclear membranes develop bubble-like blebs, chromatin condenses and migrates to nuclear membrane and undergoes internucleosomal cleavage; finally, the cell contents are packed into membrane-bounded bodies (with organelles still functioning) to be ingested by neighbour counterparts (during the apoptotic process, epitopes appear on plasma membrane marking the cell as a phagocytic target). For this reason there is no cellular leakage and no inflammation [5,6].

It has emerged from studies of apoptosis that the liver is a privileged system. In fact, although under normal conditions apoptosis occurs at a negligible rate in the liver (1–5 apoptotic hepatocytes/ 10 000 hepatocytes) [7,8], this organ is constantly exposed to a variety of potentially apoptogenic, immune, inflammatory, and metabolic stimuli. Liver apoptosis has been observed under physiological, pathological, and experimental conditions [3]. Hepatocyte loss throughout apoptosis has been observed during physiological liver cell renewal; furthermore, liver regression during starvation is accompanied by an enhanced rate of apoptosis [9]. Beside this, apoptosis is responsible for cellular depletion after the "overshoot" of cell regeneration following partial hepatectomy or the withdrawal of liver hyperplasia-inducing treatments like $\text{Pb}(\text{NO}_3)_2$ [10–12]. Apoptosis of hepatocytes is also induced by a large number of toxic compounds and sometimes precedes the onset of necrosis or coexists with it [3].

Lining the walls of the liver sinusoids, Kupffer cells are in close contact with the blood stream; in contrast, the only contact that hepatocytes have with the plasma is in the space of Disse, beyond the "sinusoidal barrier". The Kupffer cells, together with other sinusoidal cells, play a key role in the maintenance of liver function, under both physiological and pathological circumstances. The major functions of Kupffer cells include phagocytosis of foreign particles, removal of endotoxins and other noxious substances, and modulation of the immune response [13].

This study was designed to confirm whether Kupffer cells are involved in liver apoptosis induction after acute intoxication by $\text{Pb}(\text{NO}_3)_2$ and to assess any secretion mechanisms that may be implied, by investigating the real effect of $\text{Pb}(\text{NO}_3)_2$ on hepatocytes and Kupffer cells. It is worth noting that hepatocyte apoptosis is a tightly controlled process, regulated via several mechanisms including an oxidative mechanism that may account for the main apoptotic signalling pathway [14–17]. In fact, the concentration of glutathione – the most abundant antioxidant in

the cell, found in reduced, GSH, and oxidized, GSSG, redox forms – decreases upon induction of apoptosis [18].

It is well known that modulation of the apoptotic process in the liver (together with the efficient elimination of apoptotic bodies) relies mainly on Kupffer cells and is thought to be regulated by their secretion of certain cytokines and growth factors [19–21]. In addition to this, previous experimental results have already shown that the apoptotic index of hepatocytes isolated using a perfused rat liver model after treatment with $\text{Pb}(\text{NO}_3)_2$ for 1, 3, or 5 days increases with time; in contrast, co-administration with gadolinium chloride (GdCl_3), a selective Kupffer cell toxicant that suppresses their activity [22], reduces the apoptotic rate [23], suggesting a key role for liver macrophages in this process.

Results

In vivo administration of $\text{Pb}(\text{NO}_3)_2$, GdCl_3 , GdCl_3 plus $\text{Pb}(\text{NO}_3)_2$, $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ or KNO_3

In situ experiments

$\text{Pb}(\text{NO}_3)_2$ administration led to time-related modifications of liver structure (hyperplasia-apoptosis), which were totally or partially abolished by pre-treatment with GdCl_3 (i.v. injected 24 hours but not 2 or 4 hours before $\text{Pb}(\text{NO}_3)_2$) (Fig. 1). GdCl_3 administered the same day as $\text{Pb}(\text{NO}_3)_2$, reduced the hepatocyte apoptosis only moderately, but, when administered 24 hours before $\text{Pb}(\text{NO}_3)_2$, the apoptotic index was cut by half. Morphological data are confirmed by TUNEL assay; very few TUNEL positive cells were observed in livers of animals injected with 24 hours GdCl_3 pre-treatment to $\text{Pb}(\text{NO}_3)_2$ administration (Fig. 1d). A single GdCl_3 injection induced low rate apoptosis as well as same modifications of liver structure. In particular, an influx of monocytes, a decreased number of Kupffer cells and a minimal uptake activity for the remaining liver macrophages was observed. Liver repopulation of Kupffer cells and re-establishment of active phagocytosis was observed two days later. The time-course of GdCl_3 -induced Kupffer cell depletion and repopulation, (monitored by measuring colloidal carbon uptake by liver macrophages), is shown in Figure 2.

Parallel experiments were carried out by injecting animals with $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ or KNO_3 to seek whether the apoptotic liver induction was peculiar to $\text{Pb}(\text{NO}_3)_2$ -treated animals. Neither $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ nor KNO_3 induced apoptosis or mitosis in liver cells, even at longer observation times; conversely, extensive necrosis of hepatic parenchyma was observed five days after KNO_3 injection.

Flow cytometry of isolated hepatocytes

In order to quantify the morphological data above described, flow cytometry analysis of isolated hepatocytes was performed (Tables 1, 2). The use of flow cytometry

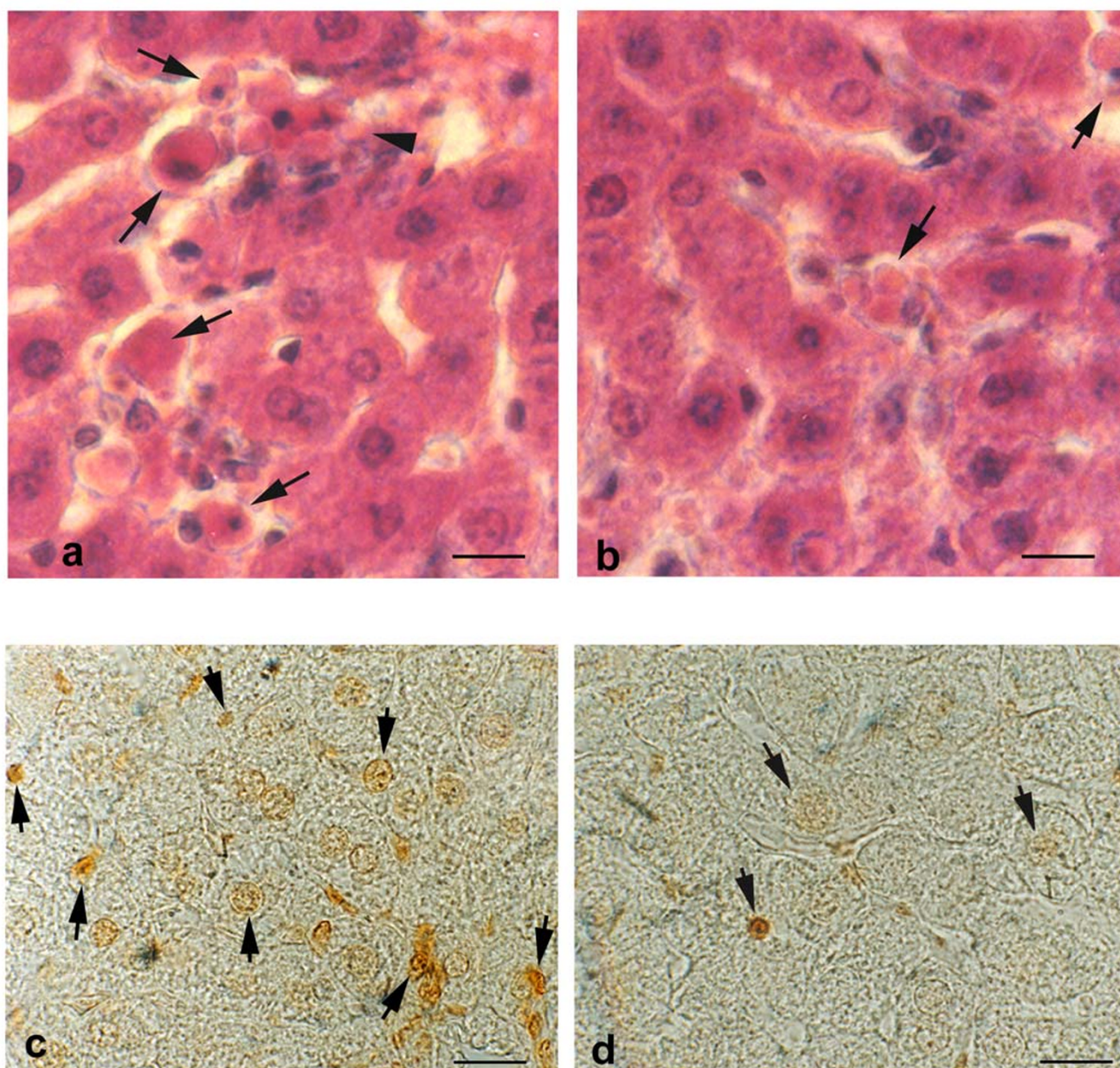


Figure 1

Light micrographs of rat liver sections, (a, b) haematoxylin eosin staining and (c, d) apoptotic detection by TUNEL staining, from (a, c) animals 5 days after a single $\text{Pb}(\text{NO}_3)_2$ injection and animals 5 (b) and 3 (d) days after $\text{Pb}(\text{NO}_3)_2$ injection 24 hours GdCl_3 pre-treated. (a) Apoptotic hepatocytes (arrows) show round shape, condensation of chromatin and cell shrinkage. One fragmented apoptotic hepatocytes (arrowhead). (b) The GdCl_3 pre-treatment reduces the hepatocytes apoptosis. In (d), the number of TUNEL-positive nuclei (arrows) in hepatic parenchyma is much lower than 5 days after a single $\text{Pb}(\text{NO}_3)_2$ injection (c, arrows). Bar = 10 µm.

allowed us the simultaneous analysis of each sample for necrosis, apoptosis and for cell cycle (from which it is possible to have the percentage of cells synthesizing DNA). Isolated hepatocytes were obtained for each treatment by

using well established enzymatic isolation procedure; cell viability in the isolated hepatocytes was always higher than 90% in each preparation from both normal untreated animals and treated animals.

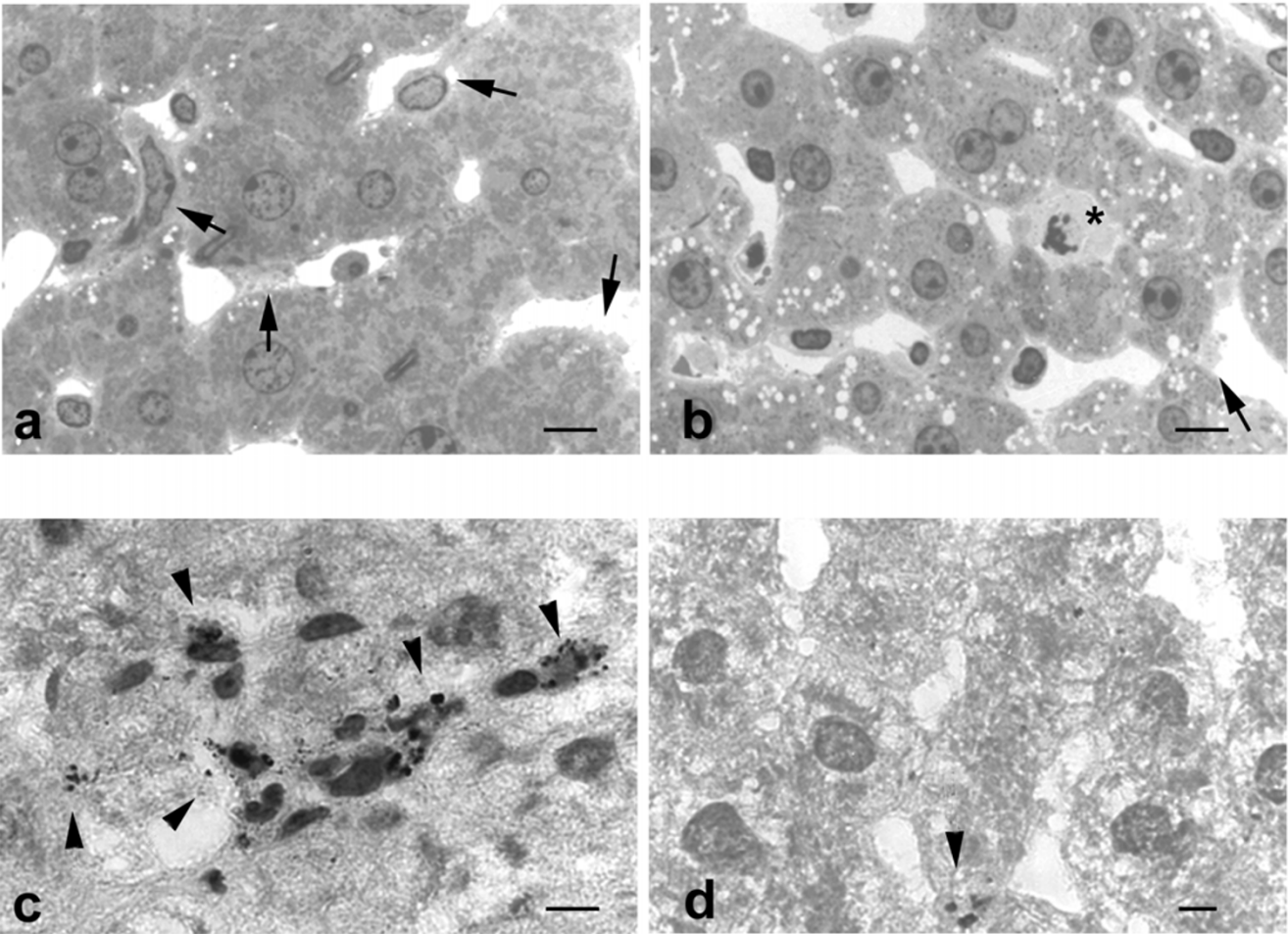


Figure 2
Light micrographs of rat liver sections (a,c) from control animals and (b,d) three days after a single $GdCl_3$ injection; (a, b) toluidine blue staining and (c, d) unstained sections of livers of $GdCl_3$ -treated rats. In normal livers large Kupffer cells, lining liver sinusoids are indicated by arrows(a) or evidenced by the colloidal carbon particles uptake by Kupffer cells (c) (arrowheads); b) three days after $GdCl_3$ administration; the majority of large Kupffer cells disappeared from liver and (d) the phagocytic activity is depressed as the scarce internalization of carbon shows (arrowhead). The asterisk (b) indicates a hepatocyte in mitosis. Bar = 10 μm .

Table 1: Time-course of necrosis, apoptosis and DNA synthesis in hepatocytes isolated from control, $Pb(NO_3)_2$ -treated, and co-administered $GdCl_3$ plus $Pb(NO_3)_2$ rats.*

	Control			Pb(NO ₃) ₂			GdCl ₃ + Pb(NO ₃) ₂ (2 hours)			GdCl ₃ + Pb(NO ₃) ₂ (4 hours)			GdCl ₃ + Pb(NO ₃) ₂ (24 hours)		
%		1 day	3 days	5 days	1 day	3 days	5 days	1 day	3 days	5 days	1 day	3 days	5 days		
Necrosis	5 (1.2)	7 (2.1)	8 (2.1)	5 (1.6)	15^(2.7)	16^(4.2)	20 (3.5)	13^(2.1)	15^(2.9)	21^(4.1)	15^(4.3)	12^(3.8)	10^(1.8)		
Apoptosis	1 (0.2)	1 (0.2)	17^(5.3)	45^(8.0)	2 (0.9)	15^(2.3)	40 (4.1)	3 (0.5)	14^(3.5)	33^(6.1)	2 (0.9)	8^(2.6)	20^(3.4)		
DNA**	4 (1.0)	3 (0.7)	10^(3.4)	9 (3.1)	5 (1.3)	12^(3.2)	6 (2.2)	6 (0.9)	12^(3.6)	7 (3.1)	1 (0.2)	8 (2.1)	5 (0.9)		

* Values are the mean (standard deviation) of at least three independent experiments of flow cytometry analysis. For each value at least 10 000 events were counted. ^Significantly different in relation to Control ($p < 0.05$). ** % of cells synthesizing DNA

Table 2: Percentage of necrosis and apoptosis in hepatocytes isolated from control, KNO₃, GdCl₃ and Pb(C₂H₃O₂)₂ treated animals.*

	Control			KNO ₃			GdCl ₃			Pb(C ₂ H ₃ O ₂) ₂		
%		1 day	3 days	5 days	1 day	3 days	5 days	1 day	3 days	5 days		
Necrosis	5 (1.2)	7 (2.1)	18 [^] (2.1)	45 [^] (1.6)	15 [^] (2.7)	16 [^] (3.2)	20 [^] (3.5)	6 (1.6)	7 (2.8)	10 (1.8)		
Apoptosis	2 (0.2)	1 (0.2)	17 [^] (3.4)	15 [^] (1.0)	2 (0.9)	10 (2.3)	12 [^] (4.1)	1 (0.1)	12 (2.8)	41 [^] (7.5)		

* Values are given as mean (standard deviation), of at least three independent experiments of flow cytometry analysis. For each value, at least 10 000 events were counted. [^]Significantly different in relation to Control (p < 0.05).

Table 3: GSH/GSSG levels in hepatocytes after *in vivo* treatment with Pb(NO₃)₂, GdCl₃ or with 24 hours pretreatment of GdCl₃ before exposure to Pb(NO₃)₂.*

Days	Untreated	PbNO ₃	GdCl ₃	GdCl ₃ /PbNO ₃
	16.05/0.20 {3}			
1		13.34/2.93 {3}	14.90/6.29 {2}	8.15/2.35 {2}
3		3.20/2.13 {10}	5.86/5.39 {10}	11.23/2.83 {6}
5		3.49/1.36 {39}	5.90/3.43 {15}	10.24/6.11 {18}
15		2.74/0.64 {20}	10.18/0.61 {10}	10.18/6.61 {10}

* The values are given as total glutathione intracellular GSH/GSSG nmol/mg protein. The relative apoptotic percentages are reported in square brackets. Standard deviations did not exceed 5%. For each point 4 animals have been used.

Apoptotic rates and DNA synthesis were significantly affected at 3 and 5 days after the Pb(NO₃)₂ injection on isolated hepatocytes (Table 1). Pre-administration of GdCl₃ reduced the apoptotic rate to a half when administered the day before but not the same day of Pb(NO₃)₂ injection. A low apoptotic rate was induced five days after a single GdCl₃ injection compared to a single Pb(NO₃)₂ injection, while an apoptotic rate similar to controls was measured one day after a single GdCl₃ injection (Table 2). GdCl₃, Pb(C₂H₃O₂)₂, and KNO₃ were toxic (i.e. induction of necrosis) for liver cells at different extent (Table 2). Neither Pb(C₂H₃O₂)₂ nor KNO₃ induced apoptosis or mitosis in liver cells, even at longer observation times; conversely, i.v. injection of KNO₃ gave rise to extensive necrosis of parenchymal cells, mostly five days after the injection (45%), probably due to the increased levels of K (Table 2).

GSH activity in hepatocytes isolated from rats treated with Pb(NO₃)₂, GdCl₃ or GdCl₃ plus Pb(NO₃)₂

To verify whether an oxidative mechanism is involved in liver apoptotic signalling after acute Pb(NO₃)₂ intoxication, the activity of both the reduced and oxidized forms of glutathione (GSH and GSSG, respectively) was measured in isolated hepatocytes. The results showed a direct relationship between apoptogenic treatment and reduction in GSH activity (Table 3). In fact, the highest apoptotic rate – i.e., 5 days of treatment with Pb(NO₃)₂ –

corresponds to the highest levels of GSH depletion, accompanied by a drop in total enzymatic activity with respect to controls. In detail, the intracellular hepatocyte content of GSH (16.05 nmol/mg of total protein) was depleted to 3.20 nmol/mg of total protein at 3 days after Pb(NO₃)₂ injection, and was drastically reduced at 15 days after the injection (i.e., 2.74 nmol/mg of protein, corresponding approximately to a 75% drop with respect to controls) (Table 3). Experimental conditions corresponding to the lowest apoptotic rates – controls, GdCl₃ and 1 day Pb(NO₃)₂ – were, on the contrary, related to the highest concentrations of GSH or the highest levels of total enzymatic activity (Table 3).

The loss of GSH due to Pb(NO₃)₂ intoxication was remarkably mitigated or abolished when GdCl₃ was administered 24 hours (but not 2 or 4 hours) before Pb(NO₃)₂ injection (again when Kupffer cells are depleted) (Table 3).

Apoptotic rates and GSH activity in hepatocytes isolated from rats treated with Pb(NO₃)₂, GdCl₃ or GdCl₃ plus Pb(NO₃)₂ in the presence or absence of pre-treatment with 2 mM BSO

To establish a possible cause-effect relationship between the intracellular levels of GSH in hepatocytes and hepatic apoptosis, rats were treated with 2 mM BSO (a GSH depleting agent that inhibits the synthesis of this

Table 4: Hepatic GSH levels after treatment with $\text{Pb}(\text{NO}_3)_2$, GdCl_3 or with 24 hours pretreatment of GdCl_3 before exposure to $\text{Pb}(\text{NO}_3)_2$.*

Weeks	Untreated / BSO	PbNO_3		GdCl_3		GdCl_3 / PbNO_3	
		3 days	5 days	3 days	5 days	3 days	5 days
-	29.00						
1	17.76 {- 3%}	13.80	11.02	14.05	16.03	16.03	18.09
2	9.65 {- 47%}	8.50	5.70	8.90	11.02	6.20	10.10
3	5.09 {- 72%}	6.65	4.30	6.42	7.90	6.10	8.80

* GSH values are given as nmol / mg protein. Animals were fed with 2 mM BSO for 1, 2 or 3 weeks before injection with the different heavy metals, and the GSH content was evaluated at 3 and 5 days after the injection. Standard deviations did not exceed 5%. In brackets is the percentage of decrement of GSH levels relative to control untreated animals. Each value is the mean of 3 independent experiments, using 3 different animals.

Table 5: Evaluation of apoptosis versus necrosis in hepatocyte cultures incubated with 10 mM $\text{Pb}(\text{NO}_3)_2$, $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ or KNO_3 and with CM from Kupffer cells.*

Treatments	Hours				
	6	8	16	24	48
Control	-	-	-	±	±
$\text{Pb}(\text{NO}_3)_2$	-	-	-	±	+++
$\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$	-	-	±	+++	++++
KNO_3	-	-	-	±	±
C.M.	-	-	-	Apoptosis (35%)	Apoptosis (48%)

* Control animals were injected with saline. The presence of necrosis is represented by +: ± <20%; + 20–40%; ++ 40–60%; +++ 60–80%; ++++ >80%. C.M. = conditioned medium collected from Kupffer cell cultures incubated for 24 h with $\text{Pb}(\text{NO}_3)_2$ (10 mM). Apoptosis was detected on slides of haematoxylin-eosin stained cells, counting at least 300 cells in at least 10 randomly selected fields.

important radical scavenging compound within 24 hours) for 1, 2 or 3 weeks before injection with $\text{Pb}(\text{NO}_3)_2$, GdCl_3 or both (Table 4). Treatment with BSO reduced GSH content by about 72% compared to untreated animals. When rats were injected, at the end of BSO administration, with $\text{Pb}(\text{NO}_3)_2$, GdCl_3 or GdCl_3 24 hours before $\text{Pb}(\text{NO}_3)_2$, an overall increase in the liver apoptotic rate was measured when compared to animals in which the synthesis of GSH was not inhibited (data not shown).

Summary of in vivo experiments

To sum up, from *in vivo* experiments it may be concluded that: i) $\text{Pb}(\text{NO}_3)_2$ but not $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ or KNO_3 induces hepatocyte apoptosis; ii) KNO_3 causes extensive necrosis of hepatocytes; iii) pre-treatment with GdCl_3 reduced modifications described for a single injection of $\text{Pb}(\text{NO}_3)_2$, when given 24 hours previously; iv) i.v. injection of $\text{Pb}(\text{NO}_3)_2$ deprived hepatocytes of GSH, whereas 24 hours GdCl_3 pre-treatment remarkably mitigated or abolished this loss; v) there is a possible cause-effect rela-

tionship between the intracellular levels of GSH in hepatocytes and hepatic apoptosis.

In vitro experiments

Apoptosis in hepatocytes and HepG2 cultures in presence of $\text{Pb}(\text{NO}_3)_2$, $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$, KNO_3 or conditioned medium collected from Kupffer cells incubated with $\text{Pb}(\text{NO}_3)_2$

Necrosis and apoptosis were evaluated at fixed time intervals (6, 8, 16, 24, 48 hours) by light microscopy in cultures of hepatocytes and Kupffer cells isolated from normal rats and incubated up to 48 hours with 10 mM $\text{Pb}(\text{NO}_3)_2$ (Table 5). More than 95% of Kupffer cells died within 24 hours of incubation with $\text{Pb}(\text{NO}_3)_2$, and only cell debris were found in the culture wells at 48 hours. Within 24 hours of culture in the presence of $\text{Pb}(\text{NO}_3)_2$ some necrotic hepatocytes were observed, whereas almost all hepatocytes were necrotic at 48 hours. Similar results were found for hepatocytes incubated with KNO_3 . 80% necrotic hepatocytes were observed at 24 hours of incubation with $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$. Apoptosis was never detected after incubation with $\text{Pb}(\text{NO}_3)_2$, $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ or KNO_3 .

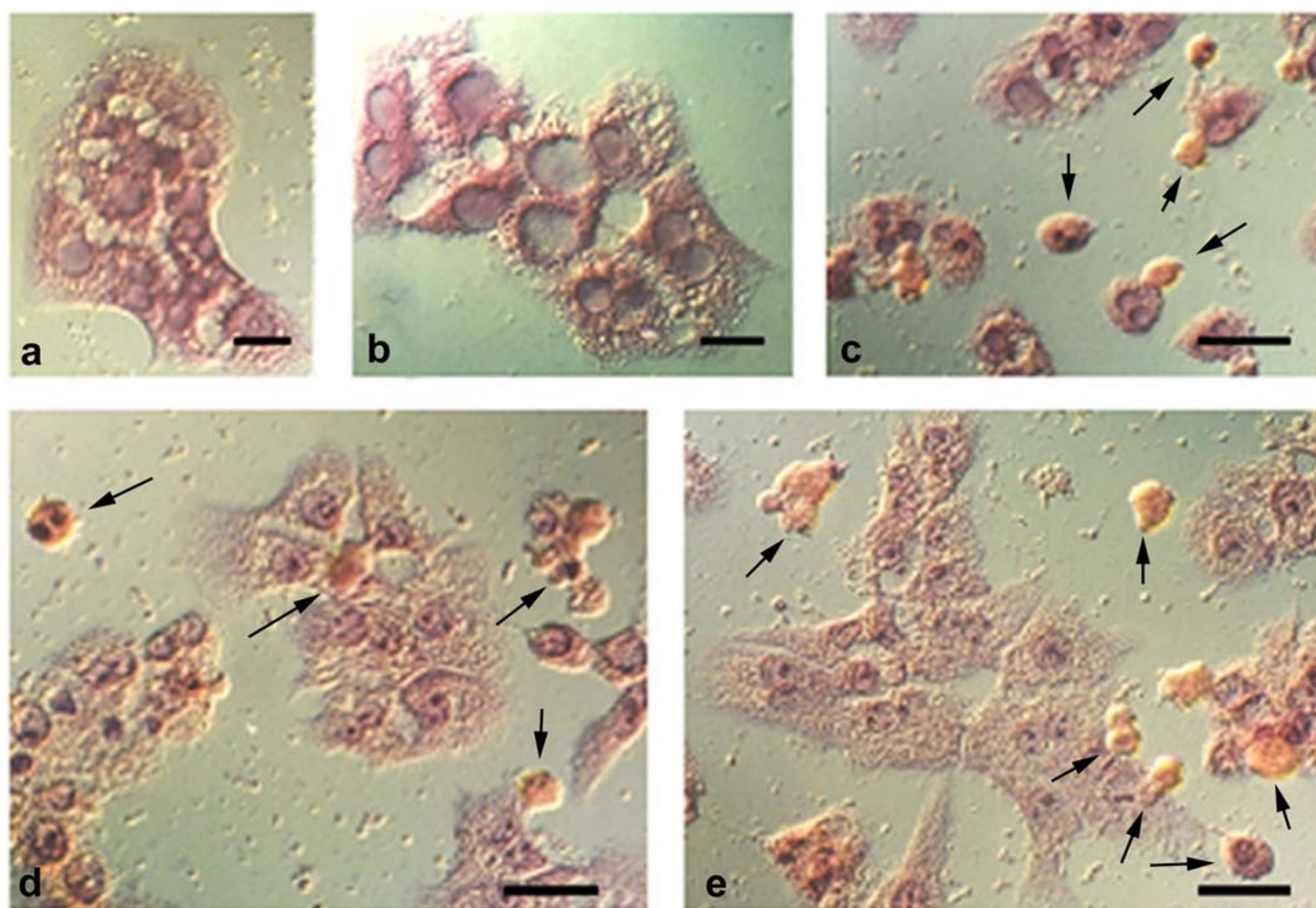


Figure 3

Nomarsky light microscopy of rat hepatocyte cultures. Cells were incubated with $\text{Pb}(\text{NO}_3)_2$ (10 mM) or with conditioned medium (CM) from Kupffer cells incubated for 24 hours with $\text{Pb}(\text{NO}_3)_2$ (a) control hepatocytes (b) 24 hours incubation with $\text{Pb}(\text{NO}_3)_2$. Apoptotic hepatocytes are not present. (c) Apoptotic cells (arrows) are present when incubated with CM from Kupffer cells incubated for 24 hours with $\text{Pb}(\text{NO}_3)_2$. (d-e) Hepatocytes pre-incubated with 1 mM BSO for 24 hours, and subsequently incubated for 24 hours (d) or 48 hours (e) with $\text{Pb}(\text{NO}_3)_2$. Deprivation of GSH leads to apoptosis also upon incubation with $\text{Pb}(\text{NO}_3)_2$. Arrows indicate apoptotic hepatocytes that are characterized by shrinkage in volume, round shape, very condensed cytoplasm and dark nucleus. Bar = 10 μm .

for up to 48 hours, but it was observed only after incubation with conditioned medium (CM) of Kupffer cells cultured for 24 hours in presence of $\text{Pb}(\text{NO}_3)_2$; values ranged from about 35% at 24 hours to about 48% at 48 hours of incubation (Fig. 3 and Table 5). Necrosis was never observed in such condition. Figure 3 shows many apoptotic hepatocytes detaching from culture dishes.

Extremely low apoptotic rates and high necrotic rates (especially for cells cultured with $\text{Pb}(\text{NO}_3)_2$) were measured for HepG2 cells incubated with $\text{Pb}(\text{NO}_3)_2$, $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ or KNO_3 for up to 24 hours. Apoptosis was detected upon incubation with Kupffer cells CM (Table 6 and Fig. 4). CM collected from control Kupffer cells, or

cells cultured for 24 hours with KNO_3 , was unable to induce apoptosis in HepG2 cells.

Apoptotic rates and GSH activity in HepG2 cells after treatment with 1 mM BSO

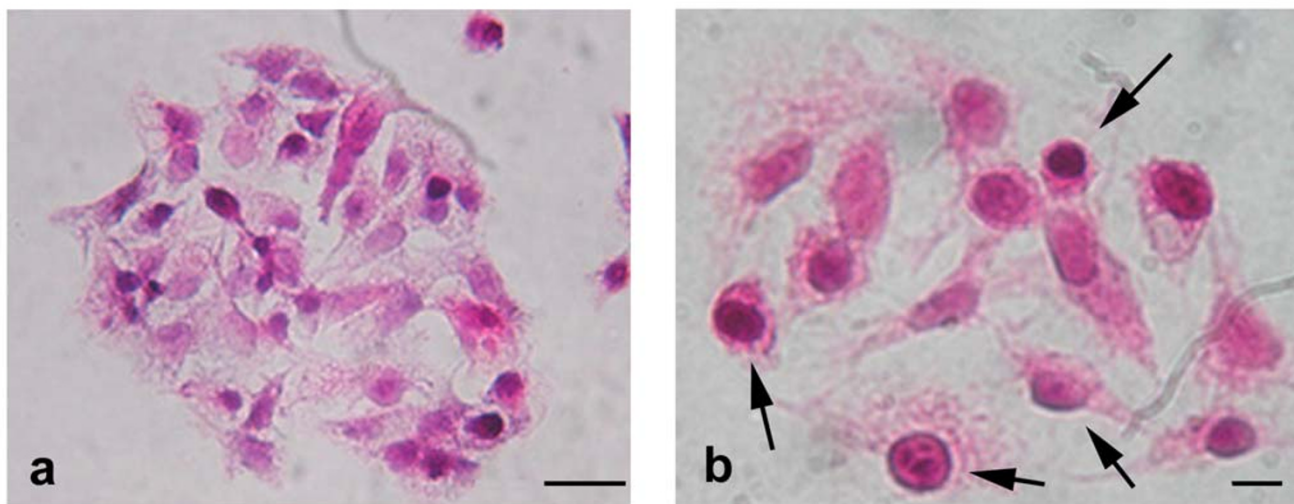
The cause-effect relationship between intracellular levels of GSH and the apoptotic rate was also investigated in HepG2 cells. When $\text{Pb}(\text{NO}_3)_2$ or KNO_3 administration in HepG2 cells for 6, 8, 16 or 24 hours was preceded by 24 hours pre-treatment with BSO (1 mM final concentration), a higher apoptotic rate than in control cells (in which GSH synthesis had not been blocked by BSO) was observed (Table 7). The apoptotic rates in the presence of

Table 6: Evaluation of apoptosis versus necrosis in HepG2 cultures incubated with 10 mM $\text{Pb}(\text{NO}_3)_2$, $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ or KNO_3 and with conditioned medium from Kupffer cells incubated with 10 mM $\text{Pb}(\text{NO}_3)_2$ for 24 hours.*

Treatments	6 hours		8 hours		16 hours		24 hours		48 hours	
	Necrosis	Apoptosis	Necrosis	Apoptosis	Necrosis	Apoptosis	Necrosis	Apoptosis	Necrosis	Apoptosis
Control	2.00	0.80	2.05	0.75	2.50	1.20	2.70	3.51	3.06	4.01
$\text{Pb}(\text{NO}_3)_2$	1.80	0.90	2.05	1.17	2.84	1.15	13.73 [^]	4.35	25.00 [^]	9.20
$\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$	4.50 [^]	2.10	18.90 [^]	1.80	35.30 [^]	3.10	81.03 [^]	7.50 [^]	92.60 [^]	15.20 [^]
KNO_3	1.42	0.18	1.20	0.53	2.69	0.70	7.71	1.19	15.30 [^]	12.58 [^]
C.M. 1	0.80	1.10	2.10	1.50	2.50	2.00	3.80	3.20	4.10	3.80
C.M. 2	1.70	3.10 [^]	3.20	4.50 [^]	2.80	10.10 [^]	3.20	18.50 [^]	3.80	25.30 [^]
C.M. 3	0.90	2.00	1.80	3.10	1.90	2.30	2.50	7.10	2.90	4.20

* Control animals were injected with saline. Apoptosis and necrosis were detected on slides of haematoxylin-eosin stained cells counting at least 150 cells in at least 10 randomly selected fields. C.M. 1 = conditioned medium collected from normal untreated Kupffer cell cultures. C.M. 2 = conditioned medium collected from Kupffer cell cultures incubated for 24 hours with $\text{Pb}(\text{NO}_3)_2$ (10 mM). C.M. 3 = conditioned medium collected from Kupffer cell cultures incubated for 24 h with KNO_3 (10 mM). Data are given as percentages. Standard deviations did not exceed 5%.

[^]Significantly different in relation to Control ($p < 0.05$).

**Figure 4**

Light micrographs of Hep G2 cells. (a) control untreated cells. (b) HepG2 cells were incubated for 24 hours with conditioned medium from Kupffer cells incubated 24 hours with $\text{Pb}(\text{NO}_3)_2$. Apoptotic cells are indicated by arrows. They appear with modified shape, i.e., elongated or pear-like, and the nucleus is more stained. Haematoxylin-eosin staining. Bar = 10 µm.

BSO were not significant, whereas the necrotic index rose considerably – values of around 40% at longer incubation times with $\text{Pb}(\text{NO}_3)_2$ and KNO_3 in GSH-deprived cells (Table 7).

Summary of in vitro experiments

In conclusion, from the *in vitro* experiments it derives that $\text{Pb}(\text{NO}_3)_2$ showed a direct necrotic but not apoptogenic

effect on hepatocytes and HepG2 cells. In fact, apoptosis was observed only when these cells had been incubated with the CM collected from Kupffer cells previously cultured with $\text{Pb}(\text{NO}_3)_2$ for 24 hours. There is also for HepG2 cells a possible cause-effect relationship between intracellular levels of GSH and apoptosis.

Table 7: Apoptotic indices in HepG2 cells incubated with 10 mM Pb(NO₃)₂ or KNO₃ after 24 hours incubation with BSO 1 mM.*

Hours	6	8	16	24	6	8	16	24
Treatments	Without BSO				With BSO			
Control	ND	ND	ND	2	ND	ND	ND	5
Pb(NO ₃) ₂	0.8	2	1.5	2.4	1.8	3.2	7.5	6.8
KNO ₃	0.3	0.6	0.9	2.2	0.9	1.6	3.1	4.8

*Apoptotic indexes are given as percentage of apoptotic cells counted on slides of haematoxylin-eosin stained cells. At least 150 cells in at least 10 randomly selected fields were counted.

Discussion

When administered to rats, Pb(NO₃)₂ induces liver hyperplasia within three days, whereas the subsequent regression from hyperplasia is due to the onset of apoptosis [11]. As a result of the administration of Pb(NO₃)₂, hepatocytes lose their contacts with the neighbouring cells and become roundish (entering the early stages of their death programme) and apoptotic cells appear in the sinusoidal lumen [12].

When the administration of Pb(NO₃)₂ coincides with the depletion of Kupffer cells (i.e., when it is combined with GdCl₃ pre-treatment, a Kupffer cells toxicant, known to suppresses Kupffer cell activity), a decrease in the apoptotic rates is observed with respect to five day-treatment with Pb(NO₃)₂, which usually correlates with the highest apoptotic rates. However, it is worth noting that modifications induced by a single injection of Pb(NO₃)₂ were reduced only when GdCl₃ was administered 24 hours (i.e., the time coinciding with the highest Kupffer cell depletion) but not 2 or 4 hours before Pb(NO₃)₂ treatment. The reason why there was liver protection from apoptosis when GdCl₃ was given 24 hours before, but not the same day as Pb(NO₃)₂, could be found in the absence of a consistent number of Kupffer cells and, as a consequence, of the soluble molecules released by them on Pb(NO₃)₂ injection. Moreover, GdCl₃ is responsible not only for Kupffer cells depletion, but also for inhibiting their phagocytic activity [22,24]. Since Kupffer cells play a key role in the liver phagocytic activity (included the phagocytosis of apoptotic cells) [25,26] and in the related signalling pathways, GdCl₃ administration might cause fewer signals to be released, leading consequently to a decreased apoptotic rate [22]. In addition, the data indicating that, the morphological modifications of liver parenchyma observed for 2 or 4 hours GdCl₃ pre-treatment were similar to those described for a single injection of Pb(NO₃)₂, further support the role of Kupffer cells in Pb(NO₃)₂-induced liver changes. On the other hand, the possible compensation for the absence of resident macrophages due to monocytic liver infiltration, will hap-

pen slighter late, i.e. at three days after GdCl₃ administration [24]. All together these data are in favour of the fact that, the apoptogenic effect of Pb(NO₃)₂ on hepatocytes may be mediated by Kupffer cells and allowed us to verify the hypothesis that the apoptogenic action of Kupffer cells could be exerted by releasing factors and/or cytokines, likely in synergy with [27]. In addition, Kupffer cells may induce hepatic apoptosis in synergy with other cells (i.e., endothelial cells), that can in turn, directly or indirectly stimulate hepatocytes to apoptosis. Kupffer cell death signals could also be combined with signals released by endothelial cells or by other non-parenchymal liver cells. It is worth noting that endothelial liver cells show the highest resistance to *in vivo* Pb(NO₃)₂ injection, whereas Kupffer cells undergo apoptosis within 24 hours from the injection [27]. It has been increasingly recognized that, under normal and pathological conditions, many hepatocyte functions are regulated by substances released by neighbouring non-parenchymal cells [28]. In fact, many mediators performing multiple paracrine and autocrine actions are secreted into the blood flow or the intercellular spaces.

The *in vitro* and *in vivo* data, excluding completely a direct apoptogenic effect of Pb(NO₃)₂ on hepatocytes, support what above discussed. In fact, a single intravenous injection of Pb(C₂H₃O₂)₂ or KNO₃ cannot determine neither hepatic apoptosis or hyperplasia – two metabolic modifications specifically induced after Pb(NO₃)₂ injection [10] – thus ruling out a direct apoptogenic action of Pb or nitrates on hepatocytes. Again, apoptosis was never observed when hepatocytes or HepG2 cells were cultured with Pb(NO₃)₂, Pb(C₂H₃O₂)₂ or KNO₃, but only when they had been incubated with the CM collected from Kupffer cells previously cultured with Pb(NO₃)₂ for 24 hours. In fact, after 24 hours Pb(NO₃)₂ incubation, necrotic but not apoptotic hepatocytes and HepG2 cells were observed; in contrast, incubation with the conditioned medium collected from Kupffer cells pre-cultured with Pb(NO₃)₂ for 24 hours was responsible for apoptosis, but not necrosis. Therefore, both *in vitro* and *in*

vivo experimental results obtained under these conditions suggest that apoptosis of hepatocytes after acute intoxication with $\text{Pb}(\text{NO}_3)_2$ is indirectly induced by soluble factors released by Kupffer cells that probably act in synergy with the secreted products of endothelial sinusoidal cells. These results are in agreement with those showing that Pb stimulates intercellular signalling between hepatocytes and Kupffer cells [29]. However, what induces Kupffer cells to release soluble mediators upon $\text{Pb}(\text{NO}_3)_2$ injection remains unknown at the moment, and Kupffer cell-derived factors still lack identification. The release of Pb/lipopolysaccharide induced by TNF-alpha (produced by Kupffer cells on hepatocytes or hepatoma cell lines) has been reported [29], in agreement with the extensive studies of the apoptogenic activity of TNF-alpha. Studies reporting Kupffer cell activity against liver metastasis by induction of Fas and Fas ligand expression on malignant glioma cells [30], or to sensitive colon cancer cells to Fas-mediated apoptosis in cirrhotic rat livers [31] further support the beneficial effects of communication between Kupffer cells and parenchymal cells.

Although the mechanism by which Kupffer cells promote hepatocyte apoptosis has not been clarified, a clue to the role of soluble factors released by liver macrophages that trigger oxidative apoptotic pathways in target cells (i.e., hepatocytes) is given by the dramatic drop in intracellular GSH activity in hepatocytes isolated from rats treated for five days with $\text{Pb}(\text{NO}_3)_2$ (i.e., after the treatment leading to the highest apoptotic rate) with respect to control levels.

In fact, the loss of GSH under those conditions is considerably mitigated or abolished when GdCl_3 is administered to rats 24 hours before $\text{Pb}(\text{NO}_3)_2$ ($\text{Pb}(\text{NO}_3)_2$ acts when Kupffer cells are depleted). These data are in agreement with those indicating that the intensity of oxidative stress exhibited by post-ischaemic lobes is closely linked to Kupffer cell activity [32]. Moreover, the same trend is observed when rats are previously fed with 2 mM BSO for 2 or 3 weeks.

In hepatocytes, like some other cells, upon oxidative stress, GSSG may either recycle to GSH or exit from cells, leading to overall glutathione depletion [33]. Cells deprived of GSH are much more prone to undergo oxidative stress since their redox balance is altered and, therefore, their ability to get rid of reactive oxygen species originated from cellular metabolism is compromised [34]. Thus, an oxidative mechanism may be a point of convergence of many different apoptogenic stimuli (i.e., H_2O_2 , tumour necrosis factor, cycloheximide and natural killer cells, all eliciting oxidative stress) into a main signalling pathway [16,32,33,35]. It is well known that NO mediates Kupffer cell-induced reduction of mitochondrial

energization in syngenic hepatoma cells [20]. In fact, oxidative apoptotic pathways include damage to mitochondria and changes in mitochondrial permeability transition, which may result in necrosis from ATP depletion or caspase-dependent apoptosis (if ATP depletion does not occur fully). Moreover, the product of the anti-apoptotic oncogene bcl-2 works throughout a radical-scavenging mechanism even in cells lacking bcl-2 [36,37]. It is worth noting that glutathione depletion can either decrease or increase death-receptor-mediated apoptosis. However, the duration of glutathione depletion before death-receptor stimulation is critical; prolonged, but not acute, glutathione depletion promotes apoptosis in mice [38,39].

Our hypothesis indicating the crucial and pre-eminent role of Kupffer cells in the onset of liver apoptosis after $\text{Pb}(\text{NO}_3)_2$ injection is summarized in Fig. 5. *In vivo* $\text{Pb}(\text{NO}_3)_2$ injection induces Kupffer cells to release factors that can act directly on hepatocytes or indirectly throughout stimulation of other cells (i.e., endothelial liver cells) inducing the death programme in hepatocytes, probably throughout oxidative stress. GdCl_3 can block the $\text{Pb}(\text{NO}_3)_2$ -induced cascade by destroying Kupffer cells, and in turn, can modify extracellular concentration of molecules released by macrophages (most probably cytokines), which are able to induce depletion of intracellular GSH levels and oxidative apoptotic signalling in hepatocytes.

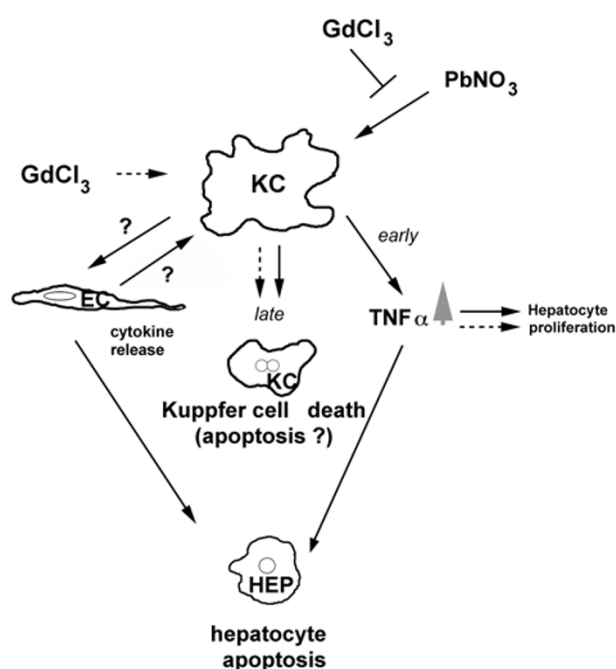
Conclusions

$\text{Pb}(\text{NO}_3)_2$ has, at most, a direct necrotic (but not apoptogenic) effect on hepatocytes and HepG2 cells, giving a clue about the regulatory role of Kupffer cells in the induction of liver apoptosis after a single $\text{Pb}(\text{NO}_3)_2$ injection without pre-treatment with GdCl_3 , probably via secreting soluble factors that trigger oxidative stress in target cells.

Methods

In vivo experiments

Adult male Wistar rats (weighing 200–250 g) were used. Animal husbandry was carried out as outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences (NIH publication 86–23 revised 1985). Animals were fed standard laboratory food and water *ad libitum*. $\text{Pb}(\text{NO}_3)_2$ (Farmitalia, Milano, Italy) was i.v. injected at a concentration of 10 μM /110 g.b.w. GdCl_3 (Aldrich Chem. Co., Brussel, Belgium) (0.75 mg/100 g.b.w.) was injected intravenously into tail veins 2, 4 or 24 hours before $\text{Pb}(\text{NO}_3)_2$ intravenous administration. Control animals were injected with saline solution or with 10 mM $\text{Pb}(\text{C}_2\text{H}_3\text{O}_2)_2$ or KNO_3 (400 μl /100 g.b.w.). The animals, anaesthetized with Farmotal (Farmitalia, Milano, Italia) (10 mg/100 g.b.w.), were sacrificed at 1, 3, 5 or 15 days after treatments.

**Figure 5**

Scheme of the role of Kupffer cells during $\text{Pb}(\text{NO}_3)_2$ -induced hepatic apoptosis. $\text{Pb}(\text{NO}_3)_2$ particles induce, as an early event, Kupffer cells (KC) to secrete molecules (probably cytokines such as $\text{TNF } \alpha$) which stimulate hepatocytes (HEP) to proliferate; the increased level of secreted molecules could initiate hepatocyte apoptosis. Activated Kupffer cells may release other molecules prompting endothelial cells (EC) to release additional cytokines, promoting apoptosis of hepatocytes. At the same time, EC can release cytokines that could promote hepatocyte apoptosis as well as activation of KC. As a late event, $\text{Pb}(\text{NO}_3)_2$ particles induce KC death. GdCl_3 particles could inhibit the effects of $\text{Pb}(\text{NO}_3)_2$ by depleting Kupffer cells, likely by inducing apoptosis.

a) Apoptosis determination

Hepatocytes were isolated by enzymatic perfusion [40]; non-viable cells, evaluated by trypan blue exclusion test, did not exceed 5% in any of the saline-injected animals. Apoptotic, mitotic and necrotic indices of hepatocytes were quantified by flow cytometry and checked by light microscopy on haematoxylin-eosin-stained hepatocytes.

An EPICSXL flow cytometer (Coulter Electronic Inc. Miami, FL, USA) with a 5-W argon laser having a 488-nm excitation wavelength was used. Hepatocytes were stained with propidium iodide (10 $\mu\text{g}/\text{ml}$) in phosphate-buffered saline containing 40 units/ml Rnase and 0.5% Tween 20. The 635-nm emission wavelength was monitored for propidium iodide emission. Histograms of relative DNA content were analysed using MultiCycle software (Phoenix

Flow Systems, San Diego, CA, USA) to quantify the percentage of cells in each stage of the cell cycle. For each flow cytometry analysis at least 10 000 events were calculated.

b) Glutathione determination

The cellular content of glutathione was evaluated on *in situ* perfused livers, and on hepatocytes isolated from the livers of rats injected with $\text{Pb}(\text{NO}_3)_2$, GdCl_3 , $\text{GdCl}_3 + \text{Pb}(\text{NO}_3)_2$, at 1, 3, 5 and 15 days after treatment. Freshly isolated hepatocytes, harvested by centrifugation at 200 g in a refrigerated centrifuge, were washed and suspended in phosphate-buffered saline. Cells were lysated by repeated cycles of freezing and thawing. Proteins were precipitated by adding sodium metaphosphoric acid (5% w/v). GSH and GSSG was measured in the clear supernatant obtained after centrifugation at 22 000 g for 15 minutes, by high-performance liquid chromatography [15]. Rat livers were perfused *in situ* with PBS to eliminate blood and snap frozen at -80°C before GSH determination as described for isolated cells. In each case, results are expressed as nmol of GSH/GSSG per mg of total proteins in the original cell extract. Proteins were determined by processing aliquots of cell lysates according to the method of Lowry et al. [41].

GSH depletion experiments were performed by feeding normal rats with D,L-buthionine sulfoximine (2 mM BSO, Sigma Chem. Co., MI, USA) in order to block GSH synthesis for 1, 2, or 3 weeks, and then treated with $\text{Pb}(\text{NO}_3)_2$, $\text{GdCl}_3 + \text{Pb}(\text{NO}_3)_2$ or GdCl_3 for 1, 3, 5 or 15 days. Control groups drank only water or 2 mM BSO for 1, 2 or 3 weeks.

c) TdT-mediated dUTP-biotin nick end labelling (TUNEL)

Apoptotic cells were detected by TUNEL, based on the specific binding of terminal deoxynucleotidyl transferase (TdT) to 3-OH ends of DNA [42].

d) Uptake of colloidal carbon

A suspension of colloidal carbon was prepared by dialyzing 10–15 ml of Indian ink (Pelikan black #17) against distilled water for 48 hours using a dialyzing membrane with a 12 000–14 000 MW cut-off. The suspension was stored at 4°C for up to 30 days prior to use and diluted in Krebs-Henseleit buffer to a concentration of 2.4 mg/ml. To test the phagocytic capacity of Kupffer cells, the diluted solution was given i.v. (0.2 ml/250 g.b.w.) 30 minutes before killing the rats. Rats were sacrificed in groups of three respectively 1, 3, 5 and 7 days after GdCl_3 administration.

In vitro experiments

a) Experiments with isolated hepatocytes, HepG2 cells and Kupffer cells

Hepatocytes, isolated as described above, and Kupffer cells, isolated according to Dini [25] were cultured in RPMI-1640 supplemented with 10% fetal calf serum, 2 mM L-Glutamine, 100 I.U./ml penicillin and streptomycin in a controlled atmosphere (5% CO₂) at 37°C. All the experiments with hepatocytes (plated on collagen-coated slides) and Kupffer cells at a density of 1×10^6 cells/ml in complete medium were performed 24 hours after plating. HepG2 cells were routinely trypsinized, plated on $7.5 \times 10^5/25$ cm² flasks, and used for experiments 3 days after trypsinization at a density of $2 \times 10^6/25$ cm² flasks.

Hepatocytes, HepG2 and Kupffer cells were incubated with Pb(NO₃)₂, Pb(C₂H₃O₂)₂, KNO₃ (30 µl of 30 mg/10 ml solution in 3 ml of medium containing 3×10^5 HepG2 cells or 1×10^6 hepatocytes or Kupffer cells) for 4, 6, 8, and 24 hours. Conditioned media from untreated Kupffer cells and Kupffer cells cultured for 24 hours with Pb(NO₃)₂, Pb(C₂H₃O₂)₂ or KNO₃ were diluted 1:2 in fresh medium containing 10% FCS and added to 24-hour-cultured hepatocytes or HepG2 cells. The apoptotic rate was evaluated by light microscopy on haematoxylin-eosin stained slides, counting at a magnification of 40 × at least 300 cells in at least 10 randomly selected fields.

b) Glutathione determination

The relationship between intracellular GSH/GSSG rates and hepatic apoptosis was tested in HepG2 cells. Cells were pre-treated 24 hours beforehand with 1 mM BSO before 24 hours incubation with Pb(NO₃)₂ or KNO₃. Controls were incubated with normal culture medium or with Pb(NO₃)₂ or KNO₃ for up to 24 hours. The apoptotic rate was evaluated by light microscopy on haematoxylin-eosin stained slides. GSH was determined as described above.

Statistical analysis

Statistical analyses were performed using Student's *t*-test for independent samples, and *p* values < 0.05 were considered significant. For data reported in Tables 1, 2, 4 and 7, normality was checked with the chi-squared goodness-of-fit test. For data reported in Tables 5 and 6, normality was checked with the Kolmogorov-Smirnov test. Homogeneity of variances was checked with both Bartlett and Cochran' tests. Data are presented as mean (standard deviation).

Authors' Contributions

PP carried out the culture experiments and the high-performance liquid chromatography. ECC performed the TUNEL assays and the isolation of hepatocytes and Kupffer cells. SC carried out the isolation of hepatocytes

and Kupffer cells and took care of the animal treatments. AC performed the culture experiments. SM participated in the writing of the manuscript. LA participated in the design of the study, drafted the manuscript and performed the flow cytometry. LD conceived the study and participated in its design and coordination. All the authors read and approved the final manuscript.

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