Original Article
Abate Cytochrome C induced apoptosome to protect donor liver against ischemia reperfusion injury on rat liver transplantation model

Zhuonan Zhuang1*, Peilong Lian2*, Xiaojuan Wu3*, Baoxu Shi4, Maoyou Zhuang5, Ruiling Zhou6, Rui Zhao2, Zhen Zhao2, Sen Guo2, Zhipeng Ji7, Kesen Xu2

1Department of General Surgery, Beijing Tsinghua Changgung Hospital Medical Center, Tsinghua University, Beijing 102218, China; 2Department of Hepatobiliary Surgery, Qilu Hospital, Shandong University, Jinan 250012, China; 3Department of Nephrology, Huai’an Hospital Affiliated to Xuzhou Medical College and Huai’an Second Hospital, Huai’an 223002, P.R. China; 4Department of Neurology, People’s Hospital of Rizhaolanshan, Rizhao 276800, China; Departments of 5Neurology, 6Clinical Lab, Rizhao First People Hospital, Rizhao 276800, China; 7Department of General Surgery, The Second Affiliated Hospital of Shandong University, Jinan 250033, China.

*Equal contributions.

Received December 16, 2015; Accepted February 15, 2016; Epub April 15, 2016; Published April 30, 2016

Abstract: Objective: Aim of this study is to protect donor liver against ischemia-reperfusion injury by abating Cytochrome C induced apoptosome on rat model. Methods: A total of 25 clean SD inbred male rats were used in this research. The rats in ischemia-reperfusion injury group (I/R group, n=5) were under liver transplantation operation; rats in dichloroacetate diisopropylamine group (DADA group, n=5) were treated DADA before liver transplantation; control group (Ctrl group, n=5); other 10 rats were used to offer donor livers. Results: In DADA therapy group, Cytochrome C expression in donor hepatocellular cytoplasm was detected lower than that in I/R group. And the Cytochrome C induced apoptosome was also decreased in accord with the lower expressions of Apaf-1 and Caspase3. Low level of cleaved PARP expression revealed less apoptosis in liver tissue. The morphology of donor liver mitochondria in DADA group was observed to be slightly edema but less than I/R group after operation 12 h. The liver function indexes of ALT and AST in serum were tested, and the results in DADA group showed it is significantly lower than I/R group after operation 12 h. The inflammation indexes of IL-6 and TNF-α expressions in DADA group were significantly lower than that in I/R group after operation 24 h. Conclusion: The dichloroacetate diisopropylamine treatment could protect the hepatocellular mitochondria in case of the spillage of Cytochrome C induced apoptosome, and protect the liver against ischemia-reperfusion injury. Thus, it may be a method to promote the recovery of donor liver function after transplantation.

Keywords: Liver transplantation, Cytochrome C, apoptosome, ischemia-reperfusion injury

Introduction

In liver transplantation, the function recovery of donor liver is one of the important evaluation indexes for the surgical success rate and survival in patients [1]. Liver function damage degree influence the prognosis of disease [2]. Hepatic ischemia/reperfusion (I/R) injury often occurs in the liver surgery, severe I/R injury could lead to liver failure [3]. Oxygen free radical, generated by ischemia-reperfusion injury, is a kind of unstable oxygen containing a single unpaired electron in the outer electrons orbit [4]. Oxygen free radicals can produce a variety of highly toxic lipid peroxides to direct damage the cells [5]. More reactive oxygen species which induce the fat mass accumulation and oxidative stress could lead to the mitochondrial dysfunction [6]. The formation of mitochondrial dysfunction included mitochondrial membrane, ATP depletion and necrosis lead to liver cell apoptosis which is easy to affect the liver function. Cytochrome C is a critical enzyme on electron transfer of biological oxidation to participate in the process of cellular respiration [7]. It is the key event of Cytochrome C release to lock in the process of cell apoptosis [8]. All of these endue the double roles to the Cytochrome C to adjust the cellular energy metabolism and apoptosis [9]. Once Cytochrome C was released into the cytoplasm in according to the mitochondria...
damaged, it can produce a high molecular weight complex consisted of Apaf-1, Cytochrome C and Caspase-9 [10]. This compounds were directly activate Caspase-3, a kind of practitioners to induce apoptosis [11]. Previous study has found that the clinical application of dichloroacetic diisopropylamine (DADA) treatment of various acute liver injury has better curative effect. The active ingredient of DADA is the gametes acid (vitamin B15), which has been showed to promote liver regeneration and resisting fatty liver in the clinical study [12]. And it is mainly used to improve liver function in the mechanism of action, inhibits pyruvate dehydrogenase kinase and increase oxygen intake to liver cells, so as to improve the energy metabolism of liver cell and so on [13]. The metabolites of DADA in organism is mainly include of diisopropylamine and glycine [14]. Under the function of glycine lyase, the glycine could cleavage into methyl folinic acid glycine, which can provide the energy to liver cell metabolism, and increase glucose oxidation to promote the Kreb’s cycle [14]. Therefore, DADA treatment can raise the utilization ratio of tissue cells in the respiratory function and oxygen to repair the damaged liver cells [13]. It could create a kind of conditions for the liver functional recovery by adjusting the energy balance in liver.

In this study, we introduced DADA treatment to protect the hepatocellular function against I/R injury on the rat liver transplantation model. It was more effect of hepatocellular mitochondria to resist the hypoxia condition after DADA therapy. In liver transplantation, we detected that the expression of apoptosome induced by Cytochrome C had been decreased during the liver ischemia reperfusion procedure.

Materials and methods

Material and reagent

The microsurgical operation instrument and the noninvasive vascular clamp were purchased from Kaiji biology company. Slicing machine was purchased from Reichert HistoSTAT, US. Incubator was purchased from Shanghai laboratory equipment company. Japanese Nikon/E200 microscope. Hitachi HT7700 transmission electron microscope. Diisopropylamine dichloroacetate, (DADA) was purchased from dandong medical and pharmaceutical industry, China.

Experimental animals

The Ethics Committee of Shandong University approved the study. 25 clean SD inbred male rats were offered by the Experimental Animal Center of Jiangsu Province. The age of all rats were selected from 8 to 10 weeks, and the body weight of rats were 240–300 g (275 g on average). Rats were randomly divided into three groups: I/R group, the rats just underwent the liver transplantation (n=10, including 5 rats provided donor liver); DADA group, the donor rats and the recipients were both treated with dichloroacetic diisopropylamine (10 mg/kg) for 3 days before operation by intraperitoneal injection (n=10, including 5 rats provided donor liver); Ctrl group: normal control group (n=5). Preoperative fasting 12 h before Rats transplantation, free drinking water.

Animal model

Donor animal model: 0.3% pentobarbital sodium was given to the rat to make anesthesia by intraperitoneal injection with the dose of 1 mL/100 g. And we used an inhalation half-open mask to maintain anesthesia during the operation. And the rats were injected by atropine 0.03 mg in muscle. After anesthesia, the donor rats were made an abdominal incision step by step, and then bared the liver by freeing the ligaments around liver. Modified the liver tissue to make sure to keep 0.4-0.5 cm superior vena cava (SVC), 0.6-0.7 cm inferior vena cava (IVC), around 0.5 cm portal vein (PV) and 0.4-0.5 cm common bile duct (CBC). We ligated the hepatic artery (HA), and inset one scaffolds separately into portal vein and common bile duct of donor liver with 1-2 mm diameter plastic pipe. The irrigation of donor liver was using 4°C Riger’s solution which was dripped into portal vein for 5 min till the color of donor liver appeared to grayish yellow in our experiment. The liver gradually turned yellow if the reperfusion was successful. Then keep the donor liver immerse into 4°C Riger’s solution.

Recipient animal model: Preoperational preparation was as the same as the procedure of donor animal model. After anesthesia, the recipient were made an abdominal incision step by step, and then bared the liver by freeing the ligaments around liver. Free and clamp the superior vena cava, inferior vena cava, portal vein hepatic artery and common bile duct by vascular clamps, and then carefully remove the
recipient liver. We continuous sutured the PV and IVC by 8-0 sutures, then unclamped the SVC and IVC to recovery the blood of vena cava. We adjust the position of diameter plastic pipes which located in portal vein and common bile duct of donor liver and connected them with the portal vein and common bile duct of recipient. Recovery the blood circulation of donor liver and checked that the color of liver gradually turned red. The rats were injected 1.6 million units of penicillin in abdominal cavity and were perfused with 4 mL Riger’s solution through the vein of abdominal wall before finishing the operation. Finally, we could stitch up its abdomen continuously. The rats of AT group only underwent the liver transplantation operation. The standard of a successful operation was that the post-operation rat could immediately regain consciousness, stood up and maintained effective breathing and heart beat [15].

**Reverse transcription (RT)-PCR analysis**

The samples of liver tissues were saved at -80°C until completely dissolved. RNA samples were diluted 100 times or appropriate multiples, and measured in 260 nm and 280 nm absorption values. RNA's concentration = OD 260 × dilution factor × 0.04 μg/μL. The range of OD260/280 is 1.8 to 2.1 as the high purity of RNA was extracted. Add non-nucleic acid enzyme with RNA, Oligo dT (18), nuclease-free double-distilled water in turns to total volume. Then mixed RNase inhibitor, Reaction Buffer, dNTPs, DTT (1 M), reverse transcriptase (AMV), and nuclease-free double-distilled water into the PCR tubes in turns. After reaction of cDNA, the samples were proceeding the amplification which the conditions is: Degenerated 30 s in 95°C, annealed 30 s in 55°C, extended to 35 s in 72°C. A total of 32 cycles were done. Primer sequences were shown below: Cytochrome C: (Forward primer: 5'-GCACTTTAATTGGAGATGATCAAA-3'; Reverse primer: 5'-CATGTGCTGAACAATCACATTGT-3').

**Western blot analysis**

Cytochrome C, Apaf-1, Caspase 9 primary antibodies (Boshide Biotechnology Company, Wuhan, China) were diluted at 1:500. Cleaved-caspase-3, and PARP primary antibodies (Cell Signaling Technology company, US) were diluted at 1:500. The cell lysates from liver tissue
were lysed in radioimmuno-precipitation assay (RIPA) protein lysate buffer. α-Tublin and β-actin were purchased from Abcam Biotechnology Company. The cytoplasm was centrifuged by 750 g, 4°C, 5 min, then collected the supernatant and centrifuged 13000 rpm, 4°C, 30 min to collect the sediment. The mitochondria protein were released by lysis of mitochondria. A total of 30 μg of protein extracts were loaded and run the gel, then transferred to nylon membranes (Immobilon-P, Millipore, Bedford, MA) for 1 h on 300 mA followed by blocking with 5% non-fat dry milk in to 0.1% TBST for 1 h. PVDF membranes were incubated with primary antibodies overnight at 4°C.

**ELISA analysis**

The blood samples of rats were collected after operation, and centrifuged it to separate the serum and stored at -80°C. All the samples were detected by ELISA assay kits which were bought from Kaiji biology Inc, (Nanjing, China). Alanine aminotransferase (ALT), aspartate aminotransferase (AST), interleukin 6 and anti-rat tumor necrosis factor (TNF-α) rat ELISA kit were
Abate Cytochrome C induced apoptosome against ischemia reperfusion injury

Figure 3. Dichloroacetate diisopropylamine decreased Cytochrome C-induced Caspase3 and PARP expressions to reduce the apoptosis in liver tissue after operation 12 h. A. RT-PCR assay showed the relative mRNA expression level of Cytochrome C was decreased after DADA treatment compared with I/R group (Cytochrome C: DADA v.s. I/R, Independent T test, \( P=0.0149 \)). B. Western blot assay detected the level of Cytochrome C in mitochondria protein of I/R group was lower than that in DADA group and control group after operation 12 h. The level of Cytochrome C in cytoplasm protein of I/R group was higher than that in DADA group and control group. C-F. Apoptosis in liver tissue detected by western blot assay showed that the expressions of cleaved-Caspase3 and cleaved PARP were both decreased in DADA group compared with that in I/R group (cleaved Caspase3: DADA v.s. I/R, Independent T test, \( P=0.0009 \); cleaved PARP: DADA v.s. I/R, Independent T test, \( P<0.0001 \)).

purchased and tested in Kaiji biology company, Nanjing, China.

**Histological and TUNEL assay**

The liver tissues of rats were fixed by formalin. Tissue sections were embedded by paraffin and proceeded the immunostaining procedure by using the streptavidin-peroxidase technique.

Hematoxylin and eosin (H&E) were conducted according to standard procedures. TUNEL Assay: The slices of liver tissue were stained by TUNEL assay with Situ Cell Death Detection Kit, Fluorescein (Roche Diagnostics, Indianapolis, IN, USA) to evaluate induction of apoptosis. The number of TUNEL-positive cells was counted and photographed.
**Statistical analysis**

All data in this experiment were statistical analyzed by SPSS 13.0 software (Inc. of Chicago, IL, US) and GraphPad Prism software (Inc. La Jolla, CA, US). Independent T test was used for statistical analyzing the difference of value between groups. \( P < 0.05 \) was considered to have statistically significant.

---

**Results**

*I/R injury promoted Cytochrome C induced apoptosome to be more expression in donor liver tissue*

During the liver transplantation, donor liver tissue was suffered a series of ischemia/reperfusion injury. According to the irrigation of donor liver tissue...
Abate Cytochrome C induced apoptosome against ischemia reperfusion injury

Liver with Riger’s solution, the hepatocellular mitochondria were stayed at a status of hypoxia condition. While the blood recirculation of donor liver was recovery (Figure 1A), the mitochondria with hypoxia condition should suffer a reperfusion injury from oxygen free radical accumulation. We observed that morphology of mitochondria after I/R procedure were dramatically swollen. Instead of a clear and whole structure of crest, the injured mitochondria appeared a damage formation with reduced, disrupted or disappeared crest (Figure 1B). The cleaved Caspase3 was found to be more expression in hepatocellular cytoplasm (Figure 1C & 1D). Cytochrome C was an critical enzyme which could activate the apoptosis procedure and could be increased during I/R injury (Figure 2A-C). Hypoxia-induced Cytochrome C could combine with Apaf-1 and Caspase3 to format the apoptosome in cytoplasm. So we could observe the expression of Apaf-1 was increased in cytoplasm after I/R injury (Figure 2D, 2E). The apoptosome played an important role in mitochondria damage and hepatocellular apoptosis.

Dichloroacetate diisopropylamine protect the mitochondria against I/R injury and decreased cytochrome C-induced apoptosis procedure in liver tissue

The rat donor liver tissue could be benefit from dichloroacetate diisopropylamine treatment and gain the ability to resist the injury of hypoxia after operation. We analyzed the relative mRNA expression levels of Cytochrome C in donor liver tissue and found that it was down-regulated after DADA treatment compared with I/R group (Figure 3A, 3B). With dichloroacetate diisopropylamine treatment, the protein expressions of cleaved-Caspase3 and cleaved PARP

Figure 5. The changes of liver serum function and inflammation factors after rat liver transplantation. A, B. The expressions of serum ALT and AST stayed a high level after operation 6 h, 12 h and 24 h compared with non-operation group. At 12 h after operation, the serum ALT and AST of DADA group were lower than that in I/R group (ALT: DADA v.s. I/R, 12 h, Independent T test, P=0.0060; AST: DADA v.s. I/R, 12 h, Independent T test, P=0.0489). C, D. The levels of serum IL-6 and TNF-α kept increasing after operation 6 h both in two operation groups. At 24 h after operation, the serum of IL-6 and TNF-α of DADA group was significantly lower than that in I/R group (IL-6: DADA v.s. I/R, 24 h, Independent T test, P=0.0074; TNF-α: DADA v.s. I/R, 24 h, Independent T test, P=0.0166).
were found to be both in a low level compared with that in I/R group (Figure 3C-F).

**Morphology changes of rat donor liver tissue after liver transplantation**

Under the dichloroacetate diisopropylamine treatment, the pathology characteristic of the rat donor liver tissue by H.E. staining showed that the liver blood sinus gap still be observed, and liver cells were slightly edema (Figure 4Ac); While in I/R group, the liver tissue was found to be dramatically edema with narrow liver blood sinus and erythrocyte sedimentation (Figure 4Ab). Dichloroacetate diisopropylamine treatment also could protect the hepatocellular mitochondria within a less injury compared with I/R group, accompanied by markedly swollen mitochondria morphology sizes, with some fault, rupture or disappeared mitochondria crest (Figure 4Bc). With less mitochondria damage, more liver cells could resist I/R injury and against the apoptosis. In DADA group, we could observe less apoptosis signal in liver tissue compared with that in I/R group by Tunnel assay (Figure 4Cb).

**The changes of liver serum function and inflammation factors after rat liver transplantation**

The liver serum function and inflammation factors were the important indication for the recovery of donor liver and the success of liver transplantation. After I/R injury, the liver function factors of recipient usually stayed in a high level after the operation. In our experiment, we found that the serum levels of ALT and AST were significantly lower in DADA group compared with that in I/R group (Figure 5A, 5B). The locally hepatocellular necrosis in donor liver tissue could induce the inflammation factors such as IL-6 and TNF-α arisen. Low levels of IL-6 and TNF-α detected in DADA group mean that this kind of dichloroacetate diisopropylamine treatment could protect the liver against I/R injury (Figure 5C, 5D).

**Discussion**

In liver transplantation, ischemia-reperfusion injury is still an critical index which effect the recovery of donor liver function and the prognosis of patients in post-operation, although the successive rate of liver transplantation is high in nowadays [16]. In operation procedure, the time of hepatic blood flow blocking, donor liver irrigation and blood supply restored period determine the extent of ischemia-reperfusion injury [17]. And a recipient with the low level of I/R injury could get fast recovery and earlier rehabilitation which is a perfect ideal state.

In our animal model, the donor liver suffered a series of ischemia period which was including the 5-min irrigational period and 15-min transplantation period to restore the blood circulation of donor liver. Then, we observed hypoxia-induced I/R injury degree of donor liver in post-operation time and found that the morphology of mitochondria were appeared to dramatically swollen, with reduced, disrupted or disappeared crest. Mitochondria seems to be the central event of the hepatocellular apoptosis procedure. Almost all of the signal pathways which are associated with apoptosis, can directly or indirectly target to the mitochondria injury or damage [6]. Cytochrome C located in mitochondria was an critical enzyme with dual function which participate both in the biology energy metabolism and apoptosis [9]. I/R injury induced mitochondria damage could lead the Cytochrome C overflow into cytoplasm to format the apoptosome, a kind of polymeric compound consisted of Apaf-1, Cytochrome C and Caspase3 [10]. In our experiment of dichloroacetate diisopropylamine therapy, the donor liver could gain the ability to resist the injury of hypoxia, and the expression of Cytochrome C in hepatocellular cytoplasm was decreased by Western Blot assay. The donor liver tissue should be protected from I/R injury in according to the down-regulation of apoptosome-induced apoptosis. We could observe the downstream proteins of cleaved Caspase3 and cleaved PARP, which expressions were both in a low level with DADA treatment. The apoptosome played an important role in mitochondria damage and hepatocellular apoptosis [18]. We observed that morphology of mitochondria after I/R procedure were dramatically swollen. Hypoxia-induced Cytochrome C could promote the apoptosome by the combination with Apaf-1 and Caspase3. With dichloroacetate diisopropylamine treatment, the pathology characteristic of rat donor liver showed that liver cells were slightly edema, and the liver blood sinus gap still be observed. These protection of mitochondria could make most liver cells resist the damage from hypoxia and against the apoptosis.
TNF-α, a kind of multiple effect to promote inflammation cytokines, can induce a variety of apoptosis mechanisms which lead to the hepatocellular damage and effect the serum liver function [19]. In the serum of rat, we detected the TNF-α expression was significantly decreased after liver transplantation 12 h in DADA group, and the same situation detected in IL-6, another inflammation factors. Thus, there should be association between DADA anti-apoptotic effect and lower TNF-α level in our rat liver transplantation model. The liver function factors of recipient usually stayed in a high level after the operation. The liver serum function and inflammation factors were the important indication for the recovery of donor liver and the success of liver transplantation. In our experiment, we found that the injury of donor liver was decreased compared with I/R group in according to the serum levels of ALT and AST, which were significantly lower in DADA group. And the serum levels of IL-6 and TNF-α in DADA group was significantly lower than that in I/R group. Previous study showed DADA could break down into diisopropylamine and dichloroacetate in body metabolism. The dichloroacetate further decomposed into glycine, which could inhibit nuclear factor kappa B activation. But we still need more experiments to clarify the relationship of DADA effective ingredient and mitochondria mechanism.

Conclusion

The dichloroacetic diisopropylamine may have the effect of resisting apoptosis by reducing the release of mitochondrial Cytochrome C and the activation of Caspase3 to abate mitochondrial apoptosis. It could decrease liver ischemia-reperfusion injury to protect the liver function and reduce inflammation. This experiment maybe provide a theoretical basis for clinical recovery treatment of liver transplantation, but still need further experimental proof.

Acknowledgements

The study was supported by the “Natural Science Foundation of China (Grant No. 81302123)”. 

Disclosure of conflict of interest

None of the authors of this study has any financial interest or conflict with industries or parties.

References

Abate Cytochrome C induced apoptosome against ischemia reperfusion injury


