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Original Article

Ferric nitrilotriacetate (Fe-NTA)-induced reactive oxidative species protects human hepatic stellate cells from apoptosis by regulating Bcl-2 family proteins and mitochondrial membrane potential

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Abstract: Reactive oxidative species (ROS)-induced apoptosis of human hepatic stellate (HSC) is one of the treatments for liver fibrosis. However, how ROS (reactive oxygen species) affect HSC apoptosis and liver fibrosis is still unknown. In our study, ROS in human HSC cell line LX-2 was induced by ferric nitrilotriacetate (Fe-NTA) and assessed by superoxide dismutase (SOD) activity and methane dicarboxylic aldehyde (MDA) level. We found that in LX2 cells Fe-NTA induced notable ROS, which played a protective role in HSCs cells apoptosis by inhibiting Caspase-3 activation. Fe-NTA-induced ROS increased mRNA and protein level of anti-apoptosis Bcl-2 and decreased mRNA protein level of pro-apoptosis gene Bax, As a result, maintaining mitochondrial membrane potential of HSCs. Fe-NTA-induced ROS play a protective role in human HSCs by regulating Bcl-2 family proteins and mitochondrial membrane potential.

Keywords: Liver fibrosis, ferric nitrilotriacetate, reactive oxidative species, Bcl-2, Bax mitochondrial membrane potential, apoptosis

Introduction

Liver fibrosis could be induced by lots of pathogenic factors including virus, alcohol, obesity, diabetes, drugs and other metabolic abnormalities [1]. Regardless of different pathogenesis, ROS is always involved in liver fibrosis [2], by activating the conversion of HSC cells to myofibroblast-like cells. Subsequently, it will activate the production of extra cellular matrix (ECM), and inhibited the degradation of ECM [3]. Mitochondria are the largest source and the primary targets of ROS. Today, there is no curative treatment for liver fibrosis, however, the ROS-induced apoptosis of HSCs may be one of the potential treatments [4]. Currently, antioxidants agents are widely used in liver diseases, including liver fibrosis. However, how antioxidants agents work is still need to be elucidated. In the study, we found Fe-NTA could induce ROS in human HSC cell lines, regulate mRNA expression of Bcl-2 family proteins, maintain mitochondrial membrane potential, and finally, protect cell apoptosis.

Materials and methods

Cell lines, culture and reagents

Human activated HSC cell line, LX-2, was provided by Prof. Friedman. Human hepatocyte cell lines, Chang liver cells, were obtained from Cell Resource Center, Shanghai Institutes of Biological Sciences, Chinese Academy of Sciences (Shanghai, China). High-sugar Dulbecco's modified Eagle's medium (DMEM) and PRMI-1640 medium were both purchased from Gibco (Gibcol BRL, CA). Fetal bovine serum was from Hyclone (Logan, Utah), calf serum was from Beijing Dingguo Biotechnology Co., Ltd (Beijing, China), trypsin was from Sigma (St. Louis, USA). Malondialdehyde (MDA) Assay Kit for cell cul-

ture supernatant was purchased from Nanjing Jiancheng Bioengineering Institute (Nangjing, China). Superoxide dismutase (SOD) Assay Kit-WST for cell lysis was obtained from Dojindo Laboratories (Kumamoto, Japan). Mito-CaptureTM Mitochondrial Apoptosis Detection Kit and Caspase-3/CPP32 Assay Kit (Colorimetric) was from BioVision (CA, USA). Annexin V-fluorescein isothiocyanate (FITC) + propidium iodide (PI) Apoptosis Detection Kit was from Beijing Biosea Biotechnology Co., LTD (Beijing, China), Trizol reagent was from Invitrogen (Carlsbad, CA) and SYBR Green Realtime PCR Kit was from Toyobo Life Science Department (Osaka, Japan). Oligo dT 18 and 10 mM dNTP were purchased from Takara Bio Inc. (Otsu, Japan). M-MLV reverse transcriptase and RNasin were obtained from Promega (Madison USA). Bcl-2 mouse anti-human monoclonal antibody and Bax rabbit anti-human monoclonal antibody were from Santa Cruz (Santa Cruz, USA). Fe-NTA was prepared using a mix of 0.1 mol/L $FeNO_3$ (Sigma, USA) and 0.1 mol/L Na_2 NAC (Fluka, USA) adjusted to pH 7.4 with $NaHCO_3$ and then, filtered with 0.22 μmol/L millipore filter.

ROS induction and measurement

The LX-2 cells and Chang liver cells in the logarithmic phase were suspended in DMEM with 10% fetal bovine serum or PRMI-1640 with 10% calf serum at 2.5×10^5 viable cells/ml. Both cells were seeded into 25 cm^2 cell culture dishes and incubated at 37°C and 5% CO_2 for 24 hours. After 24 hours, cell medium was replaced with fresh DMEM or PRMI-1640 with Fe-NTA at different concentration (0, 0.5, 1.0, 1.5 mmol/L) for another 24 hours. After treatment, the supernatant from each group was collected for MDA assay. Then, cells were washed with PBS for 2 times, then scrapped and lysised. The cell lysis was collected for SOD assay with SOD activity analysis kit. Both experiments were replicated for three times.

Cell apoptosis assay

After Fe-NTA treatment for 24 hours, LX2 cells and Chang liver cells were trypsinized with trypsin and centrifuged at 4°C and 500 g for 10 min. Then, cells were washed with PBS twice. Harvested cells were re-suspended in 200 μl binding buffer containing 10 μl Annexin V-FITC and 5 μl PI, and incubated for 30 min at room temperature. After incubation, apoptosis was analyzed with flow cytometry. The experiment above was replicated for three times.

Caspase-3 activity assay

After Fe-NTA treatment, cells were trypsinized, collected and lysised with 50 μl iced lysis buffer for 30 min with vibration per 5 min. The lysis was centrifuged at 10000 g for 1 min and the supernatant was collected into another tube. Caspase-3 substrate (DEVD-*p*NA) was added and reacted at 37°C for 2 h. Enzyme-linked immunoassay was applied to measure optical density (OD) of the sample at 405 nm. The multiple change of Caspase-3 activity of each treated group relative to that of control group = (OD of each sample-OD of background)/OD of control group.

Mitochondrial membrane potential

After Fe-NTA treatment, cells were trypsinized and suspended in 1 ml complete medium with 1‰ MitoCapture for 15 min at 37°C. Cells were washed and re-suspended in 1 ml preheating incubation buffer, mitochondrial membrane potential was determined with flow cytometry. The experiment was repeated for three times.

Bcl-2 and Bax mRNA expression

Total RNA of cells was extracted and the purification and concentration were measured by ultraviolet spectrophotometer (RNA A_{260}/A_{280} > 1.8). The specific primers for Bcl-2 and Bax were designed according to gene sequences of GenBank using Primer 5.0 software. The primers were synthesized by Sangon Biotech (Shanghai, China). Harvested RNA was reverse-transcribed to cDNA and subsequently, PCR was performed with cDNA templates. According to the instruction of SYBR Green Real-time PCR Kit, the amplification of 25 l was applied. Successively, 12.5 l of real-time PCR reagents, 0.5 l of up-stream primers, 0.5 l of down-stream primers, 2.5 l of cDNA was added into PCR tube with DEPC (diethyl pyrocarbonate) added up to 25 l. The amplification conditions were the following: at 95°C for 60 s, at 95°C for 15 s, at 60°C for 60 s followed by 40 cycles in total. The results were processed with relative quantification, namely the ratio of gene expression of treated groups to control group with β-actin as

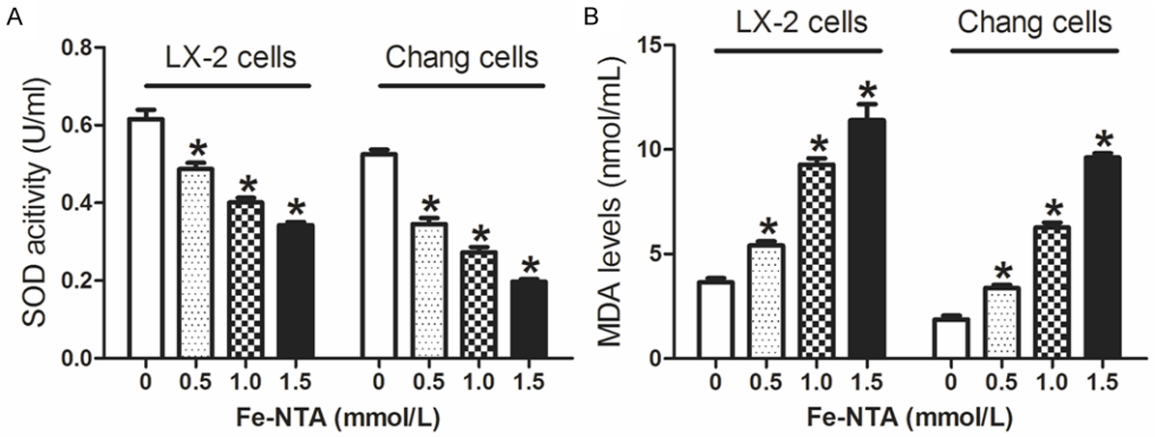

Figure 1. Fe-NTA-induced ROS in LX-2 and Chang liver cells. LX-2 and Chang liver cells were treated with Fe-NTA at indicated concentration for 24 hours, SOD activity and MDA level were measured. A. SOD activity was significantly decreased after treatment in both cell lines. B. MDA level was significantly increased after treatment in both cell lines (*P < 0.05, compared to control group).

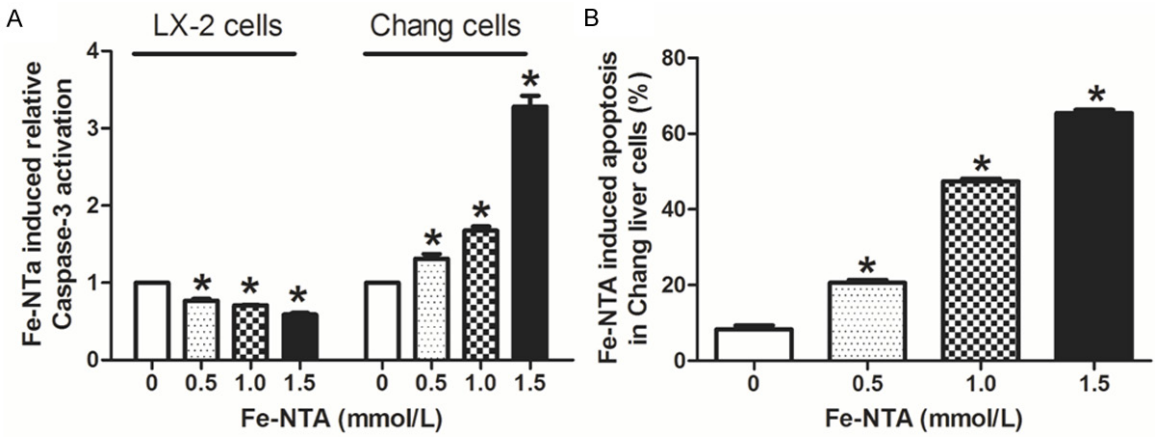

Figure 2. Fe-NTA-induced ROS inhibit Caspase-3 in LX-2 cells. LX-2 and Chang liver cells were treated with Fe-NTA at indicated concentration for 24 hours. Caspase-3 activity and cell apoptosis were measured. A. Compared to control group, Fe-NTA significantly inhibited Casepase-3 activity in LX2 cells, but enhanced Casepase-3 activity in Chang liver cells. B. Fe-NTA significantly induced cell apoptosis in Chang liver cells by Annexin V-FITC/PI assay (*P < 0.05, compared to control group).

internal reference and control group as benchmark.

Western blot assay

After Fe-NTA treatment, cell lysates were prepared for western blot (WB), using antibodies against Bcl-2 (1:3000), Bax (1:3000) and glyceraldehyde-phosphate dehydrogenase (GAPDH, 1:10000) [5].

Statistical analysis

Statistical analysis was performed with the software package SAS Version 6.12. Data were expressed as means ± standard deviation. Comparisons between groups were conducted with one-way analysis of variance (ANOVA). *P* value of less than 0.05 was regarded as statistically significant.

Results

Fe-NTA-induced ROS in both cell lines

To evaluate Fe-NTA-induced ROS, LX-2 hepatic stellate cells and Chang liver cells was administrated with Fe-NTA at indicated concentrations. As shown in **Figure 1A**, Fe-NTA significantly

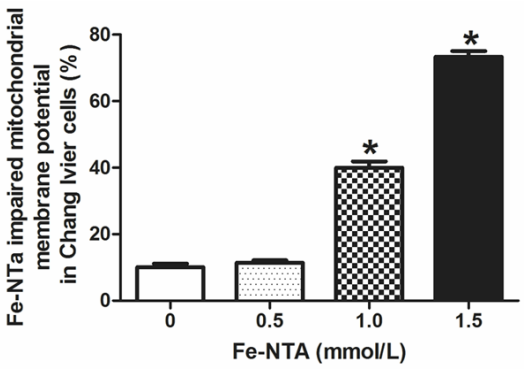

Figure 3. Fe-NTA-induced ROS impaired MMP Chang liver cell, but LX2 cells. LX-2 and Chang liver cells were treated with Fe-NTA at indicated concentration for 24 hours. MMP was measured with JC-1 staining and FACS analyzing. Fe-NTA 1.0 mmol/L or 1.5 mmol/L significantly increased cell population with collapsed MMP (*P < 0.05, compared to control group).

decreased SOD activity in LX2 cells. In control group, the SOD activity is 0.616±0.024 U/ml in LX-2 cells, however, in Fe-NTA (0.5 mmol/L, 1.0 mmol/L, 1.5 mmol/L) treated group, the SOD activity was 0.487±0.016, 0.401±0.012, 0.343±0.008 U/ml respectively ($P < 0.05$). Similarly, Fe-NTA significantly decreased SOD activity in Chang liver cells. In control group, the SOD activity is s 0.525±0.012 U/ml in Chang liver cells, however, in Fe-NTA (0.5 mmol/L, 1.0 mmol/L, 1.5 mmol/L) treated group, the SOD activity was 0.346±0.015, 0.273±0.013, 0.198±0.006 U/ml respectively ($P < 0.05$). In contrast, Fe-NTA significantly increased MDA level in both cell lines (as shown in **Figure 1B**).

Fe-NTA-induced ROS and Caspase-3 activation, cell apoptosis

Although Fe-NTA induced ROS in both cell lines, it totally unknown whether ROS involved in cell apoptosis. Caspase-3 activation is marker of cell apoptosis. As shown in **Figure 2A**, Fe-NTA significantly depressed Caspase-3 activity in LX2. Compared to control group, Caspase-3 activity in different concentration Fe-NTA groups were 0.767, 0.710 and 0.588 respectively ($P < 0.05$). However, Fe-NTA treatment for 24 hours did not decrease cell apoptosis in LX2 cells by FACS assay (data not shown, $P > 0.05$). On the contrary, Fe-NTA significantly escalated Caspase-3 activity and apoptosis in Chang liver cells. After Fe-NTA treatment, relative Casepase-3 activity raised to 1.312, 1.674 and 3.281 respectively (**Figure 2A**, $P < 0.05$). Likewise, apoptosis detected by Annexin V-FITC/PI raised from 8.333 ± 1.050% to 20.6±0.800%, 47.4±0.702%, 65.4±0.900% respectively after treatment (**Figure 2B**, $P < 0.05$). In summary, Fe-NTA-induced ROS inhibit Caspase-3 activation in HSC, but promote apoptosis in hepatocytes.

Effect of ROS on mitochondrial membrane potential (MMP)

Mitochondrial with normal potential will absorb fluorescent dye JC-1, making it renders in the mitochondria showing red fluorescence. When the mitochondrial membrane potential is impaired, JC-1 dye would disperse in the cytoplasm showing green fluorescence. Cells with fluoresce green or red can be distinguished by flow cytometry. We found that, Chang liver cells with green fluorescence increased notably after Fe-NTA treatment. The proportion of cell with green fluoresce increased 40.03% and 73.33% in Fe-NTA 1.0 mmol/L and 1.5 mm group, compared to control group 10.11% (**Figure 3**, P < 0.05). Quite the opposite, Fe-NTA failed to arouse MMP in LX2 cells. There is no difference of cell with green fluorescence between the treated groups and the control group in LX2 cells (data not shown, P > 0.05). It is demonstrated that Fe-NTA could decrease MMP in Chang liver cells, but not in LX2 cells.

Detection of Bcl-2 and Bax expression

To further explored how Fe-NTA decrease Caspase-3 activation in LX2 cells, Real-time PCR was used to detected Bcl-2 and Bax mRNA expression. PCR data showed that Fe-NTA could increase anti-apoptotic gene Bcl-2 mRNA expression levels and decrease pro-apoptotic gene Bax mRNA expression levels (**Figure 4A**). Western blot demonstrated similar results, Bcl-2 Protein levels increased after Fe-NTA treatment. In Fe-NTA groups, Bcl-2 and GAPDH gray value ratios were 0.529, 0.660, 0.826 respectively, compared to 0.350 in control group. Bax Protein levels decreased after Fe-NTA treatment. In Fe-NTA groups, Bax and GAPDH gray value ratios were 0.892, 0.967, 0.729 respectively, compared to 1.145 in control group (**Figure 4C** and **4D**, $P < 0.05$). Conversely, in Chang liver cells, Fe-NTA could reduce anti-apoptotic gene bcl-2 mRNA and

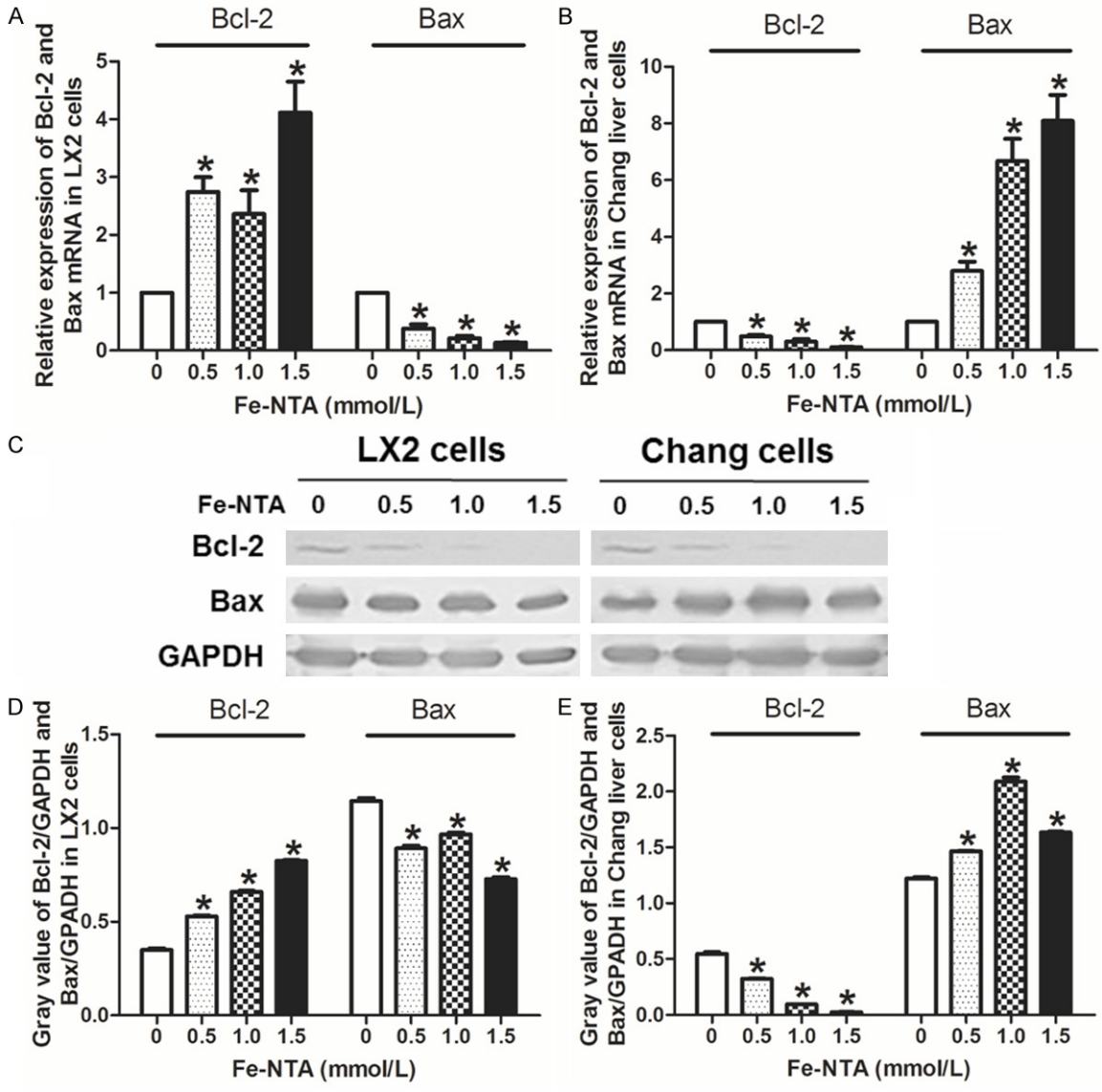

Figure 4. Fe-NTA-induced ROS effect cell apoptosis by regulating Bcl-2 and Bax. LX-2 and Chang liver cells were treated with Fe-NTA at indicated concentration for 24 hours. A. Fe-NTA significantly increased Bcl-2 mRNA level, as well as decreased Bax mRNA level in LX2 cells. B. Fe-NTA significantly decreased Bcl-2 mRNA level, as well as increased Bax mRNA level in Chang liver cells. C and D. Fe-NTA significantly increased Bcl-2 protein level, as well as decreased Bax protein level in LX2 cells. C and E. Fe-NTA significantly decreased Bcl-2 protein level, as well as increased Bax protein level in Chang liver cells.

protein levels and increase pre-apoptotic gene Bax mRNA expression levels (**Figure 4B**); Protein levels were found that expression of bcl-2 was reduced, bcl-2 mRNA and protein levels (**Figure 4E**).

Discussion

The reversibility of liver fibrosis and cirrhosis has been reported previously [6]. Liver fibrosis can be alleviated by inhibiting inflammatory factors or by inducing apoptosis of activated HSC [4]. HSC apoptosis is also discovered in animal models of liver injury. Activated HSC could promote ECM degradation by two ways [7, 8]. On one hand, the activated HSC secreted less tissue inhibitor of matrix metalloproteinase (TIMP). TIMP inhibited the degradation of collagen. On the other hand, activated HSCs produced less ECM. Therefore, it is important for inducing apoptosis of activated HSC, in the prevention and treatment of liver fibrosis [9].

Recently, lots of factors were reported to induce HSC apoptosis in rats, including fas ligand [10], endoplasmic reticulum stress [11], tumor necrosis factor (TNF)-related apoptosis ligand [12] and activated Kupffer cells [13]. ROS is well known to involve in liver fibrosis [14]. However, the relationship between ROS and HSC apoptosis is still unknown [15]. In this study, Fe-NTA-induced ROS model was established to investigate the relationship between ROS and HSC apoptosis. We found Fe-NTA could induce ROS in HSC cell lines LX2 and protect LX2 cells from apoptosis. Mechanism study demonstrate that Fe-NTA-induced ROS might increase the expression of anti-apoptosis protein Bcl-2 and decrease the expression of pro-apoptosis protein Bax, as a result, maintain MMP and help LX2 cell survive.

ROS model was established using an iron load method. Cells were treated with Fe-NTA for 24 hours [16]. The SOD activity and MDA level were measured as ROS indicator. Results showed that compared with control group, SOD activity decreased in Fe-NTA treated group, while MDA level increased in Fe-NTA treated group. Fe-NTA induced ROS in both LX2 cells and Chang liver cells in a dose-dependent manner.

ROS has different impact on apoptosis through different pathways. In some circumstances, ROS plays an anti-apoptotic role. ROS could inhibit Caspase activity by ROS, inhibit NF-B activation and increase Bcl-2 expression. Nevertheless, excessive ROS could directly induce cell death. So, ROS is a double-edged sword for cell death [17, 18]. In our study, Fe-NTA could induce cell apoptosis in Chang liver cells, but not in LX2 cells. Similarly, Novo *et al* also found that ROS could not lead to the apoptosis of fully activated human HSCs [19]. However, further studies were needed to determine whether the contradictory result is due to different cell lines or different ROS level [20].

Mitochondria is not only the major organelle for ROS production, but also the main target of ROS. On the other hand, Mitochondria also play an important role in the initiation of intrinsic cell apoptosis [21]. In the intrinsic apoptosis pathway, mitochondrial membrane potential was firstly impaired, and cytochrome C was released, then Caspase-9 and Caspase-3 was activated, cell apoptosis is set off. Once the mitochondrial membrane potential is collapsed then apoptosis will be irreversible [22]. We found that Fe-NTA at different concentrations failed to impair MMP of LX2 cells. The result illustrated that ROS might inhibit MMP collapse and impeded the initiation of HSC apoptosis. Further study found that Fe-NTA induced ROS down-regulated intracellular caspase-3 activity, compared to the control group, showing a dose-dependent manner. This data suggested ROS might inhibit caspase-3 activity in HSC. In summary, Fe-NTA-induced ROS may play an anti-apoptosis role in HSC, which might increase ECM and accelerate liver fibrosis. In contrast, Fe-NTA induced oxygen stress could significantly decrease MMP, activate Caspase-3, thereby initiating cell apoptosis in liver cells.

To understand the downstream events of oxygen stress effects on apoptosis, we further detect Bcl-2 family protein expression. Bcl-2 family is composed of two kinds of proteins: anti-apoptosis proteins and pro-apoptosis proteins. Bcl-2 protein, an anti-apoptosis protein, is mainly distributed in the mitochondrial membrane and cytoplasm, blocking all early signs of apoptosis, including MMP reduction and Caspase family proteins activation. Conversely, Bax, a pro-apoptosis protein, is distributed in the cytoplasm, promoting several apoptosis process [23]. It was found that Bcl-2 gene silencing cells were more sensitive to TNF-induced apoptosis. Bcl-2 expression in HSC was significantly increased [19] in patients with hepatitis C related liver cirrhosis, suggesting that Bcl-2 may be an anti-apoptotic proteins, which help HSC involved in fibrosis and cirrhosis.

Our study found that Fe-NTA induced ROS could increase mRNA and protein levels of Bcl-2, while increase mRNA and protein levels of Bax in HSC. However, Fe-NTA-induced ROS play an opposite role on Bcl-2 and Bax in human liver. Therefore, we considered that ROS could maintain MMP in HSC but decline MMP in liver cells by regulating mitochondrial apoptosis pathway Bcl-2 family proteins.

To sum up, our study found that Fe-NTA induced ROS played anti-apoptotic role in human HSC. Increased ROS regulated Bcl-2 family proteins and maintained MMP, then inhibited caspase-3 activation. Thus, we hypothesized that antioxidants agents could reduce ROS in both cell

lines, and promote apoptosis in HSC, as well as prevent apoptosis in hepatocytes by regulating bcl-2 family proteins and MMP. This study provided a piece of evidence that antioxidant agents could be a potential treatment for liver fibrosis.

Disclosure of conflict of interest

None.

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