

RAPID COMMUNICATION

## Ethanol induced mitochondria injury and permeability transition pore opening: Role of mitochondria in alcoholic liver disease

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### Abstract

**AIM:** To observe changes of mitochondria and investigate the effect of ethanol on mitochondrial permeability transition pore (PTP), mitochondrial membrane potential (MMP,  $\Delta\Psi_m$ ) and intracellular calcium concentration in hepatocytes by establishing an animal model of alcoholic liver disease (ALD).

**METHODS:** Fourty adult male Wistar rats were randomly divided into two groups, the model group (20) was administered alcohol intragastrically plus an Oliver oil diet to establish an ALD model, and the control group (20) was given an equal amount of normal saline. The ultramicrostructural changes of mitochondria were observed under electron microscopy. Mitochondria of liver was extracted, and patency of PTP, mitochondrial membrane potential ( $\Delta\Psi_m$ ), mitochondrial mass and intracellular calcium concentration of isolated hepacytes were detected by flow cytometry using rhodamine123 (Rh123), Nonyl-Acridine Orange and calcium fluorescent probe Fluo-3/AM, respectively.

**RESULTS:** Membrane and cristae were broken or disappeared in mitochondria in different shapes under electron microscopy. Some mitochondria showed U shape or megamitochondrion. In the model group, liver mitochondria PTP was broken, and mitochondria swelled, the absorbance at 450 nm, A540 decreased ( $0.0136 \pm 0.0025$  vs  $0.0321 \pm 0.0013$ , model vs control,  $P < 0.01$ ); mitochondria transmembrane potential ( $239.4638 \pm 12.7263$  vs  $377.5850 \pm 16.8119$ ,  $P < 0.01$ ) was lowered; mitochondrial mass ( $17.4350 \pm 1.9880$  vs  $31.6738 \pm 3.4930$ ,  $P < 0.01$ ); and  $[Ca^{2+}]_i$  was increased in liver cells ( $7.0020 \pm 0.5008$  vs  $10.2050 \pm 0.4701$ ,  $P < 0.01$ ).

**CONCLUSION:** Chronic alcohol intake might lead to broken mitochondria PTP, decreased mitochondria membrane potential and injury, and elevated intracellular  $Ca^{2+}$  production. Ethanol-induced chondriosome injury may be an important mechanism of alcoholic diseases.

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**Key words:** Alcoholic liver disease; Chondriosome; Apoptosis; Ultra microstructure; Membrane potentials; Permeability transition pore; Transmembrane potential; chondriosome mass;  $Ca^{2+}$

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### INTRODUCTION

Alcohol abuse used to be a principal factor for the cause of serious liver disease in western countries. In China the incidence of alcoholic liver disease (ALD) has increased. Alcohol abuse has been considered the second leading cause of hepatic lesion after virus hepatitis. The accurate mechanism of ethanol-induced hepatic cells injury is still not completely clear. Some researchers found that ethanol could activate lipid peroxidation, leading to liver injury. Since the mitochondrion is one of the fundamental parts of reactive oxygen species (ROS) formation, we investigated mitochondrial changes in ALD in order to prove the correlation between mitochondrion injury and hepatocytes apoptosis induced by ethanol and reveal the role of ethanol-induced mitochondrion injury in the pathogenesis of ALD, hoping that by this study we could improve the prevention and treatment of ALD.

### MATERIALS AND METHODS

#### Materials

Healthy male Wistar rats, weighing  $150 \pm 5$  g, were purchased from the Experimental Animal Center of Shandong University. Olive oil (unsaturated fatty acid content > 90%) was a product of Algemesi Co.,

Spain. Erguotou (a liquor of 60°) was a product of Beijing Hongxing Erguotou Alcohol Factory. HEPES, Rhodamin123 and Fluo-3/AM were purchased from Sigma Co., USA. DMEM was obtained from GIBCO. FBS was purchased from Hangzhou Sijiqing. NAO was obtained from GENMED.

### **Animals and protocol**

Forty adult Wistar rats (4 wk old), weighing ( $150 \pm 5$ ) g, were fed in separate cages with 5 in each. After feeding normally for a week, these rats were randomly divided into two groups. Twenty were given intragastric alcohol as the ALD model group whose food was composed of ethanol 46% (intragastric administration twice a day at an eight-hour interval), protein 18%, carbohydrate 12%, fat 14% (mainly olive oil, unsaturated fatty acid 9%) by energy as well as vitamin complex and essential minerals at the same time. In the first four weeks, the rats were lavaged with 30% ethanol (4 g/kg per day), 40% ethanol (5 g/kg per day) in the following four weeks, 50% ethanol (6 g/kg per day) from wk 9 to 12. Another twenty rats in the control group were given an equal volume of normal saline and the composition of food was the same as the model group until the end of the 12<sup>th</sup> wk.

### **Preparation and observation of samples under electron microscopy**

Liver tissue (1 mm<sup>3</sup>) from the right lobe of the liver near the porta hepatis was removed and pre-fixed for 2 h at 4°C in 4% paraformaldehyde solution with 0.1 mol/L phosphate buffer (pH 7.3) and 2.5% glutaraldehyde. The tissue was then rinsed in the same buffer and post-fixed in 1% osmium tetroxide in 0.1 mol/L phosphate buffer (pH 7.3). Post-fixation was followed by dehydration in ethanol, embedding in Epon 812 and polymerization. Tissues were cut using an LKB 2088 ultramicrotome, stained with 1% uranyl acetate and lead citrate, and examined under a transmission electron microscope (JEM-2000 EXII, 80 KV, Japan).

### **Patency of mitochondrial PTP**

**Isolation and purification of mitochondria:** Approximately 5 g of the liver harvested from sacrificed rats was homogenized in a ten-fold volume of isolation buffer (0.33 mmol/L sucrose, 0.025 mmol/L EDTA). After filtering with double-ply gauze to remove tissue dregs, the liver homogenate was centrifuged for 10 min at  $800 \times g$ . The supernatant was further centrifuged for 10 min at  $8200 \times g$ . The second supernatant was discarded and the sediment was resuspended with washing buffer (0.33 mmol/L sucrose). The suspension obtained was centrifuged for another 10 min at  $8200 \times g$  to get the purified mitochondria.

**Detection of mitochondrial PTP opening:** The purified mitochondria were diluted to 0.3 mg/mL with test solution (230 mmol manicol, 70 mmol sucrose, 3 mmol Hepes, pH 7.4). The absorbance of mitochondria was detected at 570 nm using an ultraviolet spectrophotometer at 20°C to find the state of mitochondrial PTP opening.

### **Isolation and purification of hepatocytes**

Hepatocytes were isolated with the procedure of *in situ* two-step perfusion of Seglen<sup>[1]</sup>. After anesthetizing with pentobarbital sodium, the abdominal cavity of the rats was opened from the middle abdominal wall. Plastic duct was inserted into the portal vein after exposure. The livers were perfused first with Ca<sup>2+</sup>- and Mg<sup>2+</sup>- free D-Hanks solution for 20 min (20-40 mL/min) at 37°C to remove blood and Ca<sup>2+</sup> in the liver, and next with 0.01% (w/v) collagenase solution at 37°C when the fluid flowed from the inferior vena cava became clear. The membrane of gently fully digested liver was removed with curved forceps, then gently the hepatocytes and sinusoid endothelial cells were dispersed in Dulbecco's modified Eagle's medium. After filtering through 200-screen mesh to remove cell aggregates and tissue fibers, the cell suspension was washed thrice in DMEM/H with low-speed centrifugation ( $50 \times g$ , 5 min  $\times$  3) to obtain purified hepatocytes.

### **Detection of mitochondrial membrane potential of hepatocytes**

Rh123 powder was prepared with methanol stored at -20°C as a 1 g/L solution. The storing solution was diluted to 5 mg/L with PBS when used. After incubating in Rhodamin123 (final concentration 2.5 mg/L) for 1 h at 37°C, hepatocytes ( $1 \times 10^6$ ) were washed thrice in preheating PBS. After resuspending in 300  $\mu$ L PBS, filtered through 200-screen mesh,  $10^4$  cells were measured with flow cytometry using the CellQuest software (maximum absorbing wave length 590 nm, excitation wave length 488 nm).

### **Detection of mitochondrial mass**

After incubated in 1 mL NAO staining solution at 37°C for 20 min, the hepatocytes ( $1 \times 10^6$ ) were centrifuged at  $300 \times g$  for 5 min. The precooling GENMED preserving fluid was added into the supernatant, mixed with cell granulations and analyzed with flow cytometry using the CellQuest software (maximum absorbing wave length 519 nm, and excitation wave length 495 nm).

### **Detection of intracellular calcium concentration**

After incubating in 1 mL Fluo-3/AM (5  $\mu$ mol/L) at 37°C for 40 min, the hepatocytes ( $1 \times 10^6$ ) were washed 3 times with PBS, resuspended in 300  $\mu$ L PBS and filtered through a 200-screen mesh, and  $10^4$  cells were collected and detected with flow cytometry using the CellQuest software (maximum absorbing wave length 506 nm, and excitation wave length 490 nm).

### **Statistical analysis**

All values were expressed as mean  $\pm$  SD and analyzed with the SPSS Version. All experiments were repeated at least three times.  $P < 0.05$  was considered statistically significant.

## **RESULTS**

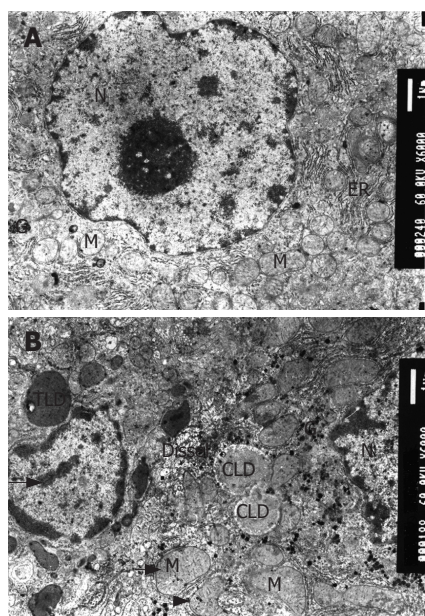
### **Ultrastructural changes of hepatocyte mitochondria under electron microscopy**

There were rich organelles with lots of ribosomes, endoplasmic reticulum, mitochondria and few lipid

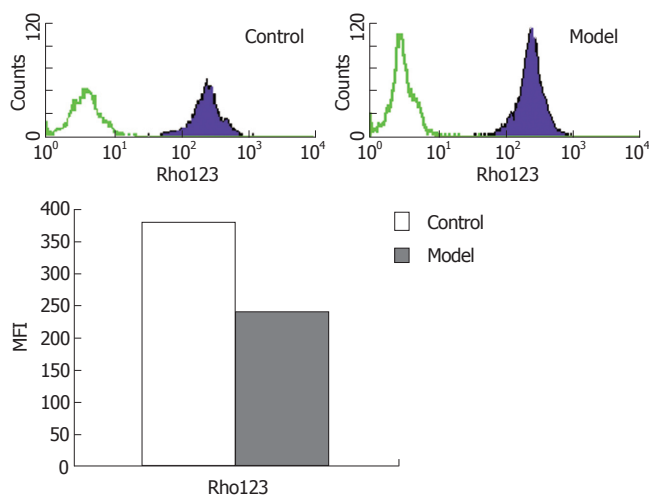
**Table 1** Absorbance comparison of liver mitochondria in two groups of rats

Groups	1	2	3	4	5	6	7	8	$\bar{x} \pm s$
Control	0.0305	0.0304	0.0331	0.0334	0.032	0.0314	0.0325	0.0336	$0.0136 \pm 0.0025$
Model	0.0167	0.0169	0.0161	0.0123	0.0119	0.012	0.0113	0.0116	$0.0321 \pm 0.0013^b$

<sup>b</sup> $P < 0.01$  vs control group.



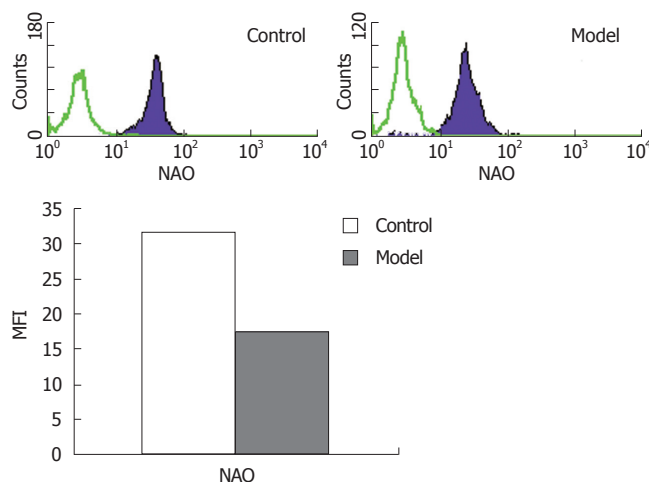
**Figure 1** Mitochondria appearance under electron microscope (EM  $\times 6000$ ); **A**: Mitochondria in normal group; **B**: Mitochondria in model group. M: mitochondria, G: glycogen, N nucleus, ER: endoplasmic reticulum, LD: lipid droplet. The long arrow shows abnormally distributed chromatin in nuclei, the short one is megamitochondrion and the arrow head is U-type mitochondria.



**Figure 2** Mitochondrial membrane potential detected by flow cytometry.

droplets in hepatocytes in the control group under electron microscopy. Nuclei of hepatocytes were round with clear membranes and well-distributed nuclear chromatin. The perinuclear cisterna and nucleopore were clear (Figure 1A).

Many lipid droplets with equal size and electron density and chromaffin lipid droplets could be seen in the model group. The endoplasmic reticulum became swollen. The membrane was ruptured and cristae were broken or disappeared in the mitochondria resulting in different shapes, and the electron density of their ground



**Figure 3** Mitochondria mass detected by flow cytometry.

substance decreased. Some mitochondria were U-type or megamitochondrion. The glycometabolism was abnormal, and glycogen increased with various densities. Many myelin figures of metabolites and few lysosomes could be observed. Nuclear material presented irregular profiles with a pseudo-inclusion body found within. Nuclear chromatin was abnormal in distribution and occasionally gathered at the perimeter. The number of plasmocytes increased (Figure 1B).

We had established the ALD model successfully, and confirmed our viewpoint that alcohol could cause structural and morphological damage in mitochondria.

**Patency of PTP in hepatocytes mitochondria**

The results of A540 detected in model group ( $0.0136 \pm 0.0025$ ) are obviously lower than in the control group ( $0.0321 \pm 0.0013$ ),  $P < 0.01$ , which demonstrated that the PTP in liver mitochondria of rats were opened in the model group (Table 1).

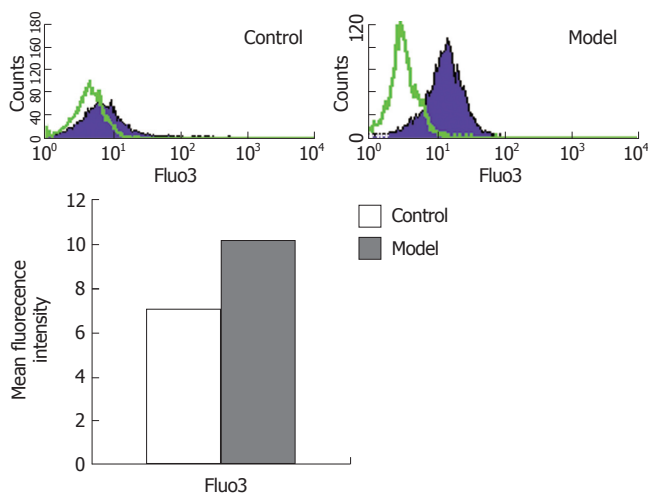
The data indicated that ethanol could increase mitochondria membrane permeability, resulting in mitochondria swelling and damage.

**Mitochondrial membrane potential of hepatocytes detected by Rh123**

The mean fluorescence intensity (MFI) of Rh123 in the model group was  $239.464\% \pm 12.726\%$  while in the control group was  $377.585\% \pm 16.812\%$ , which demonstrated that the mitochondrial membrane potential ( $\Delta\psi_m$ ) in the model group decreased evidently with a statistically significant difference ( $P < 0.01$ ) (Figure 2).

**Changes of liver mitochondria mass**





**Figure 4** Intracellular calcium concentrations in hepatocytes detected by flow cytometry.

The MFI of diphosphatidyl glycerol in the model group was  $31.6738\% \pm 3.4930\%$  while the control group was  $17.4350\% \pm 1.9882\%$ ,  $P < 0.01$  (Figure 3).

#### Intracellular calcium concentration in hepatocytes

The MFI of Fluo-3/AM in the model group was  $7.0020\% \pm 0.5008\%$  while the control group was  $10.2050\% \pm 0.4701\%$ , which demonstrated that the intracellular calcium concentration in hepatocytes in the model group increased evidently with a statistically significant difference ( $P < 0.01$ ) (Figure 4).

## DISCUSSION

Excessive alcohol consumption can result in multiple organ injury, of which alcoholic liver disease (ALD) is the most common. With economic development and improvement of living conditions of the people, the incidence of liver diseases caused by alcohol abuse has been increasing in China, although its pathogenesis remains obscure. In the past, most researches about ALD focused on oxidative stress, cytokines unbalance, level alteration of proteins and genes and so on. Recently, more attention has been on the function of mitochondria in the pathogenesis of ALD, but the mode of mitochondrial damage has not been clarified. In our study, we presented data showing structure and function damage, membrane permeability transition as well as membrane potential alteration of mitochondria, hoping to reveal the correlation between mitochondrial damage and the early stage of ALD.

#### Figure and structure alteration of liver mitochondria induced by ethanol

Mitochondria have long been known to be very important organelles of the eukaryocyte. They produce ATP to supply energy directly for life activity of the mammal cells. Moreover, mitochondria are considered the key organelle in the initiation of apoptosis since mitochondrial disintegration not only leads to a loss of their function but also causes the release of proapoptotic factors. Emerging

evidence suggests that mitochondrial dysfunction caused by oxidative stress is an early event that plays an important role in the pathogenesis of ethanol induced apoptosis.

In this study, we found that the mitochondria of the model group were obviously damaged, and membrane and cristae were broken or disappeared under electron microscopy. We also observed U-form mitochondria (deformed mitochondria) in different sizes and megamitochondria. In hepatocytes, there are some myelin figures, chromatin distribute abnormally and some can be edge-gathering, indicating the existence of apoptosis. All of these further confirmed the damage in the structure and function of mitochondria. Meanwhile, the structural and functional damage of mitochondria play a very important role in accelerating hepatocyte aging and apoptosis induced by ethanol.

#### Mitochondria PTP opening influenced by ethanol

In recent years, the knowledge of apoptosis has shifted gradually from the mode of nucleolus-central to mitochondria-central adjustment<sup>[2]</sup>. In apoptosis, the basic change in mitochondria is mitochondrial permeability transition (MPT) and decreased transmembrane potential and other transformation of apoptosis, leading finally to cell apoptosis.

PTP is a nonselective passage with a high conductance which stretches across the outer and inner membrane of mitochondria. Physiologically, the diameter of PPT is  $0.2\text{--}0.3\ \mu\text{m}$ , large enough to allow micromolecule solute to pass<sup>[3]</sup>. By the effect of  $\text{Ca}^{2+}$ , ADP, ATP and so on, PTP switches alternately. This condition may play a role in material exchange between mitochondria and cytoplasm. But pathologically, the opening diameter is obviously large ( $1.8\text{--}2.6\ \mu\text{m}$ ) so that the solute smaller than  $1.5\ \text{kDa}$  can pass through PPT to the cytoplasm, causing mitochondrial swelling and transmembrane potential disappearance<sup>[4]</sup>. PTP opening has a self-enlarging phenomenon that after receiving the death signal, can perform positive feedback in the same cell. It means that once PTP maintains opening, it will no longer depend on the previous stimulant signal, and the cell will die irreversibly. Our experiment indicated that long-term alcohol abuse of ethanol can make PTP open, the mechanism is possibly because in hepatocytes, anti-oxidation materials such as materials containing sulfhydryl are reduced, and/or lipid peroxidation intensifies, causing injury to the mitochondrial membrane. Thus, the permeability of the membrane increases, mitochondria swell, and finally the mitochondria are injured.

#### Relationship between ethanol and mitochondria transmembrane potential

The permeability of the mitochondrial outer membrane is comparatively large, material smaller than  $15\ \text{kDa}$  can pass through it freely, so substances of the outer compartment of the mitochondria are similar to cytoplasm. The permeability of the mitochondrial inner membrane is small enough to allow the solute smaller than  $1.5\ \text{kDa}$  to pass through. Oxadative phosphorylation to supply energy takes place in mitochondria. In this process, energy produced by the tricarboxylic acid cycle is transmitted to

electrons, which shuttles along respiratory enzymes of the inner mitochondrial membrane, thus manifesting the generation of ATP. The proton pump is localized on the inner membrane, it pumps protons from groundmass to the intermembrane in order to form the transmembrane potential between outer and inner membrane of the mitochondria  $\Delta\psi_m$ <sup>[5]</sup>. When protons return, they pass energy to ADP and Pi to generate ATP. So  $\Delta\psi_m$  plays a crucial role in keeping the function of mitochondria. Further studies of apoptosis indicate that there is mitochondrial polarization and  $\Delta\psi_m$  disruption in injured cells induced by apoptosis repulsion, moreover these changes take place before alteration due to apoptosis in cells. All of these indicate that  $\Delta\psi_m$  alteration is the early stage of apoptosis<sup>[6]</sup>.

In our experiment, we measured mitochondrial transmembrane potential ( $\Delta\psi_m$ ) changes during culture using Rhodamine123 (specific fluorescence mark) staining and flow cytometry. Results reveal that long-term alcohol consumption can cause transmembrane potential significantly lower than the control group, indicating that mitochondrial depolarization and  $\Delta\psi_m$  alteration must be an early-onset, and even a crucial event in the cell death induced by ethanol.

#### **Mechanism of mitochondrial quality degeneration induced by ethanol**

The mitochondrial quality is the amount of all the intracellular mitochondria and their content, and it is an important index of mitochondrial injury. Nonyl-Acridine Orange (NAO) is fluorescent can bind specifically to unoxidizable phospholipids. The inner membrane of mitochondria, and the fluorescence intensity is proportional to the quality of phospholipids in the mitochondrial inner membrane<sup>[7]</sup>. In our experiment, we used NAO flow cytometry to measure the quality of ALD mitochondria. The biological values are to show the quantity of mitochondria with normal respiratory efficiency, and to indicate the degree of injury to the mitochondria. The results show that mitochondrial quality of the model group is obviously lower than that of the control group. This indicates that in the process of ALD, the mitochondrial quality is lowered.

#### **Effect of $Ca^{2+}$ in hepatocyte apoptosis and ALD injury**

Physiologically, hepatocytes are surrounded with a high concentration of  $Ca^{2+}$ , and the concentration difference between outer and inner cell is 3-4 orders of magnitudes. Distribution of  $Ca^{2+}$  is uneven intracellularly, the content of  $Ca^{2+}$  is higher in mitochondria and endoplasmic reticulum than in cytoplasm and caryon<sup>[8]</sup>. Such a concentration gradient between organelle and cytoplasm is a precondition that  $Ca^{2+}$  can be an intracellular messenger. Slow accumulation of  $Ca^{2+}$  in mitochondria causes over-

loading of  $Ca^{2+}$  so as to make PTP open, leading to a prompt decrease of the membrane potential ( $\Delta\psi_m$ ), swelling of mitochondria, and finally apoptosis<sup>[9]</sup>.

In our study, we found that the  $Ca^{2+}$  level of the model group is significantly higher than that of the control group. It indicates that  $Ca^{2+}$  plays an important role in the process of ALD. The  $Ca^{2+}$  signal may induce mitochondrial damage through several possible ways as follows: (1) the elevation of  $Ca^{2+}$  density in hepatocytes makes PTP open, causing  $Ca^{2+}$  to transfer from cytoplasm to mitochondria. This damage is reversible; (2) the over-loading of  $Ca^{2+}$  in mitochondria maintains PTP open, leading to a decrease of  $\Delta\psi_m$ , swelling of mitochondria, and finally apoptosis. So our study indicates that the  $Ca^{2+}$  signal transduction system plays an crucial role in the pathogenesis of ALD.

In summary, our study demonstrates that in the process of hepatocyte injury, ethanol metabolism induce mitochondrial membrane depolarization and permeability changes in cultured hepatocytes. It indicates that mitochondrial damage must be of pathophysiological importance in the process of ALD. The pathway is possibly  $Ca^{2+}$  transferring from cytoplasm to mitochondria, increasing mitochondrial permeability, decreasing mitochondrial membrane potential, finally leading to hepatocyte injury.

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