

## **Iron Overload Induced Apoptotic Cell Death in Isolated Rat Hepatocytes Mediated by Reactive Oxygen Species**

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### **Abstract**

Isolated rat hepatocytes in culture were incubated with different concentrations of iron-sorbitol (50, 100, 150, and 200  $\mu$ M) to assess the changes in reactive oxygen species (ROS) and lipid peroxidation leading to apoptotic hepatocyte cell death. The viability of hepatocytes was declined depending on the iron concentration. One hour incubation of the cells with 100  $\mu$ M iron resulted in decreased of the hepatocyte viability down to 50% ( $EC_{50}$   $\mu$ M). Cellular glutathione (GSH) was depleted depending on the concentration of iron added to the hepatocytes in culture. Decline in cellular GSH was associated with elevation in reactive oxygen species (ROS) generation and formation of thiobarbituric acid reactive substances (TBARS) as index of lipid peroxidation. TBARS concentration was elevated in hepatocytes exposed to >100  $\mu$ M of iron for 40 min. A significant increase in ROS formation was also observed in cells incubated with 75  $\mu$ M of iron for 60 and 120 min. The consequences of ROS-mediated damages to hepatocytes were observed by DNA fragmentation, nuclear staining by propidium iddide and finally with induction of apoptotic hepatocyte cell death. Terminal deoxynucleotie transferase-mediated dUTP nick end labeling i.e. TUNEL assay (In situ- cell death-detection kit) and nuclear staining were also used to confirm apoptosis. These data clearly show that iron overload can cause apoptotic cell death in isolated hepatocytes and generation of ROS precedes other changes related to oxidative stress.

**Keywords:** Apoptosis; Iron overload; Hepatocyte; Oxidative Stress.

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### **Introduction**

Iron in excessive amounts may be toxic particularly to the liver since it is the major site of iron storage. Increased levels of iron in laboratory animals fed high levels of iron containing diet (Sochaski et al., 2002) and in pregnant women (Hininger et al., 2001) taking iron tablets has been reported.

With massive iron overload, such iron-induced alterations may cause cell death, also known as sideronecrosis. Studies on different complications related to iron overload revealed that inheritant hemochromatosis is associated with apoptosis in hepatocytes (Zhao et al., 1997). Iron catalyzed oxidative damage to cellular components is probably responsible for development of hepatic fibrogenesis and carcinogenesis (Kowdley, 2004).

The mechanisms by which excess iron exerts its cytotoxic effects include enhanced

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formation of free radicals and lipid peroxidation in organelle membranes. Lipid peroxidation can lead to structural and functional alterations in lysosomes, mitochondria and the endoplasmic reticulum. Different chemical forms of iron have been shown to initiate lipid peroxidation in liver cells (Khan et al., 1995; Valerio and Petersen, 1998; Knutson et al., 2000; Milckak and Bicker, 2002).

The ability of iron to catalyze the formation of reactive oxygen species (ROS), including the hydroxyl radical ( $\cdot\text{OH}$ ), has also been reported (Halliwell, 1992; Kehrer, 2000; Ryan and Aust, 1992). According to Jacob and co-workers (1997) inorganic iron is responsible for acceleration of endothelial cell apoptosis due to ROS generated of via Fenton reaction. In this connection the role glutathione and  $\alpha$ -tocopherol in iron-induced hepatotoxicity has been elucidated (Milchak and Bricker, 2002). Hence, glutathione plays a major role in maintaining cell viability following iron exposure and probably there is a synergistic relationship between GSH and other antioxidants. It has been reported that chronic iron exposure can result in elicitation of unique pattern of stress protein expression which may modulate hepatic long-term responses to iron overload (Brown et al., 2007).

Despite the bulk of information on iron-overload complications, the relationship between the cytotoxicity events and apoptotic cell death is not fully understood. In this study, changes in parameters related to oxidative stress were monitored in isolated rat hepatocytes loaded with iron to better understand the mechanisms underlying iron-induced hepatotoxicity.

## Experimental

### *Chemicals*

Bovine serum albumin (BSA), collagenase type II, 2, 7-dichloro fluorescein diacetate (DCF-DA), EGTA, 8-hydroxy quinoline (8-HQ), reduced glutathione (GSH)

Iron-sorbitol (Jectofer™) containing ferrous iron (50 mg/ml) in 2 ml ampoules was from Astra, UK.

Iron (III), and thiobarbituric acid (TBA) were from Sigma Chemical Co. St. Louis, USA.

In situ cell death detection kit, i.e., TdT-

mediated dUTP nick end labeling (TUNEL) assay was the product of Roche Diagnostic, Germany. All other solvents and reagents were of the highest grade commercially available.

### *Hepatocyte isolation*

Male adult rats of Wistar strain, 200-250 g were obtained from Pasteur Institute of Iran. Animals were allowed to acclimatize for 1 week and provided with rodent chow pellets and tap water ad libitum. Hepatocytes were isolated by collagenase perfusion of the liver according to Seglen (1995). Briefly, the abdomen of the animal was opened under pentobarbital (60 mg/kg BW) anesthesia and the portal vein was cannulated with a Teflon catheter. Liver was perfused with EGTA (0.2 mM)-containing buffer free of  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$ , pH 7.3 at 37 °C. This was followed by a second perfusion with 100 ml of second perfusion buffer containing collagenase (0.12%, pH 7.4). The flow rate was adjusted to 30 ml/min for 15-25 min. The perfused liver was then transferred to a Petri dish containing washing buffer, gently dispersed in the buffer pre-cooled on ice.

Isolated cells were filtered through four layers of gauze, washed three times with washing buffer precipitated every time by centrifugation. After the final cell washing, the cells were counted using a hemacytometer and initial cell viability was determined using 0.2% (w/v) trypan blue exclusion test according to Moldeus et al., 1995. Viability exceeding 90% was found in all preparations used in this study. The number of cells was estimated as  $4\text{-}5 \times 10^6$  cells/liver and the cells were metabolically active for about 10 h.

### *Incubation condition*

Hepatocyte suspension ( $10^6$  cells/ml) was loaded with different concentrations of iron-sorbitol (Jectofer™), viz. 0, 100, 150, 200 or 250  $\mu\text{mol}$  in Erlenmeyer glass flasks. 8HQ (100  $\mu\text{mol}$ ) was also added to the culture media as the iron carrier as suggested by Jacob and co-workers (1977). Flasks were capped with rubber stoppers before incubation for 2 h at 37 °C in a water bath shaker under continuous 10%, 85%  $\text{N}_2$  and 5%  $\text{CO}_2$ . The cytotoxicity effects of iron was determined in hepatocyte preparation incubated with iron-sorbitol at different time intervals viz.

30, 60, 120 and 180 min. The concentration at which iron caused 50% reduction ( $EC_{50}$   $\mu$ M) in isolated hepatocytes viability after incubation for 2 h was determined by trypan blue exclusion assay.

#### *Histological studies*

The first signs of apoptosis in hepatocytes treated with iron were noticed under the fluorescence microscope after staining with either acridine orange or ethidium bromide. Hepatocytes incubated with different concentrations of iron, viz. 0 (control), 50, 100, and  $\mu$ M for 60 min in this assay. Then 10  $\mu$ l of cell suspension was mixed with stain and smear was prepared covered with cover slip and observed under fluorescent microscope.

#### *Nuclear staining by propidium iodide*

Samples from hepatocytes incubated with 100  $\mu$ M iron for 60 min were taken and stained with annexin V and propidium iodide. The distribution pattern of cells assessed in a flow cytometry resulted in four types of histograms related to distribution and differentiation of cells. This assay was first set up with thymocytes which were incubated with dexamethasone for 6 h and considered as positive control.

#### *Measurement of glutathione level*

Hepatocyte glutathione was measured by HPLC according to the procedure described by Harvey et al., 1989. Briefly, following exposure to different concentrations of iron, the cells were washed and scraped in cold phosphate-buffered saline. After centrifugation, cell pellet was lysed in ice-cold 0.6 N perchloric acid containing 2 mM EDTA. Precipitated proteins were removed by centrifugation and the supernatant was diluted (1:1 v/v) in mobile phase buffer and then pH was adjusted to 2.0-2.3 with 6 N NaOH. Ten  $\mu$ l of each sample was injected into the column for chromatography equipped with an electrochemical detector.

#### *Lipid peroxidation*

Formation of thiobarbituric acid reacting substances (TBARS) was determined as a marker of lipid peroxidation according to the procedure described by Buege and Aust, 1978. After

incubation of cells with iron for a period of 10 or 40 min, the levels of TBARS were measured in 1.0 ml aliquots of hepatocyte suspension ( $10^6$  cells/ml) using thiobarbituric acid (TBA) reagent. The absorbance of the reaction product was recorded at 532 nm in a Shimatzu-UV-3000 spectrophotometer.

#### *Reactive oxygen species (ROS)*

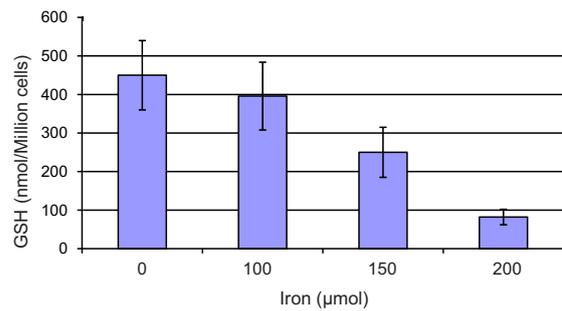
The rate of ROS generation induced by iron in hepatocytes was determined following incubation with different concentrations of iron. The level of ROS was measured based on the procedure described by Le Bel et al., 1992. Briefly, 1 ml of hepatocyte suspension was pre-incubated with iron for 30 min, and then centrifuged at  $50\times g$  for 1 min to collect the cells. The cells were re-suspended and then incubated with DCF-DA for 10 min at 37°C. The fluorescence intensity was recorded at excitation of 500 and emission of 520 nm wavelengths in a Shimatzu RF-5000 spectrofluorimeter. The concentration of DCF generated in hepatocytes was determined from standard curve using known concentrations of DCF.

#### *Detection and quantification of DNA fragmentation*

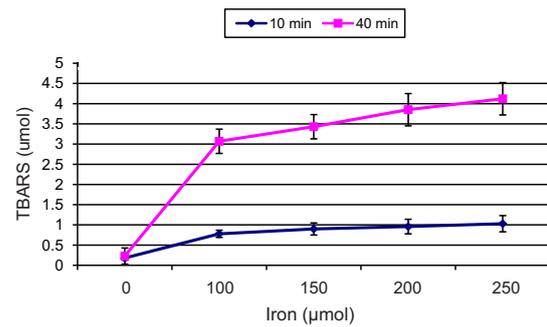
Hepatocytes were incubated in 24-well culture plates in a CO<sup>2</sup> incubator for 1 h. DNA was then prepared for gel electrophoresis. Electrophoresis was carried out in 2% (w/v) agarose gel in 40 mM Tris-acetate buffer (pH 7.4) at 90 V for 1 h. The fragmented DNA was visualized by staining with ethidium bromide.

#### *TUNEL assay*

The role of iron in inducing DNA fragmentation in isolated hepatocytes was further confirmed by TUNEL assay using a commercial kit. In this technique, the terminal deoxynucleotidyl transferase (TdT) binds to the 3'-OH end and synthesizes a polynucleotide at the nick end. Then the biotinylated nucleotides interacts with avidin-peroxidase that can be detected histochemically (Iwasa et al., 1996). The assay was first standardized with thymocytes treated with dexamethasone (positive control). Accordingly, untreated thymocytes were used and considered as negative control. The assay



**Figure 1.** Changes in glutathione in isolated hepatocytes incubated with Iron. Data are 'Mean±SE' of three assays carried out in duplicate. Controls were incubated in absence of iron.



**Figure 2.** The level of TBARS in isolated hepatocytes incubated with iron. Data are 'Mean±SE' of three assays carried out in duplicate. The concentration of TBARS was expressed as µM.

was then applied for assessment of apoptosis in hepatocytes due to iron. Hepatocytes suspension was fixed on slides, labeled and then processed for microscopic observation.

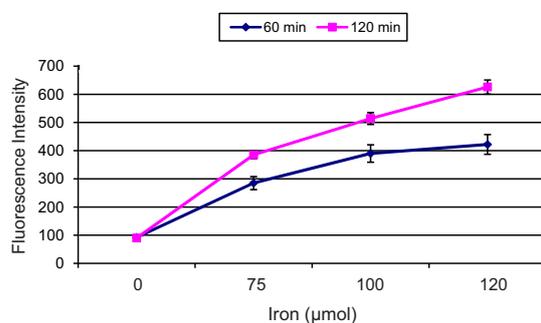
## Results

### Cytotoxicity

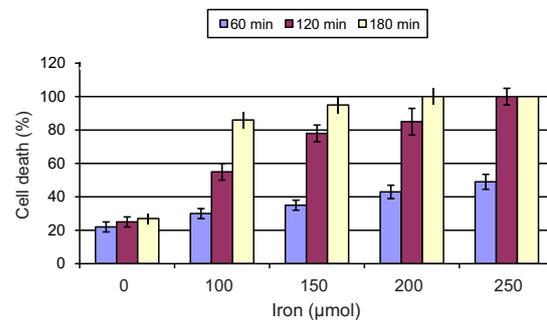
Isolated hepatocytes were incubated with different concentrations of iron-sorbitol. This resulted in a concentration-dependent decrease in cell viability. The concentration of iron sufficient to cause a 50% decrease in hepatocytes viability ( $EC_{50}$  µM) at different time intervals (60, 120 and 180 min) is summarized in Figure 1. The  $EC_{50}$  for iron was found to be 100 µM for hepatocytes incubated for 2 h (Figure 1). Based on this data, higher concentration of iron was used to induce

apoptosis in isolated hepatocytes. iron caused a remarkable decrease in cellular GSH levels depending on the iron concentration added to the hepatocyte cultures (Figure 2).

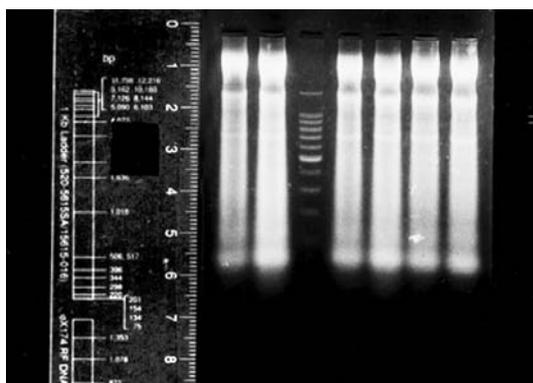
Figure 3 shows the changes in the rate of lipid peroxidation in isolated hepatocytes incubated with different concentrations of iron for 10 or 40 min. There were no significant changes in the levels of TBARS in hepatocytes incubated with different concentrations of iron for 10 min, whereas, similar concentrations of iron incubated for 40 min caused a remarkable increase in lipid peroxidation products. As shown in Figure 4, changes in reactive oxygen species (ROS) was associated with elevation in lipid peroxidation products. The pattern of DNA fragmentation in isolated hepatocytes incubated for 1-h with 100 µmol of iron as compared to control group has



**Figure 3.** Reactive oxygen species in isolated hepatocytes incubated with iron. Data are 'Mean±SE' of three assays carried out in duplicate. "ROS" formation was expressed as fluorescence intensity units.



**Figure 4.** Cytotoxicity of iron in isolated hepatocytes. Isolated hepatocytes incubated with different concentrations of iron-sorbitol and sampling was carried out at different time intervals to determine cell viability by trypan blue exclusion assay. Data are 'Mean±SE' of three analyses carried out in duplicate.  $IC_{50}$  for iron was found to be 100 µmol.



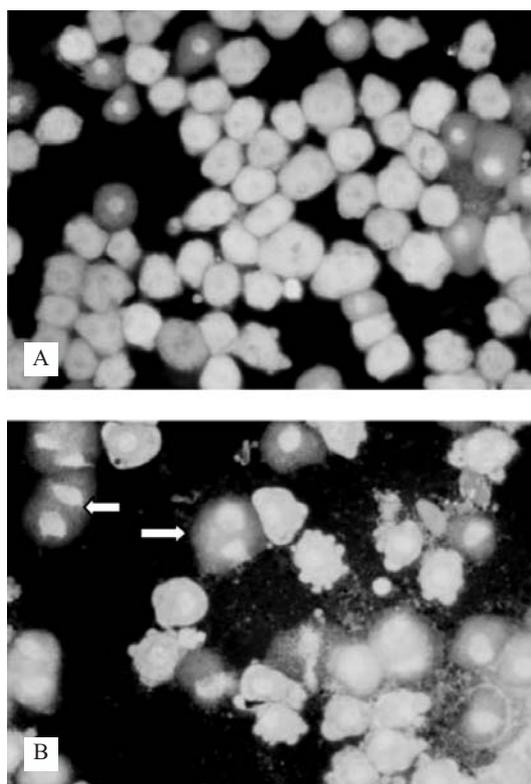
**Figure 5.** DNA fragmentation in hepatocytes incubated with iron.

From left to right lane 1, 2 and 4-7 show DNA fragments obtained from hepatocytes incubated for 1 h with iron at a concentration of 100  $\mu\text{mol}$ . Lane 3 from left shows DNA standard ladder.

been demonstrated in Figure 5. Flow cytometry performed on hepatocytes incubated with 100  $\mu\text{mol}$  of iron resulted in four types of histograms. Data related to the total cells, distribution according to the differential staining, cells stained with annexin-V and those stained with propidium iodide were assessed. The results of flow cytometry indicated that depending on the duration of incubation of hepatocytes with iron, the number of apoptotic cell bodies are increasing. Figure 6 shows the effect of two concentrations (50 and 100  $\mu\text{mol}$ ) of iron on nuclear and stained with acridine orange and ethidium bromide. The number of apoptotic cell phenotypes were found to be increased in cells incubated with higher concentrations of iron. Reductions in cytoplasmic volume and condensation of nuclei were the prominent criteria observed in apoptotic cells. The nuclei stained with ethidium bromide appeared as red color. Figure 7 shows the results of TUNEL assay. In this figure, apoptotic cells appeared in brown color due to DAB precipitation in hepatocytes exposed to 100  $\mu\text{mol}$  of iron for 60 min.

### Discussion

iron storage diseases are believed to cause organ damages through generation of reactive oxygen species, ROS (Qu et al., 2001; Knight et al., 2001). ROS is probably an early signal that

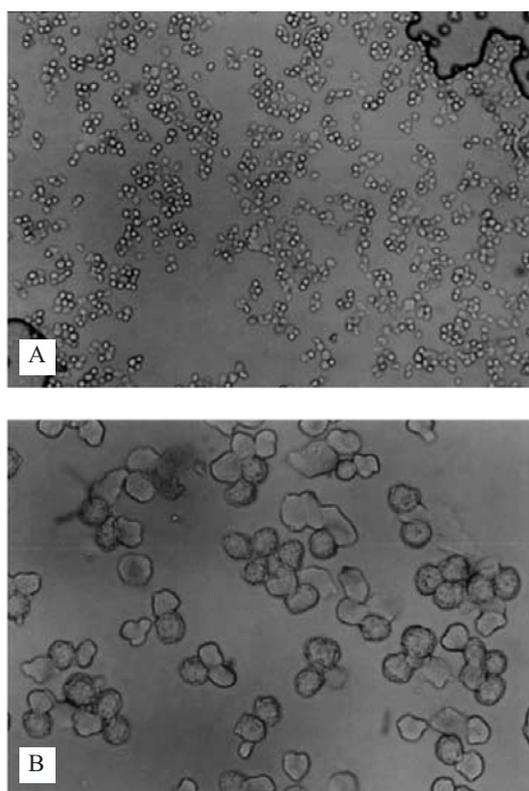


**Figure 6.** Acridine orange and ethidium bromide staining of apoptotic cell bodies.

Part-A shows hepatocytes incubated for 1 h with 50  $\mu\text{mol}$  and Part-B shows cells treated with 100  $\mu\text{mol}$  of iron. Arrow heads show apoptotic cell deaths. Reduction in cytoplasmic volume and condensation of nuclei were prominent criteria observed in apoptotic cells. The nuclei stained with ethidium bromide appear as red color.

mediates apoptosis (Simon et al., 2000; Castisti and Vercesi 1999). ROS generation is associated with oxidation of target molecules among which unsaturated fatty acids (USFAs) are probably more vulnerable to oxidative stress. Necrosis occurs when cell damages overcome the defense mechanisms, whereas alternatively cells may undergo apoptosis pathways. The release of ROS is one of the major signaling pathways involved in many different forms of apoptotic cell death in many cell types (Kroemer et al., 1995). The role of ROS in initiation of such damages was further approved when it was shown that damages caused by iron were reversed when iron-chelating agent such as deferoxamine was used (Demling et al., 1991).

In this study, short-term (2 h) incubation of freshly isolated rat hepatocytes with iron



**Figure 7.** Nuclear DNA end labeling (TUNEL assay) in hepatocyte treated with iron. Hepatocytes exposed to 100  $\mu\text{mol}$  iron for 1 h. Apoptotic cells appear in brown color due to DAB precipitation in hepatocytes exposed to 100  $\mu\text{mol}$  of iron for 1 h. Part A and part B show 10 $\times$  and 40 $\times$  magnification.

resulted in changes in a panel of markers related to oxidative stress leading to apoptotic cell death. As it was expected, relatively small fraction (~20%) of hepatocytes isolated by collagenase perfusion underwent cell death in untreated groups. Nevertheless, the bulk of lipid peroxidation products formed during incubation of hepatocytes with iron indicated the extent of the damages caused to the membranes.

ROS generation in isolated hepatocytes was an early response to iron that was dependent on the dose and time of cell exposures to iron. Moreover, as it was expected increased ROS was associated with elevation of lipid peroxidation products. Oxidative damages to cell membrane or its organelles, particularly lysozyme and mitochondrial membrane could be responsible for marked decrease in cell viability. Comparing these results with ROS data may suggest that ROS

is generated even with lower iron concentration (75  $\mu\text{mol}$ ) and 1 h before the number of hepatocytes reached to 50%. It appears that changes in ROS and lipid peroxidation precede GSH depletion which occurred due to iron doses above  $\text{EC}_{50}$  (Figure 2).

The pattern of distribution of hepatocytes exposed to iron according to flow cytometric data further indicated a decline in cell viability in treated groups. Flow cytometry data using annexin-V confirmed modifications in cell membrane particularly in rearrangement of phosphatidyl serine from the inner plasma membrane. Depletion of intracellular glutathione in hepatocytes incubated with iron by approximately 90% adds to the oxidative stress which can potentate iron cytotoxicity. The protective role of glutathione is better explained when it was shown that lipid peroxidation and cytotoxicity resulted from iron to cells in culture are reversed by exogenous GSH (Milchak and Bricker, 2002).

DNA fragmentation is used to distinguish between necrotic and apoptotic hepatocyte cell death. The pattern of chromatin condensation and DNA fragmentation induced by iron clearly showed the process of apoptosis in the hepatocytes (Figure 5). The results of TUNEL assay showing the nick end of the fragments of DNA also confirmed the apoptotic pattern of DNA fragmentation. It is worth mentioning that iron-related change in hepatocytes was depending on the concentration of iron in the cell culture. There was a gradual change in parameters studies, and upon increased levels of iron the ratio of necrotic cells to apoptotic cell bodies was increased.

Recently, it has been reported that under certain condition, where the hepatitis C virus polyprotein is expressed in transgenic mice, iron overload may leads to hepatocellular carcinoma (Furutani et al., 2006). Similar morphological changes also observed in vivo in iron-overload induced rats. Typical features of apoptosis including condensation of the nuclei, increased nuclear permeability, and membrane bleb formation were observed in our laboratory in liver of rats directly injected with iron formulation (Unpublished data). The results presented in this paper indicate that the iron induced apoptotic cell death is mediated

by oxidative stress. ROS generation is probably the first event that occurs in hepatocytes loaded with iron. Overall our results provide further evidences about the mechanism(s) by which iron overload induces hepatotoxicity.

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