

Role of mitochondria in cell apoptosis during hepatic ischemia-reperfusion injury and protective effect of ischemic postconditioning

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Abstract

AIM: To investigate the role of mitochondria in cell apoptosis during hepatic ischemia-reperfusion injury and protective effect of ischemic postconditioning (IPC).

METHODS: A rat model of acute hepatic ischemia-reperfusion was established, 24 healthy male Wistar rats were randomly divided into sham-operated group, ischemia-reperfusion group (IR) and IPC group. IPC was achieved by several brief pre-reperfusion followed by a persistent reperfusion. Concentration of malondialdehyde (MDA) and activity of several antioxidant enzymes in hepatic tissue were measured respectively. Apoptotic cells were detected by TdT-mediated dUTP-biotin nick end labeling (TUNEL) and expression of Bcl-2 protein was measured by immunohistochemical techniques. Moreover, mitochondrial ultrastructure and parameters of morphology of the above groups were observed by electron microscope.

RESULTS: Compared with IR group, the concentration of MDA and the hepatocellular apoptotic index in IPC group was significantly reduced ($P < 0.05$), while the activity of antioxidant enzymes and OD value of Bcl-2 protein were markedly enhanced ($P < 0.05$). Moreover, the injury of mitochondrial ultrastructure in IPC group was also obviously relieved.

CONCLUSION: IPC can depress the synthesis of oxygen free radicals to protect the mitochondrial ultrastructure and increase the expression of Bcl-2 protein that lies across the mitochondrial membrane. Consequently, IPC can reduce hepatocellular apoptosis after reperfusion and has a protective effect on hepatic ischemia-reperfusion injury.

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INTRODUCTION

Hepatic ischemia-reperfusion injury (IRI) is a common pathological process of traumatic surgical diseases in the liver, such as severe liver trauma, extensive hepatic lobus excision, liver transplantation, shock and infection. When hepatic IRI

happens, a series of metabolic and structural and functional disorder of hepatic tissue cells would occur, which directly influence the prognosis of patients^[1,2]. At present, the study about the mechanism and intervention method of hepatic IRI has been carried out extensively. It has been confirmed that apoptosis as a way of cell death is significant for maintaining normal cell development and stabilization, and is closely related to the initiation and development of many clinical diseases, and it also participates in IRI of tissues and organs^[3-6]. Mitochondria as one of the organelle of cells play an important role in providing energy, adjusting osmotic pressure, calcium balance, and pH value, and cell signaling. Mitochondria perform their function by production of ATP and reactive oxygen species (ROS) which are also known as signals regulating gene expression and triggering cell death^[7,8]. At present, mitochondria/ cytochrome C apoptotic pathway has attracted close attention of scholars. Many stimulators such as ROS, Ca²⁺ and cytokines could activate cysteine aspartate-specific proteases (Caspase) by inducing cytochrome C release^[9-12]. But the study on the changes of the structure and function of mitochondria in hepatic IRI and the adjusting function of mitochondria in hepatocellular apoptosis was rarely reported at home and abroad. We established a hepatic IRI model in liver of rats, and observed the influence of ischemic postconditioning (IPC) on cell apoptosis in hepatic IRI and the adjustment function of mitochondria. This experiment lays a theoretical foundation for adopting a more reasonable method to restore the blood flow after hepatic ischemia.

MATERIALS AND METHODS

Materials

Twenty-four healthy male Wistar rats, weighing 200-250 g, were supplied by the Experimental Animal Center of Wuhan University. Malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-PX) assay kits were purchased from Nanjing Jiancheng Bioengineering Co.Ltd, China. Mouse anti-rat Bcl-2 monoclonal antibody and SP assay kit were provided by Beijing Zhongshan Biotechnology Co.Ltd, China. Terminal deoxynucleotidyl transferase (TdT) mediated dUTP nick end labeling (TUNEL) *in situ* cell death detection kit was the product of Boehringer Mannheim Co.Ltd, USA.

Animal model

The animals were fed standard laboratory chow. Before the day of experiment, the animals were fasted overnight, but allowed free access to water. They were anesthetized by sodium pentobarbital (30 mg/kg). Hepatoduodenal ligament was separated after entry into the belly, the first porta hepatis was exposed and a rat local hepatic IRI model was established with reference to the previous method^[13]. Blood flow of caudate and left lobe of the liver was blocked with non-trauma mini artery clamp, causing 70% liver ischemia. However, the blood flow of right lobe was not blocked to prevent blood clot in portal vein and gastrointestinal tract. Twenty-four rats were randomly divided into three groups (eight in each group) and

subjected to the following treatments. (1) Sham-operated group: only hepatoduodenal ligament was separated after entry into the belly, blood flow of porta hepatis was not blocked. (2) Ischemia-reperfusion group (IR): the livers were undergone ischemia for 60 min followed by reperfusion for 120 min. (3) Ischemic postconditioning group (IPC): the livers were subjected to porta hepatis occlusion for 60 min, then treated for 2 min, 3 min, 5 min, 7 min by brief reperfusion consecutively, each was separated with occlusion for 2 min, followed by a persistent reperfusion for 95 min, which made the total reperfusion time the same as the other two groups.

MDA and antioxidant enzyme measurement

Concentration of MDA and activity of SOD, CAT and GSH-PX in hepatic tissue were measured according to the commercial kit manual.

Immunohistochemical staining

Fresh hepatic tissues, cut from left lobe of the liver, were fixed in formalin for 12-24 h and then embedded in paraffin wax. A 4- μ m thick sections were cut and mounted onto slides. They were deparaffinized by passing through xylene and graded series of ethanol, followed by rinsing in tap water and 0.01 mmol/L phosphate buffered saline (PBS) respectively. Endogenous peroxidase activity was quenched by treating the sections with 30 mL/L hydrogen peroxide for 10 min. Nonspecific binding was blocked by incubating sections in PBS containing 10 g/L bovine albumin for 10 min. Then the sections were incubated for an hour in primary antibody (mouse anti-rat Bcl-2 monoclonal antibody). After rinsed in PBS, the sections were treated sequentially with biotin-conjugated second antibody for 10 min and then with streptavidin-peroxidase for another 10 min with PBS rinsing after each step. The sections were stained subsequently with freshly prepared DAB reagent for 3 min, terminated by rinsing in water, then immersed in hematoxylin for 3-5 min and 0.5 mmol/L HCl for 10 s. Finally, after passing through xylene and graded series of ethanol, the sections were covered with coverslips for light analysis. The sections were examined with HAIPS-2 000 image analysis and the Absorbency value was used to evaluate the content of Bcl-2 protein.

Hepatic TUNEL staining

In situ terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) of fragmented DNA was performed on paraffin slices using the *in situ* cell death detection kit as described in the commercial kit manual. Positively labeled nuclei (brown color) were identified from negatively unstained nuclei (blue color). The number of positive nuclei was determined by counting (magnification \times 400) all the positively labeled nuclei present in five random visual fields under a microscope. The percentage of TUNEL positive nuclei to all nuclei counted was used as apoptotic index (AI). The

apoptotic cells had the following characteristics: single cells, no inflammation, curling of cell membrane, brown particulate or fragmented nuclei.

Morphopathological observation of ultrastructural organization

One mm³ fresh hepatic tissue, cut from left lobe of the liver, was pre-fixed with glutaraldehyde at the volume fraction of 2.5 % and post-fixed with 1 g/L osmic acid. Then the specimens were immersed in propylene oxide after dehydration with gradient ethanol, embedded with epoxy resin and made into ultrathin sections. The sections were stained subsequently with lead-uranium and the changes of ultrastructural organization were observed under H-600 transmission electron microscope. Finally, the sections were measured for cross-sectional area (A), cross-sectional perimeter (P) of mitochondria (using Image Pro Plus, USA), and computed specific surface of mitochondria following the formula: $\delta=4P/(\pi A)$.

Statistical analysis

All data were expressed as mean \pm SD. Comparisons between groups were analyzed with one-way ANOVA and Student-Newman-Keuls *q* test. *P* values less than 0.05 were considered statistically significant.

RESULTS

MDA concentration and antioxidant enzyme activity

The concentration of MDA in hepatic tissue was rapidly elevated after reperfusion in IR and IPC groups against sham group ($P<0.05$), while the activity of SOD, CAT and GSH-PX was significantly reduced ($P<0.05$). Compared with IR group, the concentration of MDA in IPC group was markedly reduced and activity of each antioxidant enzyme was significantly enhanced ($P<0.05$) (Table 1).

Table 1 Measurement of MDA concentration and antioxidant enzyme activity (mean \pm SD, *n*=8)

Group	MDA (nmol/mg)	SOD (U/mg prot)	CAT (U/mg prot)	GSH-PX (U/mg prot)
Sham	3.42 \pm 1.12	241.47 \pm 19.52	91.66 \pm 8.16	124.17 \pm 24.37
IR	17.56 \pm 4.80 ^a	132.99 \pm 22.11 ^a	40.24 \pm 7.73 ^a	51.75 \pm 16.43 ^a
IPC	10.18 \pm 3.56 ^{ab}	176.84 \pm 20.50 ^{ad}	64.81 \pm 7.19 ^{ad}	89.70 \pm 28.15 ^{ad}

^a $P<0.05$ vs sham, ^d $P<0.05$ vs IR.

Expression of Bcl-2 protein

The A value of Bcl-2 protein in IR group (0.067 \pm 0.011) was significantly lower than that in sham group (0.096 \pm 0.017) ($P<0.05$). After postconditioning, the expression of Bcl-2 protein was more eminent (A value 0.138 \pm 0.016) than that in IR group ($P<0.05$) (Figure 1A, B, C).

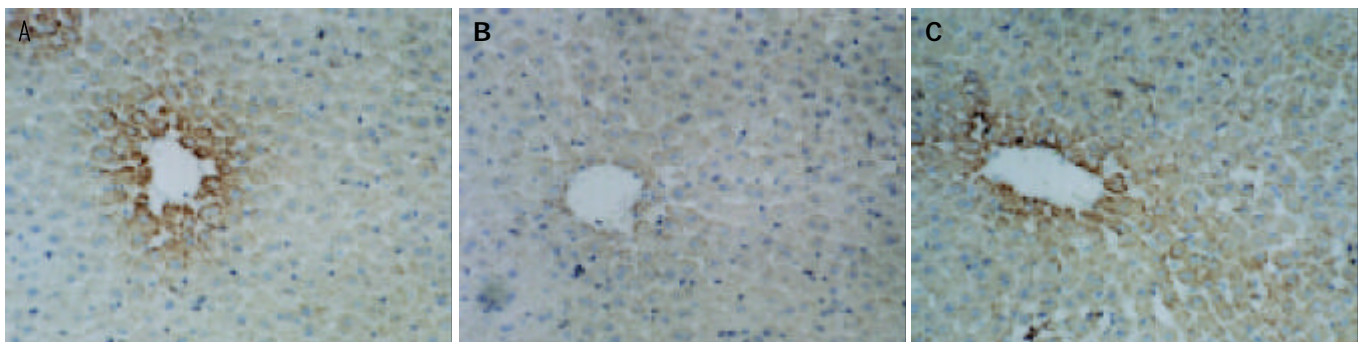


Figure 1 Bcl-2 expression in sham, IR and IPC groups (\times 200). A: Bcl-2 expression in sham group, B: Bcl-2 expression in IR group, C: Bcl-2 expression in IPC group.

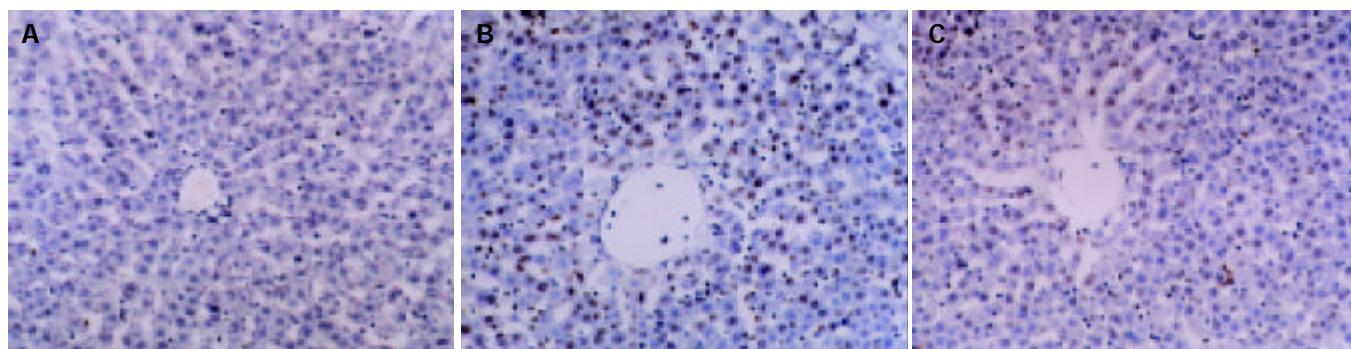


Figure 2 TUNEL staining in sham, IR and IPC groups ($\times 200$). A: TUNEL staining in sham group, B: TUNEL staining in IR group, C: TUNEL staining in IPC group.

Effect of IPC on hepatocellular apoptosis

Under light microscope, TUNEL staining indicated that cell injury occurred primarily in the form of apoptosis at the early stage of reperfusion and apoptotic cells were mainly around the central vein (Figure 2A, B, C). Under electron microscope, the early manifestations of apoptosis, such as swelling and rounding of cells, cell exfoliation, and aggregation of chromatin in the edge, as well as the typical manifestations of apoptosis, such as the concentration of cytoplasm and nucleus, and formation of apoptotic body, could be seen (Figure 3). The apoptotic index in IR group (46.53 ± 3.47) was significantly higher than that in sham group (2.05 ± 0.83) ($P < 0.05$). After postconditioning, the apoptotic index (23.16 ± 2.67) was significantly reduced compared with IR group ($P < 0.05$).

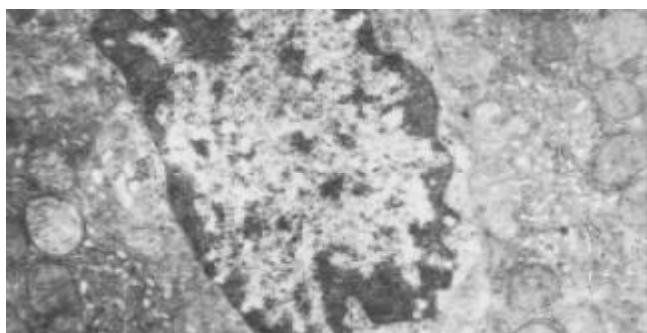


Figure 3 Morphological change of hepatocellular apoptosis in IR group (TEM $\times 15\ 000$).

Ultrastructural changes of mitochondria

In sham group, mitochondrial structure was normal. Mitochondria were orderly arranged in the form of round or ellipsoid without swelling. Mitochondrial membrane was intact and cristae were arranged in the form of concentric ring or vertical line,

congested and clear (Figure 4A). In IR group, mitochondria were swollen obviously. Mitochondrial membrane was vague or partly ruptured and cristae were obviously loose and dissolved, a lot of vacuoluses were formed (Figure 4B). In IPC group, mitochondrial structure was basically normal. Mitochondria were orderly arranged, mitochondrial membrane was basically intact, cristae were congested and there was no vacuolization (Figure 4C).

Quantitative analysis of mitochondrial morphology

The cross-sectional area and perimeter of mitochondria were markedly increased after reperfusion in IR and IPC groups against sham group ($P < 0.05$), while the specific surface of mitochondria was significantly reduced ($P < 0.05$). Compared with IR group, the cross-sectional area and perimeter of mitochondria in IPC group were markedly reduced and the specific surface was significantly increased ($P < 0.05$) (Table 2).

Table 2 Changes of parameters of mitochondrial morphology (mean \pm SD, $n=8$)

Group	Area (μm^2)	Perimeter (μm)	Surface (μm^{-1})
Sham	76.2 ± 8.1	22.7 ± 4.6	0.39 ± 0.04
IR	278.3 ± 22.2^a	60.9 ± 5.9^a	0.17 ± 0.05^a
IPC	150.9 ± 16.4^{ac}	42.1 ± 4.3^{ac}	0.26 ± 0.05^{ac}

^a $P < 0.05$ vs sham, ^c $P < 0.05$ vs IR.

DISCUSSION

Under the influence of many kinds of stress factors such as ROS, Ca^{2+} overloading, and toxin, mitochondrial ultrastructure and its function were easily damaged, then the active substance originally in mitochondria related to apoptosis including cytochrome C, was released into cytoplasm^[14-18]. Recent progress in studies on apoptosis has revealed that cytochrome

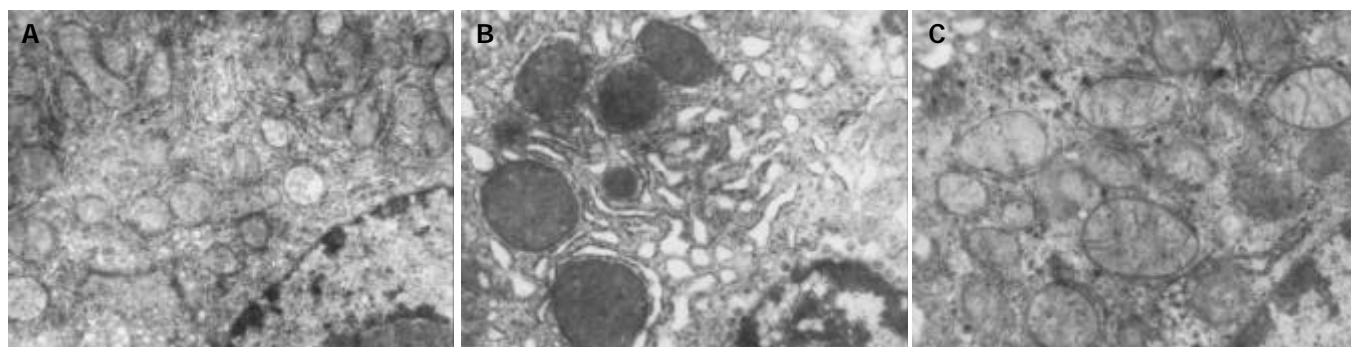


Figure 4 Mitochondrial ultrastructure in sham, IR and IPC groups (TEM $\times 15\ 000$). A: Mitochondrial ultrastructure in sham group, B: Mitochondrial ultrastructure in IR group, C: Mitochondrial ultrastructure in IPC group.

C is a pro-apoptotic factor. It is released at early stage of apoptosis and, by combining with some cytosolic proteins, could activate conversion of the latent apoptosis-promoting protease to its active form^[19]. Once cytochrome C was released, cells would die through two ways. One was through quick apoptotic mechanism. Released cytochrome C and apoptotic protease activating factor-1 (Apaf-1) and Caspase-9 were combined, and then CytC-Apaf-1-Caspase-9 compound was formed, which would lead to the activation of pro-Caspase-9, and subsequently, downstream Caspase-3 was activated, and apoptotic cascade reaction was promoted. At the same time, part of the unreleased cytochrome C was steadily combined with cytochrome b-c1 and C oxidase, and adequate ATP was produced to provide energy for apoptosis^[20-23]. The other was through necrotic process. Permeability transition pore (PTP) opened, mitochondrial matrix was swollen and ruptured due to hyperosmotic effect, the transmembrane potential ($\Delta \Psi_m$) of the original mitochondrial membrane quickly collapsed, cytochrome C was released in great amount, electron transferring respiratory chain was blocked, ATP content was radically reduced, effective supply of energy could not be realized and cell necrosed. Consequently, mitochondria could determine the cells to go into apoptosis or necrosis^[24]. This experiment proved that early injury of cells after reperfusion occurred mainly in the form of apoptosis. The end product MDA of lipid peroxidation in liver tissue increased after reperfusion, while SOD, CAT and GSH-PX as the major antioxidant enzyme and free radical scavenger were obviously decreased, indicating their capacity of preventing cells from the injury caused by ROS. Excessive oxygen free radicals could directly react with unsaturated fatty acid on the surface of mitochondrial membrane, which led to the destruction of its structure and function. Cytochrome C and other apoptosis-promoting substances were released in great amount, which triggered the apoptosis of cells.

The quantitative analysis of the stereo-form of mitochondria indicated that the major morphological change of mitochondrial injury was swelling. It was considered previously that the apoptotic cells manifested condensed chromatin but intact mitochondria. Now much more evidence revealed that significant changes of mitochondria such as swelling, megamitochondria, mitochondrial pyknosis and disrupted outer-membrane could take place in many apoptotic models^[25,26]. Specific surface is the ratio of average area of granular membrane to its volume. It is the objective index to reflect the swelling degree of mitochondria. Our study found that, after reperfusion, the cross-sectional area and perimeter of mitochondria were obviously increased, but specific surface was markedly smaller. The swelling of mitochondria was obvious, and this was in accordance with the changing tendency of mitochondrial ultrastructural organization observed under electron microscope. After postconditioning, the degree of mitochondrial swelling injury was obviously relieved and hepatocellular apoptotic index was also decreased, indicating that the structural and functional changes of mitochondria in apoptotic cells were correlated with the initiation and development of cell apoptosis, suggesting that mitochondrial dysfunction might be the key event in the development of cell apoptosis. Moreover, IPC had a protective effect on the mitochondrial ultrastructure and function.

The exact mechanisms underlying this postconditioning-produced protection remain unclear. It is most likely, though still speculative, that the IPC scheme (reiterative brief pre-reperfusion followed by continuous reperfusion) adopted in this experiment to some extent equaled a controlled slow intermittent reoxygenation, which might reduce the burst production of oxygen free radicals at the early stage of reperfusion and stimulate the release of antioxidant enzymes and free radical scavengers, thus producing protective effects. Compared with IR group, in IPC group MDA in the liver tissue

was decreased and every antioxidant enzyme was increased conspicuously, which reduced the injury to mitochondrial membrane lipid peroxidation by toxic oxygen radicals, protected the integrity of the structure and function of mitochondria, and prevented the apoptosis of liver parenchyma cells. Cave *et al.*^[27] reported that, in an isolated perfused heart model, compared with sudden reperfusion (remove artery snare occluder abruptly), controllable and slow reperfusion could decrease the occurrence of ventricular fibrillation. The postconditioning treatment in the present study was essentially comparable in some senses to those controlled and slow reoxygenation.

In addition, in order to know the function of mitochondria in apoptotic regulatory pathway, we also measured the change of the content of Bcl-2 protein in liver tissue. Bcl-2 protein as the major representative of apoptotic restraining proteins in Bcl-2 family, functioned through signal-anchor sequence of carboxyl terminal stretching cross mitochondrial membrane^[28-31]. Bcl-2 protein could regulate the permeability of PTP on the surface of mitochondrial membrane, or itself could participate in the formation of the specific pathway directly. This could maintain the stability of transmembrane potential and inhibit the release of cytochrome C and other apoptosis-promotion substances, and consequently inhibit the activation of Caspase in cytoplasm and block apoptotic cascade reaction^[32-35]. Our experiment confirmed that after postconditioning, the A value of Bcl-2 protein was obviously higher than that of IR group, and apoptotic index was obviously decreased. This showed that IPC could decrease apoptosis in liver IRI by up-regulating trans-mitochondrial membrane Bcl-2 protein expression. But the detailed mechanism needs to be further studied.

To sum up, in hepatic IRI, various stress factors can activate the apoptotic pathway mediated by mitochondria, which leads to the increase of apoptosis, and the decrease of the total number of parenchyma cells, and thus the hepatic tissue and liver function are severely damaged. Mitochondrial apoptotic pathway participates in hepatic IRI, and is a ring-point in this complicated mechanism. IPC can protect the mitochondrial ultrastructural organization and function through the inhibition of synthesis of excessive oxygen free radicals after reperfusion and increase trans-mitochondrial membrane Bcl-2 protein expression. Consequently, IPC can inhibit cell apoptosis and reduce hepatic IRI. IPC should carry out after operation and before comprehensive blood perfusion. It is easy to perform and the time is adequate. Undoubtedly, it has direct clinical application value.

REFERENCES

- 1 **Lei DX**, Peng CH, Peng SY, Jiang XC, Wu YL, Shen HW. Safe upper limit of intermittent hepatic inflow occlusion for liver resection in cirrhotic rats. *World J Gastroenterol* 2001; **7**: 713-717
- 2 **Kiemer AK**, Heinze SK, Gerwig T, Gerbes AL, Vollmar AM. Stimulation of p38 MAPK by hormonal preconditioning with atrial natriuretic peptide. *World J Gastroenterol* 2002; **8**: 707-711
- 3 **Oshiro T**, Shiraishi M, Muto Y. Adenovirus mediated gene transfer of antiapoptotic protein in hepatic ischemia-reperfusion injury: the paradoxical effect of Bcl-2 expression in the reperfused liver. *J Surg Res* 2002; **103**: 30-36
- 4 **Chen MF**, Chen JC, Chiu DF, Ng CJ, Shyr MH, Chen HM. Prostacyclin analogue (OP-2507) induces delayed ex vivo neutrophil apoptosis and attenuates reperfusion-induced hepatic microcirculatory derangement in rats. *Shock* 2001; **16**: 473-478
- 5 **Sileri P**, Schena S, Morini S, Rastellini C, Pham S, Benedetti E, Cicalese L. Pyruvate inhibits hepatic ischemia-reperfusion injury in rats. *Transplantation* 2001; **72**: 27-30
- 6 **Ikebe N**, Akaike T, Miyamoto Y, Hayashida K, Yoshitake J, Ogawa M, Maeda H. Protective effect of S-nitrosylated alpha (1)-protease inhibitor on hepatic ischemia-reperfusion injury. *J Pharmacol Exp Ther* 2000; **295**: 904-911
- 7 **Susin SA**, Zamzami N, Castedo M, Hirsch T, Marchetti P, Ma-

- cho A, Daugas E, Geuskens M, Kroemer G. Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *J Exp Med* 1996; **184**: 1331-1341
- 8 **Kluck RM**, Bossy-Wetzel E, Green DR, Newmeyer DD. The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 1997; **275**: 1132-1136
- 9 **Marchetti P**, Susin SA, Decaudin D, Gamen S, Castedo M, Hirsch T, Zamzami N, Naval J, Senik A, Kroemer G. Apoptosis-associated derangement of mitochondrial function in cells lacking mitochondrial DNA. *Cancer Res* 1996; **56**: 2033-2038
- 10 **Petit PX**, Susin SA, Zamzami N, Mignotte B, Kroemer G. Mitochondria and programmed cell death: back to the future. *FEBS Lett* 1996; **396**: 7-13
- 11 **Hengartner MO**. The biochemistry of apoptosis. *Nature* 2000; **407**: 770-776
- 12 **Desagher S**, Martinou JC. Mitochondria as the central control point of apoptosis. *Trends Cell Biol* 2000; **10**: 369-377
- 13 **Nauta RJ**, Tsimoyiannis E, Uribe M, Walsh DB, Miller D, Butterfield A. Oxygen-derived free radicals in hepatic ischemia and reperfusion injury in the rat. *Surg Gynecol Obstet* 1990; **171**: 120-125
- 14 **Liu X**, Kim CN, Yang J, Jemmerson R, Wang X. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome c. *Cell* 1996; **86**: 147-157
- 15 **Sola S**, Brito MA, Brites D, Moura JJ, Rodrigues CM. Membrane structural changes support the involvement of mitochondria in the bile salt-induced apoptosis of rat hepatocytes. *Clin Sci* 2002; **103**: 475-485
- 16 **Reichert AS**, Neupert W. Contact sites between the outer and inner membrane of mitochondria-role in protein transport. *Biochim Biophys Acta* 2002; **1592**: 41-49
- 17 **Xue L**, Borutaite V, Tolkovsky AM. Inhibition of mitochondrial permeability transition and release of cytochrome c by anti-apoptotic nucleoside analogues. *Biochem Pharmacol* 2002; **64**: 441-449
- 18 **Siskind LJ**, Kolesnick RN, Colombini M. Ceramide channels increase the permeability of the mitochondrial outer membrane to small proteins. *J Bio Chem* 2002; **277**: 26796-26803
- 19 **Skulachev VP**. Cytochrome c in the apoptotic and antioxidant cascades. *FEBS Lett* 1998; **423**: 275-280
- 20 **Marcocci L**, Marchi U, Salvi M, Milella ZG, Nocera S, Agostinelli E, Mondovi B, Toninello A. Tyramine and monoamine oxidase inhibitors as modulators of the mitochondrial membrane permeability transition. *J Membr Biol* 2002; **188**: 23-31
- 21 **Kim TS**, Jeong DW, Yun BY, Kim IY. Dysfunction of rat liver mitochondria by selenite: induction of mitochondrial permeability transition through thiol-oxidation. *Biochem Biophys Res Commun* 2002; **294**: 1130-1137
- 22 **Rodrigues T**, Santos AC, Pigoso AA, Mingatto FE, Uyemura SA, Curti C. Thioridazine interacts with the membrane of mitochondria acquiring antioxidant activity toward apoptosis-potentially implicated mechanisms. *Br J Pharmacol* 2002; **136**: 136-142
- 23 **Li P**, Nijhawan D, Budihardjo I, Srinivasula SM, Ahmad M, Alnemri ES, Wang X. Cytochrome c and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell* 1997; **91**: 479-489
- 24 **Green DR**, Reed JC. Mitochondria and apoptosis. *Science* 1998; **281**: 1309-1312
- 25 **Frey TG**, Mannella CA. The internal structure of mitochondria. *Trends Biochem Sci* 2000; **25**: 319-324
- 26 **Wakabayashi T**. Structural changes of mitochondria related to apoptosis: swelling and megamitochondria formation. *Acta Biochim Pol* 1999; **46**: 223-237
- 27 **Cave AC**, Collis CS, Downey JM, Hearse DJ. Improved functional recovery by ischaemic preconditioning is not mediated by adenosine in the globally ischaemic isolated rat heart. *Cardiovasc Res* 1993; **27**: 663-668
- 28 **Nguyen M**, Branton PE, Walton PA, Oltvai ZN, Korsmeyer SJ, Shore GC. Role of membrane anchor domain of Bcl-2 in suppression of apoptosis caused by E1B-defective adenovirus. *J Bio Chem* 1994; **269**: 16521-16524
- 29 **Decaudin D**, Geley S, Hirsch T, Castedo M, Marchetti P, Macho A, Kofler R, Kroemer G. Bcl-2 and Bcl-XL antagonize the mitochondrial dysfunction preceding nuclear apoptosis induced by chemotherapeutic agents. *Cancer Res* 1997; **57**: 62-67
- 30 **Castedo M**, Hirsch T, Susin SA, Zamzami N, Marchetti P, Macho A, Kroemer G. Sequential acquisition of mitochondrial and plasma membrane alterations during early lymphocyte apoptosis. *J Immunol* 1996; **157**: 512-521
- 31 **Zamzami N**, Susin SA, Marchetti P, Hirsch T, Gomez-Monterrey I, Castedo M, Kroemer G. Mitochondrial control of nuclear apoptosis. *J Exp Med* 1996; **183**: 1533-1544
- 32 **Marchetti P**, Castedo M, Susin SA, Zamzami N, Hirsch T, Macho A, Haeflner A, Hirsch F, Geuskens M, Kroemer G. Mitochondrial permeability transition is a central coordinating event of apoptosis. *J Exp Med* 1996; **184**: 1155-1160
- 33 **Zamzami N**, Marchetti P, Castedo M, Hirsch T, Susin SA, Masse B, Kroemer G. Inhibitors of permeability transition interfere with the disruption of the mitochondrial transmembrane potential during apoptosis. *FEBS Lett* 1996; **384**: 53-57
- 34 **Akao Y**, Maruyama W, Shimizu S, Yi H, Nakagawa Y, Shamoto-Nagai M, Youdim MB, Tsujimoto Y, Naoi M. Mitochondrial permeability transition mediates apoptosis induced by N-methyl(R) salsolinol, an endogenous neurotoxin, and is inhibited by Bcl-2 and rasagiline, N-propargyl-1(R)-aminoindan. *J Neurochem* 2002; **82**: 913-923
- 35 **Li S**, Zhao Y, He X, Kim TH, Kuharsky DK, Rabinowich H, Chen J, Du C, Yin XM. Relief of extrinsic pathway inhibition by the Bid-dependent mitochondrial release of Smac in Fas-mediated hepatocyte apoptosis. *J Bio Chem* 2002; **277**: 26912-26920

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