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# Hyperammonia induces specific liver injury through an intrinsic $\text{Ca}^{2+}$ -independent apoptosis pathway

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## Abstract

**Background:** Numerous pathological processes that affect liver function in patients with liver failure have been identified. Among them, hyperammonia is one of the most common phenomena. The purpose of this study was to determine whether hyperammonia could induced specific liver injury.

**Methods:** Hyperammonemic cells were established using  $\text{NH}_4\text{Cl}$ . The cells were assessed by MTT, ELISA, and flow cytometric analyses. The expression levels of selected genes and proteins were confirmed by quantitative RT-PCR and western blot analyses.

**Results:** The effects of 20 mM  $\text{NH}_4\text{Cl}$  pretreatment on the cell proliferation and apoptosis of primary hepatocytes and other cells were performed by MTT assays and flow cytometric analyses. Significant increasing in cytotoxicity and apoptosis were only observed in hepatocytes. The cell damage was reduced after adding BAPTA-AM but unchanged after adding EGTA. The expression levels of caspase-3, cytochrome C, calmodulin, and inducible nitric oxide synthase were increased and that of bcl-2 was reduced. The  $\text{Na}^+\text{-K}^+\text{-ATPase}$  activities in hyperammonia liver cells was no significant difference compared with the control group, but was decreased in astrocytes.  $\text{NH}_4\text{Cl}$  pretreatment of primary hepatocytes promoted the activation of mitochondrial permeability transition pores and the mitochondria swelled irregularly.

**Conclusions:** Hyperammonia induces specific liver injury through an intrinsic  $\text{Ca}^{2+}$ -independent apoptosis pathway.

**Keywords:** Liver injure, Hyperammonia, Calcium overload, Mitochondrial damage

## Background

Acute liver failure is a multisystem disorder associated with acute renal failure, hypotension, sepsis, coagulopathy, encephalopathy, and cerebral edema [1]. Some researchers have considered that a few hepatocytes are required to restore the liver mass after profound liver injury, while other liver-repopulation and transplantation studies have indicated that bone-marrow stem cells might have the capacity to differentiate into hepatocytes [2-5]. However, the regenerative capacity is insufficient after chronic liver injury [6]. Currently, most researchers consider that the

activation, proliferation, migration, differentiation, and survival of cells in the regenerating liver are controlled by a large number of growth factors and cytokines, which expressed at the sites of injury or reach the liver via the circulatory system [7]. Furthermore, a recent study found that severe lactic acidosis was harmful for cirrhotic patients [8]. Our preclinical studies showed that the clinical symptoms of some patients with liver failure, who did not present with symptoms of hepatic encephalopathy (HE) and were treated with blood ammonia-lowering drugs, were greatly relieved. The primary disease process in the liver is complicated because of numerous metabolic disturbances throughout the body [9]. Therefore, we inferred that a reduction in the blood ammonia level could promote the functions of synthesis, secretion, and transformation in liver cells and simultaneously

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relieve the damage to liver cells. Recent studies showed that increasing ammonia concentrations had deleterious effects on the functions of the central nervous system and the elevation of arterial ammonia was associated with high mortality in patients with acute liver failure [10,11]. Ammonia is a neurotoxin involved in the pathogenesis of neurological disease associated with hyperammonia [12]. Hyperammonia following acute and chronic liver diseases may lead to HE, which is accompanied by the failure of energy metabolism [13], disturbances of neurotransmission in the brain, and changes in  $\text{Na}^+\text{-K}^+\text{-ATPase}$  [14,15]. Moreover, our study showed that there was no change in  $\text{Na}^+\text{-K}^+\text{-ATPase}$  using gene chip assays, but arginine disappeared. Although the liver can convert ammonia to nontoxic urea through the urea cycle [16], the urea synthesis capacity is reduced in patients with liver disease, leading to a reduced capacity to detoxify ammonia in the liver. Besides, hyperammonia is also produced in urea cycle disorders and other conditions leading to either defective ammonium removal or overproduction of ammonium beyond the capacity of liver clearance [17]. Therefore, we thought that ammonia might induce liver injury through another mechanism. However, there are few reports about whether increasing blood ammonia can lead to the damage of hepatocyte function observed in the present study. We also found that  $\text{NH}_4\text{Cl}$  induced specific liver injury compared with other cell types and apoptosis of primary hepatocytes was significantly increased compared with control cells. In this study, we hypothesized that hyperammonia might directly induce a series of changes leading to liver injury. To verify this assumption, a hyperammonia cell model was established to investigate the effects of  $\text{NH}_4\text{Cl}$  on liver damage and further examine the effects of  $\text{NH}_4\text{Cl}$  on hepatocyte apoptosis.

## Methods

### Cell lines, cell culture, and $\text{NH}_4\text{Cl}$ treatment

Primary hepatocytes were purchased from the Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). A-549 lung cancer cells, MCF-7 breast cells, BGC-823 gastric cancer cells, SKOV3 ovarian cancer cells, C6 glioma cells, and HepG2 HepG2.2.15 hepatocarcinoma cells were all preserved in our laboratory. All cells were maintained in RPMI1640 medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100  $\mu\text{g}/\text{ml}$  streptomycin at  $37^\circ\text{C}$  under 5%  $\text{CO}_2$ . The cells were treated with  $\text{NH}_4\text{Cl}$  at 0.5, 5, 10, and 20 mmol/L.

### Enzyme levels in media

The supernatant was extracted after exposed to 0 and 20 mM  $\text{NH}_4\text{Cl}$  for 12, 24 and 48 h respectively. The detection of each indicator was carried out in accordance with the requirements of the ELISA kit. The OD

values were detected at 450 nm with a Microplate reader (MultiskanMK3).

### Viability measurement

Viability was tested by using MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl -tetrazolium bromide) [18]. After different concentrations of  $\text{NH}_4\text{Cl}$  exposure, MTT was added to a final concentration of 0.5 mg/ml. After 4 h MTT-incubation, supernatants were removed and 150  $\mu\text{L}$  DMSO was added in each well, darkly shaken for 15 min. The absorption was measured by a microplate reader at a wavelength of 490 nm.

### Apoptosis of different cell lines evaluated by flow cytometry

Cells were treated with  $\text{NH}_4\text{Cl}$ , harvested after 24 hours and suspended in annexin V-binding buffer. Thereafter, the cells were incubated by FITC for 15 min in the dark, and propidium iodide was added. Thereafter, all samples were analyzed by a FACSCalibur flow cytometer with CellQuest software.

### Assay of Na-K ATPase specific activity

Total membrane fractions were prepared from control and  $\text{NH}_4\text{Cl}$ -treated hepatocytes and C6 cells. Reaction mixtures for Na-K-ATPase activity assay was as the manufacturer's instructions (Jianchen Biology Engineering Institute, China). Enzymatic activity was measured as a function of liberated inorganic phosphate ( $\text{P}_i$ ) by the colorimetric reaction. The color developed after 10 min at  $37^\circ\text{C}$  and was read at a wavelength of 850 nm with a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA).

### Arginine levels after primary hepatocyte injury induced by $\text{NH}_4\text{Cl}$

Primary hepatocytes were treated with 10 mmol/L  $\text{NH}_4\text{Cl}$  for 24 h. We added 100ul sample cell fluid 350  $\mu\text{L}$  extraction liquid ( $V_{\text{methanol}}: V_{\text{chloroform}} = 3:1$ ) and 50  $\mu\text{L}$  L-2-Chlorophenylalanine as an internal standard and vortexed for 10 s. The samples were centrifuged for 10 min at 12,000 rpm and  $4^\circ\text{C}$ . Then we transferred 0.35 mL from the supernatant into a fresh 2 mL GC/MS glass vial. Next 80 mL methoxyamination reagent was added and shaken for 2 h at  $37^\circ\text{C}$ , followed by addition of 0.1 mL BSTFA reagent and shook for 1 h at  $70^\circ\text{C}$ . GC-MS analyses were performed when the temperatures cooled to room temperature.

### Effects of BAPTA-AM and EGTA on primary hepatocyte injury induced by $\text{NH}_4\text{Cl}$

Primary hepatocytes seeded in 96-well plates were treated with media containing various concentrations of  $\text{NH}_4\text{Cl}$  for 1 h, followed by addition BAPTA-AM ( $1 \times 10^{-5}$  mol/L).

Next,  $\text{NH}_4\text{Cl}$  and EGTA were added simultaneously. The control group was treated with solvent alone. MTT solution was added after BAPTA-AM for 6 h. Absorbance was measured at 450 nm using a microplate reader.

#### Detection of mitochondrial permeability transition pore (mPTP) opening

Primary hepatocytes were seeded in 48-well plates. After treatment with 20 mmol/L  $\text{NH}_4\text{Cl}$  for 24 h, 500  $\mu\text{l}$  GENMED reagent A preheated in  $37^\circ\text{C}$  was added to each well and then removed reagent A carefully. Then add 200  $\mu\text{l}$  dyeing working fluid (5  $\mu\text{l}$  reagent B plus 500  $\mu\text{l}$  reagent C) was added to each well incubating at  $37^\circ\text{C}$  in cell culture box for 20 min darkly. Next we wash the cells with liquid A twice. Then images were collected as described. The cells were observed by fluorescence microscopy using an excitation wavelength of 488 nm and an emission wavelength of 505 nm. The peak shifting to the left indicated the increased activity of MPTP pore.

#### Analysis of mitochondrial morphology by transmission electron microscopy

Primary hepatocytes were treated with 0, 10, and 20 mM  $\text{NH}_4\text{Cl}$  for 48 h. Then the cells were harvested, fixed in 2.5% glutaraldehyde, and embedded in propylene oxide and epoxy resin overnight at  $37^\circ\text{C}$ . The embedded cells were cut into ultrathin sections, double-stained with uranyl acetate and lead citrate, and observed by transmission electron microscopy.

#### RNA extraction and quantitative real-time PCR

Total RNA was extracted from primary hepatocytes after 12, 24, and 48 h of  $\text{NH}_4\text{Cl}$  treatment using the TRIzol reagent (Takara, Japan). First-strand cDNA was synthesized from 1  $\mu\text{g}$  total RNA using a PrimeScript RT Reagent Kit With gDNA Eraser. The cDNA was used to detect the expression levels of caspase-3, cytochrome C (Cyt C), calmodulin, and inducible nitric oxide synthase (iNOS). Quantitative real-time PCR was performed using SYBR Premix Ex Taq II in a Step One Plus system.

#### Western blot analysis

To extract the total proteins, livers or cells which were treated with  $\text{NH}_4\text{Cl}$  were lysed on ice for 30 min in lyses buffer [19], and centrifuged at 12000 g for 10 min, the supernatant were recovered. After denaturation, 50  $\mu\text{g}$  proteins were separated on 10% SDS/PAGE gels and then transferred to nitrocellulose membranes by using a transfer cell system (Bio-Rad, California, USA). Membranes were blocked for 1 h at room temperature with 5% nonfat dried milk powder/Tris-buffered saline Tween-20 (TBST) and then with primary antibodies incubated overnight at  $4^\circ\text{C}$ . Immunoblots were washed 3 times with TBST and were incubated with secondary antibodies conjugated with horseradish peroxidase against mouse IgG or rabbit IgG for 1 h at room temperature. Immunoreactive proteins were visualized using the infrared laser scanning imaging system (CDYSSEY CLx; General Electric Company).

#### Statistical analysis

Statistical analysis was performed with SPSS 13.0 software (SPSS Inc., Chicago, IL, USA). Data are expressed as means  $\pm$  SD, and three individual experiments were carried out in triplicate. The Student's t-test was used to compare data between two groups. One-way ANOVA and Dunnett's test were used to compare data between three or more groups.  $P < 0.05$  was considered to indicate a statistically significant result.

## Results

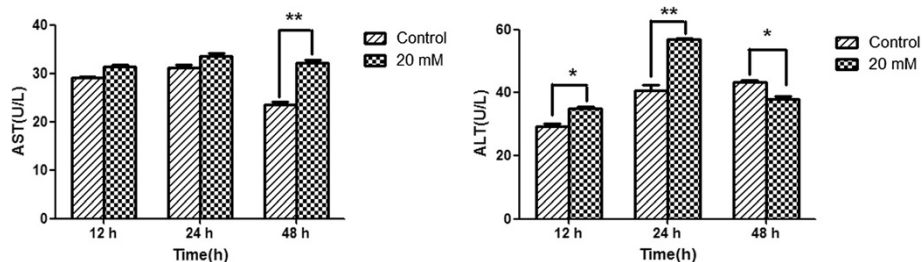
### Hyperammonia induces specific inhibition of liver regeneration

#### Liver damage caused by ALT and AST in vitro

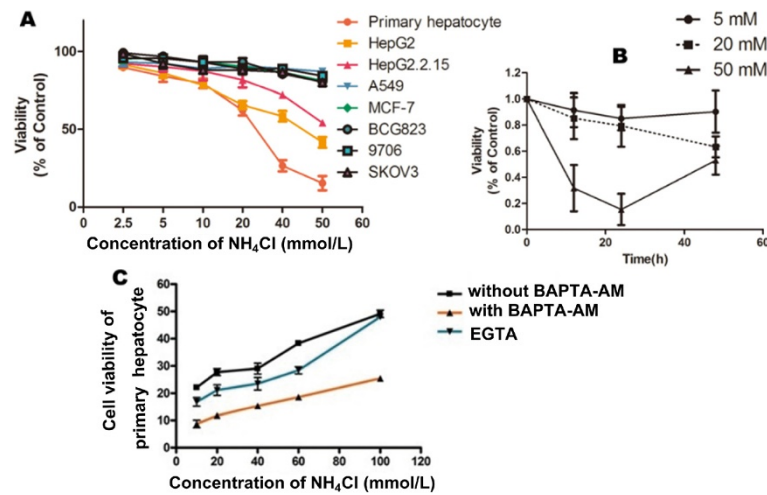
The expression of ALT, markers of liver injury, (Control group:  $43.44 \pm 0.70$ , 20 mM ammonia group:  $37.87 \pm 0.91$ ) was in a relatively decreased within 48 h (Figure 1A). Significantly increased expression AST (Control group:  $23.59 \pm 0.51$ , 20 mM ammonia group:  $32.04 \pm 0.66$ ) was detected only at the time of 48 h (Figure 1B).

#### Effects of $\text{NH}_4\text{Cl}$ on cell growth in cell lines

The MTT assay showed that the liver cells was inhibited by  $\text{NH}_4\text{Cl}$  (Figure 2A), but had no effect on A549, MCF-7,



**Figure 1** Liver damage caused by ALT and AST in vitro. Values are expressed Mean  $\pm$  SD, \* $P < 0.05$  versus control group, \*\* $P < 0.001$  versus control group. ALT: glutamate pyruvate transaminase; AST: Aspartate aminotransferase.



**Figure 2** Effects of NH<sub>4</sub>Cl on the cytotoxicity of primary hepatocytes and different cell lines. Cytotoxicity was measured by MTT assays. **(A)** The levels of MTT reduction were quantified and compared with the control at each concentration. The results showed that NH<sub>4</sub>Cl induced specific cytotoxicity toward regenerating hepatocytes. **(B)** The viability of primary hepatocytes after exposed to 5, 20 and 50 mM ammonia for 48 h. A dramatic time-dependent loss was found in cell viability when exposure to 20 mM and 50 mM ammonia, however the viability of cells recovered when exposed to 5 mM ammonia for 48 h. **(C)** Effects of BAPTA-AM and EGTA on primary hepatocyte injury induced by hyperammonia. The cell viability of the hyperammonia group without BAPTA-AM or EGTA was clearly lower than those in the groups with BAPTA-AM or EGTA addition. Each value indicates the mean  $\pm$  SEM normalized by the control for each concentration. Data were obtained from three independent experiments.

BCG823, SKOV3. At 24 h, we determined that primary hepatocytes (IC<sub>50</sub>: 27.26 mmol/L) were significantly more sensitive to NH<sub>4</sub>Cl than HepG2 cells (IC<sub>50</sub>: 43.00 mmol/L) or HepG2.2.15 cells (IC<sub>50</sub>: 62.78 mmol/L). A dramatic time-dependent loss was found in cell viability when exposure to 20 mM and 50 mM ammonia, however the viability of cells recovered when exposed to 5 mM ammonia for 48 h (Figure 2B). Considering these results, we decided to use the 20 mM ammonia for the following experiments.

#### NH<sub>4</sub>Cl-induced apoptosis in primary hepatocytes

As shown in Figure 3A, the apoptosis assays evaluated by flow cytometry demonstrated that the apoptosis rate was increased to  $25.97 \pm 1.46\%$  in primary hepatocytes after treating with 20 mmol/L NH<sub>4</sub>Cl for 24 h compared with that in the control group, which exhibited approximately  $3.30 \pm 0.44\%$  spontaneous apoptosis.

#### Roles of caspase-3, Cyt C, and bcl-2 in liver regeneration

To examine whether hyperammonia induces liver injury through an apoptosis pathway, we examined the effects of NH<sub>4</sub>Cl on apoptosis by measuring the levels of caspase-3, Cyt C, and bcl-2. The results of western blot are consistent with QT-PCR. The energy barrier caused by ammonia increased the expression of caspase-3, Cyt C, and accompanying reduced bcl-2 expression (Figures 4 and 5A). As depicted in Figures 5 and 6A, the mRNA expression of caspase-3 and Cyt C were significantly increased after NH<sub>4</sub>Cl treatment, which exhibited maximal extent when treated with 20 mmol/L NH<sub>4</sub>Cl

at 48 h. The protein expression were significantly changed after treating with NH<sub>4</sub>Cl for 48 h and then returned to the control levels at 72 h. The results showed that the expression of caspase-3 ( $2.18 \pm 0.31$ ) and Cyt C ( $0.55 \pm 0.14$ ) were most increased when the concentration of NH<sub>4</sub>Cl was 10 mmol/L, while the expression of bcl-2 ( $0.55 \pm 0.14$ ) was most significantly decreased at 48 h.

#### Differences in liver damage induced by hyperammonia between liver cells and other cells

##### Activity of Na-K ATPase

To elucidate the differences in Na-K-ATPase activity between liver cells and astrocytes, we measured the Na-K-ATPase activity in membrane fractions of liver cells and astrocytes treated with 20 mM NH<sub>4</sub>Cl. In the liver cells, the Na-K-ATPase activity was no significant difference (control group:  $11.4 \pm 4.5$  nmol Pi/ $\mu$ g protein/min NH<sub>4</sub>Cl-treated group  $12.8 \pm 2.9$  nmol Pi/ $\mu$ g protein/min). However, in the astrocytes, the Na-K-ATPase activity was significantly increased in NH<sub>4</sub>Cl-treated group (control group:  $13.9 \pm 3.6$  nmol Pi/ $\mu$ g protein/min NH<sub>4</sub>Cl-treated group:  $47.6 \pm 2.9$  nmol Pi/ $\mu$ g protein/min).

##### Arginine level after primary hepatocyte injury induced by NH<sub>4</sub>Cl

Compared with the control group, the arginine level was decreased in the NH<sub>4</sub>Cl-treated group ( $0.0003023 \pm 0.3271$ ), representing  $69.16 \pm 0.4179\%$  of the arginine level in the control group ( $0.0004372 \pm 0.2146$ ).

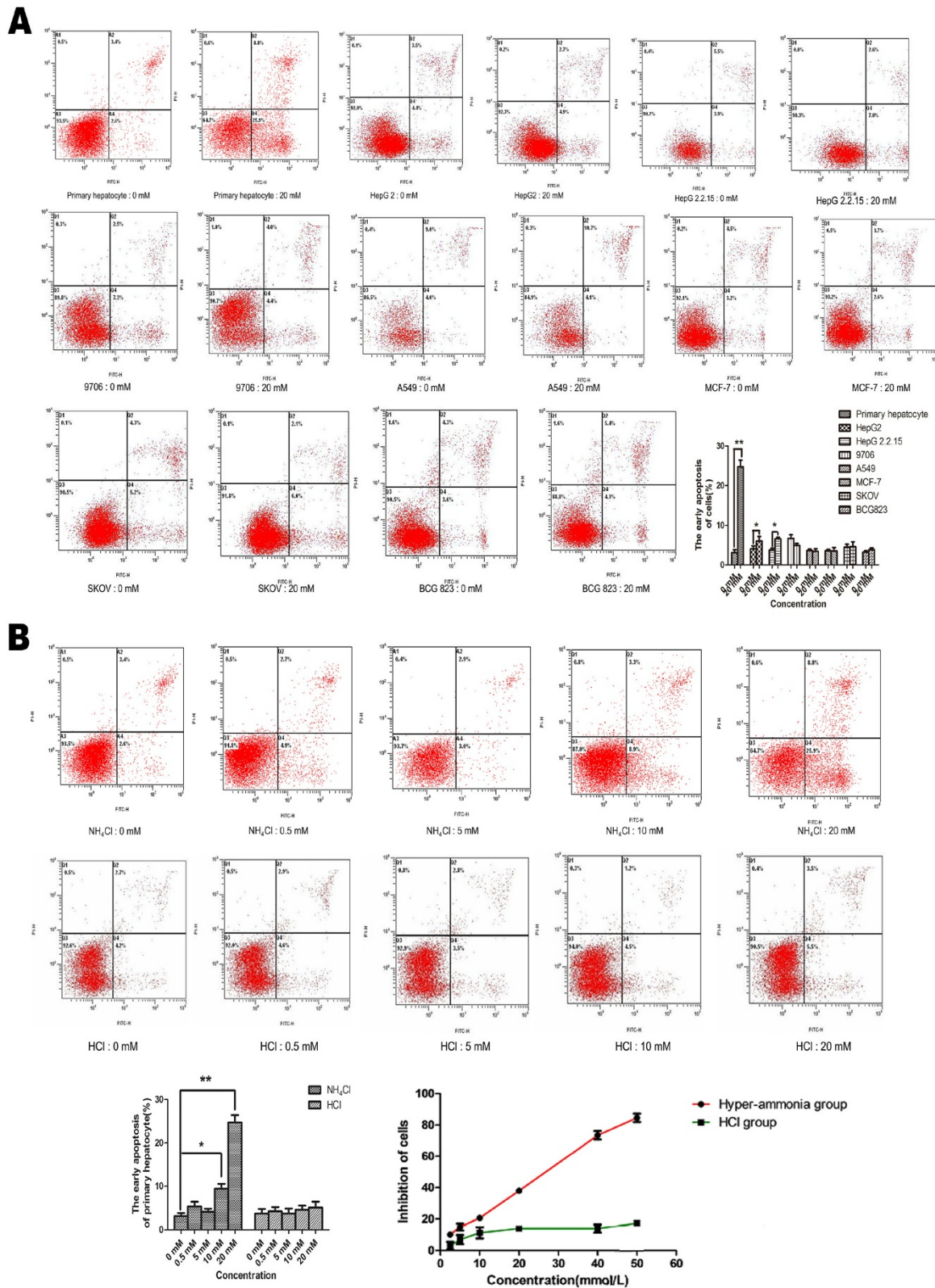


Figure 3 (See legend on next page.)

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**Figure 3 Flow cytometry analysis of apoptosis in different cell lines.** Flow cytometry was used to assess changes in the rate of apoptosis in the primary hepatocytes and other different cell lines with  $\text{NH}_4\text{Cl}$  treatment. **(A)** The results showed that the apoptosis rates in the hyperammonia group of primary hepatocytes ( $24.7 \pm 1.71$ ) were significantly greater than those in the control group ( $3.1 \pm 0.71$ ), while the hyperammonia groups of HepG2.2.15 (0 mM  $3.73 \pm 0.47$ ; 20 mM:  $6.60 \pm 0.36$ ) and HepG2 cells (0 mM:  $4.07 \pm 0.76$ ; 20 mM:  $6.03 \pm 0.76$ ) showed increased rates, but they were less than those in the primary hepatocytes. The apoptosis in the other cell lines with hyperammonia (9706: 0 mM  $6.7 \pm 0.91$ ; 20 mM:  $4.9 \pm 0.51$ ; A549: 0 mM  $3.63 \pm 0.40$ ; 20 mM:  $3.40 \pm 0.70$ ; MCF-7: 0 mM  $3.57 \pm 0.35$ ; 20 mM:  $3.53 \pm 0.86$ ; SKOV3: 0 mM  $4.47 \pm 0.75$ ; 20 mM:  $4.60 \pm 1.23$ ; BCG-823: 0 mM:  $3.2 \pm 0.36$ ; 20 mM:  $3.83 \pm 0.45$ ) showed no difference. **(B)** Flow cytometry revealed that the apoptotic hepatocytes in the hyperammonia group were increased compared with the control group, especially when the concentrations of  $\text{NH}_4\text{Cl}$  were 0.5 and 20 mmol/L. In addition, there were no significant differences among the HCl groups. For all experiments, each value indicates the mean  $\pm$  SEM normalized by the control. \* $p < 0.05$ ; \*\* $p < 0.01$ , one way ANOVA.

### Hyperammonia induces mitochondrial damage

#### Hyperammonia induces the change of morphologic features of mitochondria

To investigate the mechanism of the liver damage induced by hyperammonia, we examined the morphological features of mitochondria by transmission electron microscopy. We observed the changes in the mitochondria depending on the ammonia exposure with 10 and 20 mmol/L. The mitochondria showed irregular swelling, fractured cristae, and vacuolar degeneration (Figure 6A).

#### Effects of BAPTA-AM and EGTA on primary hepatocyte injury induced by hyperammonia

We observed that the cell viability was increased ( $51.83 \pm 2.30\%$ ) after BAPTA-AM treatment (Figure 2C) compared with the control group ( $76.51 \pm 1.96\%$ ) which was only treated with 100 mmol/L  $\text{NH}_4\text{Cl}$ . Each value is shown as the mean  $\pm$  SEM, after normalization by the control for

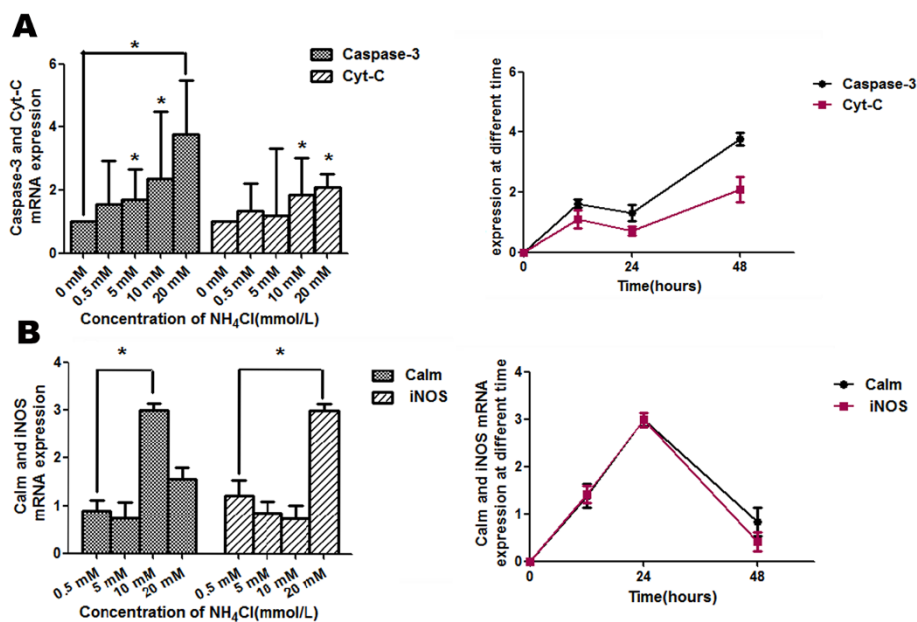
each concentration. In contrast, the cell viability of primary hepatocytes was unchanged after EGTA addition.

#### Hyperammonia triggers mPTP opening

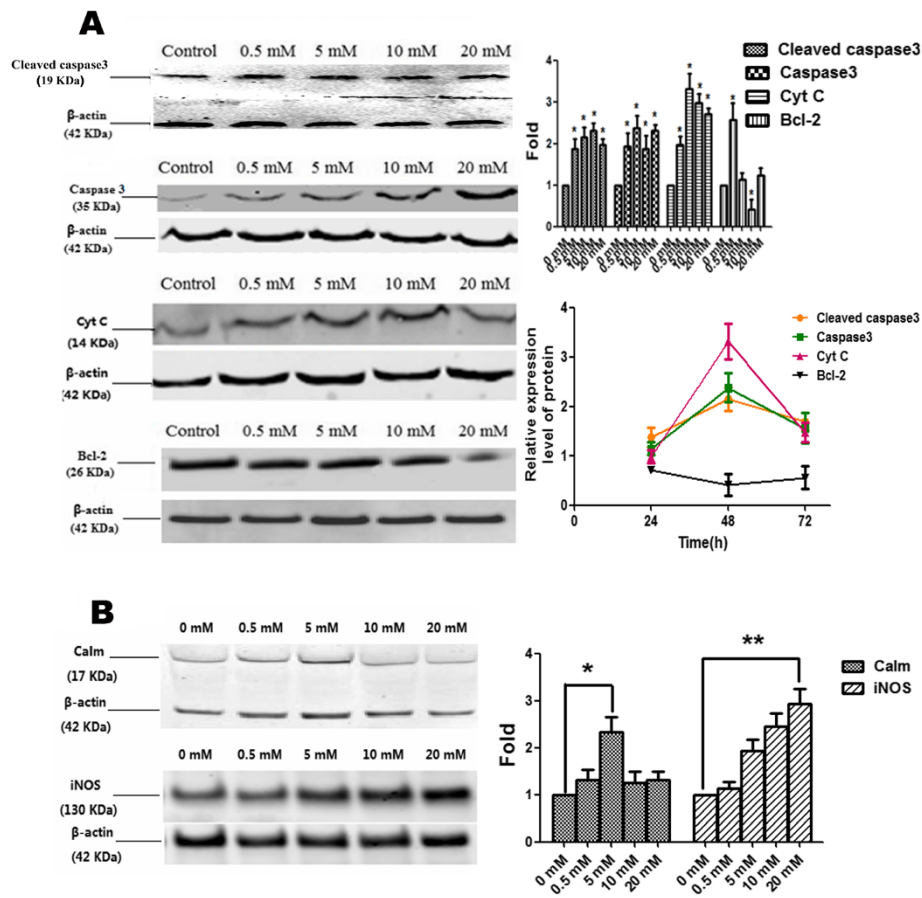
Treatment of hepatocytes with 20 mM ammonia (48 h) caused the position of peak shift move to left, but the position of peak shift has little change in control group (as shown in Figure 4B: C1 and T1). Comparison of C1 and C2, the position of peak shift move to left enhanced membrane channel activity (Figure 6B: T1, T2 and T3).

#### Hyperammonia induces apoptosis through an intrinsic $\text{Ca}^{2+}$ -independent apoptosis pathway

We examined the effects of  $\text{NH}_4\text{Cl}$  on apoptosis through an intrinsic  $\text{Ca}^{2+}$ -independent apoptosis pathway by measuring levels of calmodulin and iNOS. The results of WB are consistent with QT-PCR. The energy barrier caused by ammonia increased the expression of calmodulin and iNOS



**Figure 4 Analysis of mRNA levels for caspase-3, Cyt C, calmodulin and iNOS, in primary hepatocytes.** Real-time PCR analyses were performed to detect the mRNA expression levels of caspase-3 and Cyt C **(A)**, calmodulin, iNOS **(B)** in liver cells following treatment with  $\text{NH}_4\text{Cl}$  for 12, 24 and 48 h. The data were obtained from three independent experiments. For all experiments, each value indicates the mean  $\pm$  SEM normalized by the control for each time point and concentration. \* $p < 0.05$ ; \*\* $p < 0.01$ .



**Figure 5** Effect of NH<sub>4</sub>Cl on the expressions of cleaved caspase-3, caspase-3, Bcl-2, Cyt C, calmodulin, and iNOS. Ammonia-induced apoptosis of liver cells was accompanied by increased levels of cleaved caspase-3, caspase-3 and Cyt C and a decreased level of bcl-2 (A), and these phenomena were clearly observed after treating with NH<sub>4</sub>Cl for 48 h. To clarify the mechanism of the apoptosis, the protein levels of calmodulin and iNOS (B) were detected by western blotting analyses and all of these levels were increased. For all experiments, each value indicates the mean  $\pm$  SEM normalized by the control for each time point and concentration. \*p < 0.05; \*\*p < 0.01.

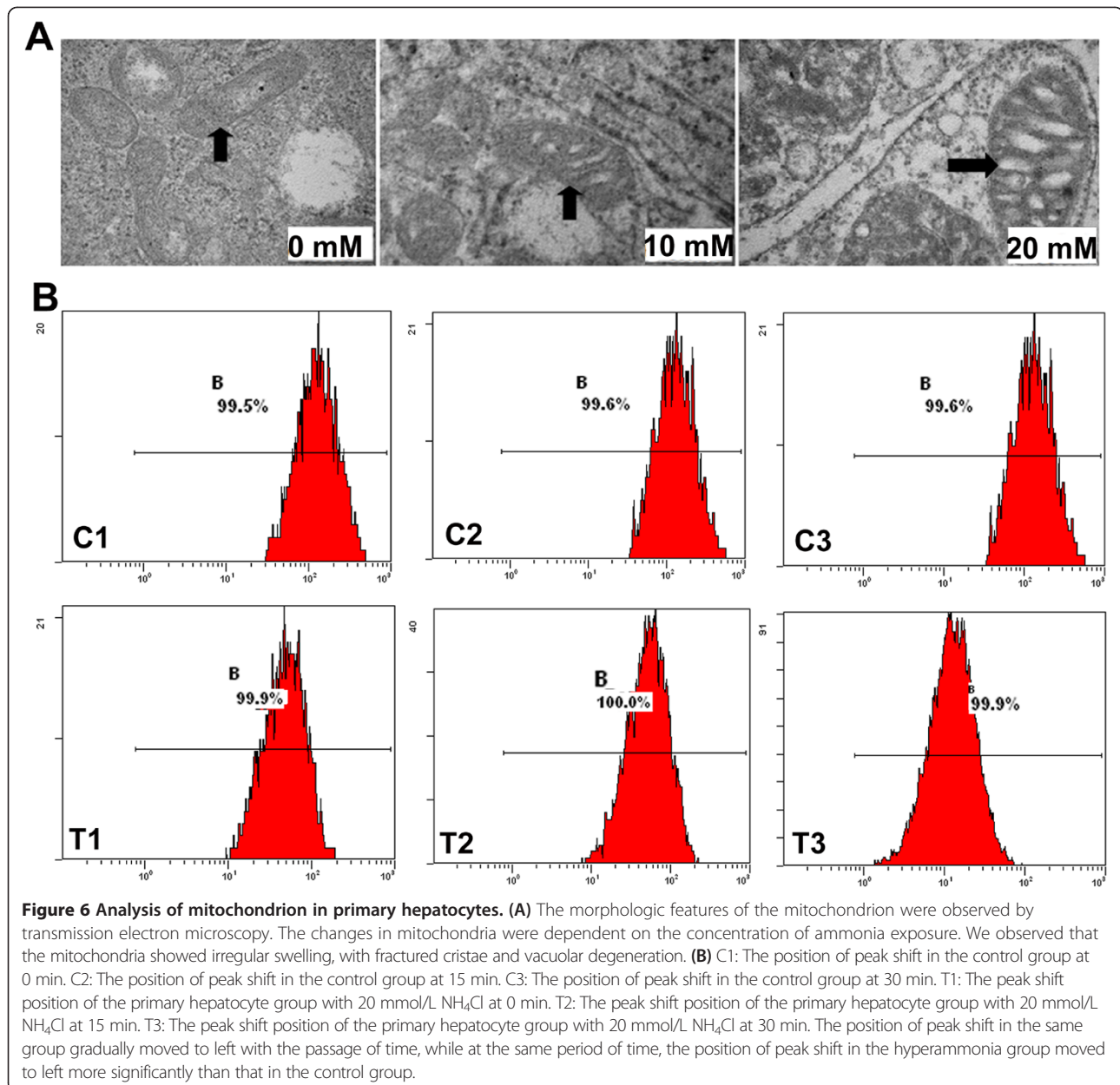
(Figure 5B). As depicted in Figures 4 and 5B, the mRNA expression of the molecules did not change after treatment with NH<sub>4</sub>Cl for 12 h ( $p > 0.05$ ; data obtained from three independent experiments) and were significantly increased after treating with 20 mmol/L NH<sub>4</sub>Cl for 24 h. The protein expressions were significantly changed after NH<sub>4</sub>Cl treatment for 48 h and then returned to the control levels at 72 h. The results showed that the most significantly increased expressions of calmodulin ( $2.34 \pm 0.31$ ) and iNOS ( $2.46 \pm 0.14$ ) were observed after treatment with NH<sub>4</sub>Cl at 10 mmol/L for 48 h.

## Discussion

Numerous pathological processes which affect liver function in patients with liver failure have been identified. Among them, hyperammonia is one of the most common phenomena. It is well known that many growth factors and cytokines have explicit effects on regulating liver regeneration [7]. However, it remains unclear whether hyperammonia has an effect on liver injury. Currently,

most researchers believe that ammonia is a kind of toxicant which can inhibit many cell functions, such as cell regeneration. In our studies, we showed that hyperammonia could induce specific liver injury, while liver damage could elevate the concentration of blood ammonia.

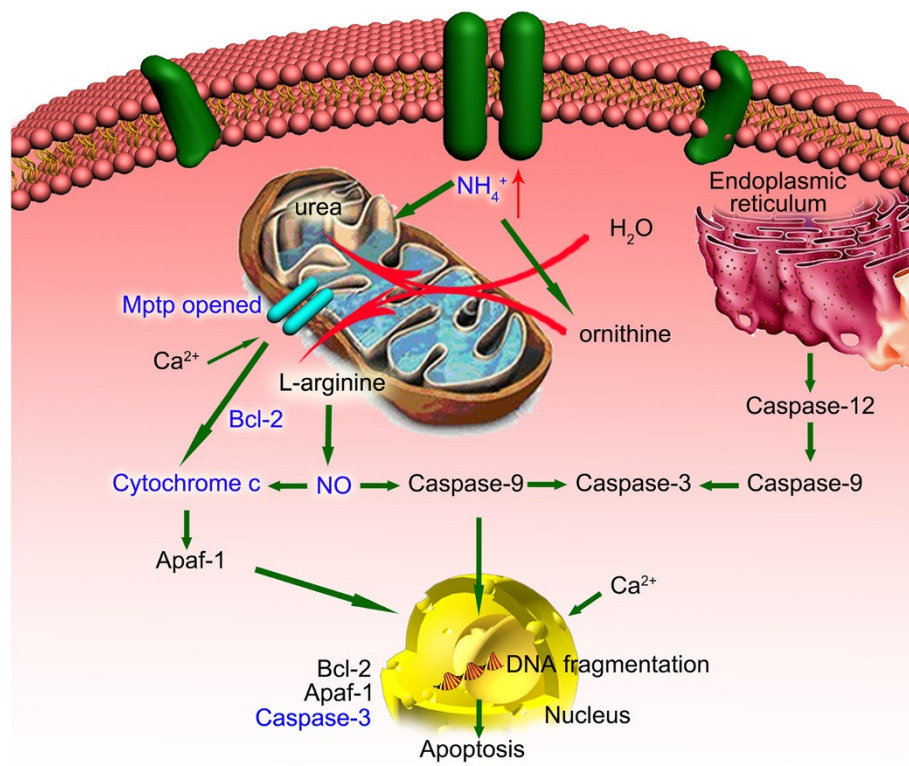
In agreement with previous work [20,21], our study demonstrated that NH<sub>4</sub>Cl induced liver damage, resulting in considerable cytotoxicity, and showed that the ALT and AST levels were increased because of the cytotoxicity against regenerating hepatocytes, which are biomarkers of pathological changes in the liver [22]. We also investigated the toxicity of ammonia to hepatocytes. With the increased concentration and time of ammonia exposed to hepatocytes, significant differences were found in the cell viability, indicating hepatocytes were sensitive to ammonia toxicity (Figure 2A and B). Many studies have focused on the neurotoxicity of ammonia, however, ammonia is not toxic to all cells. Hassel T et al. [23] found different cells show different growth inhibition when exposed to the same concentrations of ammonia. It suggested that the



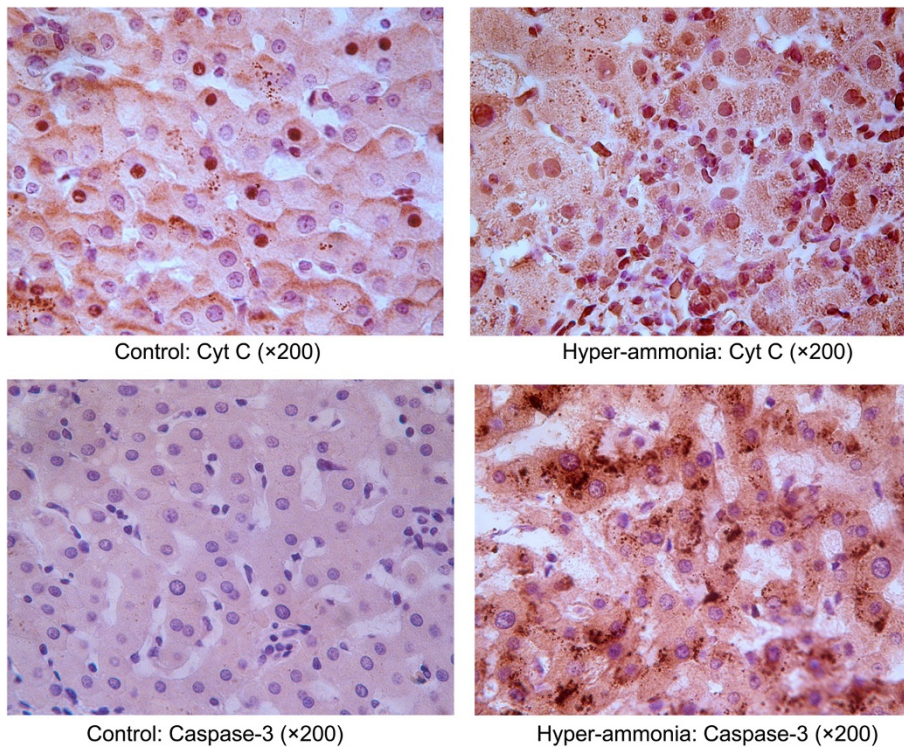
effect of ammonia on cells has significant cell specificity. Our study demonstrated only hepatocytes were inhibited, as evaluated by exposing various cell lines to  $\text{NH}_4\text{Cl}$ . Furthermore, the apoptosis rates in the hyperammonia group of primary hepatocytes were significantly higher than those in the control group, especially when the concentrations of  $\text{NH}_4\text{Cl}$  were 20 mmol/L. However, the apoptosis rates for hyperammonia of other cell lines showed no differences. To eliminate the influence of the pH value in our study, we adjusted the different  $\text{NH}_4\text{Cl}$  concentrations with dilute hydrochloric acid solution to the same pH values. We found that the pH value had no effects on the cytotoxicity and apoptosis (Figure 3B).

Investigators now believe that hyperammonia is a major factor in HE, which is associated with liver disease [24]. HE represents a broad continuum of neuropsychological dysfunctions in patients with acute or chronic liver disease. The pathophysiology of the disease is complex, which involves overproduction and reduced metabolism of various neurotoxins, particularly ammonia [25]. Currently, there are different theories about HE [26,27], and the majority of studies have focused on ammonia poisoning. In the present study, we found that hyperammonia could lead to different expression of some important genes, such as glucose transporter (GLUT-1), glycine transporter (GLYT-1),  $\text{Na}^+\text{-K}^+\text{-ATPase}$  [28,29]. However, in our





**Figure 7** Apoptotic pathway caused by the cytochrome.



**Figure 8** Expression of caspase-3 and Cyt C in hepatocytes.

previous study, we found that  $\text{Na}^+\text{-K}^+\text{-ATPase}$  showed no difference under hyperammonia conditions in gene chip assays, while arginine was decreased [30]. Furthermore, we found that the activity of  $\text{Na}^+\text{-K}^+\text{-ATPase}$  in hepatocytes under hyperammonia did not significantly differ from the control level, but it was decreased in astrocytes. Therefore, we consider that the mechanism of the liver damage induced by hyperammonia differs from that in HE.

To investigate the mechanism of the liver damage induced by hyperammonia, we measured the mitochondrial in hepatocytes. With increasing concentration of ammonia, the morphologic of mitochondrion began to be abnormalities (swelling and turn round, Crista disorder, vacuolar degeneration) (Figure 6A). To further examine the function of mitochondrion, we measured the mPTP by calcein fluorescence, we found that ammonia induced opening of mPTP after exposed to ammonia (Figure 6B). The mPTP, which represents the increase in the permeability of mitochondrion, is a protein pore which is formed in the inner membrane of the mitochondria under certain pathological conditions [31,32].

mPTP was opened when  $\text{Ca}^{2+}$  was transferred into the mitochondria and Cyt C was released [33-35]. To further confirm the hyperammonia affecting the dysfunction of mitochondrion in hepatocytes, we found that the cytotoxicity of  $\text{NH}_4\text{Cl}$  toward hepatocytes was significantly reduced when adding BAPTA/AM, which is an effective intracellular  $\text{Ca}^{2+}$  chelator [36]. However, the cytotoxicity was unchanged by adding EGTA, which can only bind to extracellular calcium. To confirm the results, we detected the expression levels of calmodulin and iNOS by real-time PCR and western blotting analyses. We found that both of these molecules were increased, especially for treatment with 10 mmol/L  $\text{NH}_4\text{Cl}$ . Moreover, we found arginine was decreased. It is well known that arginine is the direct precursor of nitric oxide (NO) and urea [37]. NO may be important as a signaling factor in many cells [38], and plays a role in apoptosis. A high concentration of NO, which is produced from iNOS (Figure 7), has been shown to suppress cell proliferation and induce cell apoptosis through  $\text{Ca}^{2+}$ /calmodulin-dependent pathway [39]. To validate this view, we detected the expression levels of calmodulin and iNOS by real-time PCR and western blotting analyses in hepatocytes under hyperammonia, and showed that both of these levels were increased.

At the present time, Cyt C is generally accepted as a key step in the apoptotic cascade [40,41]. The apoptosome recruits are considered to be the major enzymes in the commitment to apoptosis [42,43]. In our study, we found that the apoptosis rates in the hyperammonia group of primary hepatocytes were significantly increased. To further examine the apoptosis of cells, we detected the levels of caspase-3 and Cyt C by Real-time PCR and Western blot. We observed that both the Cyt C and caspase-3 levels were increased in ammonia treated group compared with the

control group (Figures 4 and 5A), indicating ammonia inducing apoptosis and liver damage in hepatocytes. Moreover, we found higher expression levels of caspase-3 and Cyt C in the tissues of acute liver failure patients (Figure 8).

## Conclusions

In summary, based on the model of liver cell damage resulting from  $\text{NH}_4\text{Cl}$ , the present study has revealed that increased  $\text{NH}_4\text{Cl}$  may intensify liver injury, in a manner which was specific for concentration and time. The mechanism possibly involves mitochondrial damage through activation of an intrinsic  $\text{Ca}^{2+}$ -independent apoptosis pathway. It appears that therapeutic approaches to inhibit the concentration of  $\text{NH}_4\text{Cl}$  and rebalance apoptosis might be efficacious in preventing  $\text{NH}_4\text{Cl}$ -induced liver damage.

## Competing interests

The authors declare that they have no competing interest.

## Authors' contributions

QCK contributed to in the conception, study design. JLL, 1) have made substantial contributions to acquisition of data, or analysis and interpretation of data; 2) have been involved in drafting the manuscript or revising it critically for important intellectual content; and 3) have given final approval of the version to be published. ZJY contributed to collection of test samples, interpretation of the data and critical review of the final manuscript version. QYW, DLL, BJ, YBZ, SS, YFW, SSL and LB provided purely technical help. YWY provided writing assistance. All authors read and approved the final version of the manuscript.

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