Cytochrome *c* modulates the mitochondrial signaling pathway and polymorphonuclear neutrophil apoptosis in bile duct-ligated rats

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Abstract. It has been observed that polymorphonuclear neutrophils (PMN) increase in number and function during obstructive jaundice (OJ). However, the precise mechanisms underlying PMN apoptosis during OJ remain poorly understood. The aim of the present study was to investigate the modulation of cytochrome *c* (Cytc) on the mitochondrial signaling pathway in bile duct-ligated (BDL) rats and the effect on PMN apoptosis following the intravenous administration of Cytc. Rats were randomly divided into four groups: A control group, a sham group, a BDL group and a BDL + Cytc group (rats with common bile duct ligation as well as Cytc intravenous injection). Blood samples were collected from the inferior vein cava for biochemical analysis and separation of the PMN. PMN apoptosis was evaluated using flow cytometry. The mitochondrial membrane potential ($\Delta\Psi$m) of PMN was detected by rhodamine-123 staining. The Cytc protein expression levels were examined using western blotting. PMN mitochondria were observed using transmission electron microscopy. The results of the present study revealed that the PMN apoptosis rate in rats decreased gradually from 12 to 72 h following BDL to levels that were significantly lower than those of the control group and the sham group. Compared with the corresponding time point of the BDL group, the BDL + Cytc group showed a significantly increased PMN apoptosis rate. The mean fluorescence intensity (MFI) of $\Delta\Psi$m decreased from 12 to 72 h following BDL, and was significantly increased compared with the control and sham groups. MFI in the BDL + Cytc group was higher compared with that in the BDL group. Cytc expression levels increased in the mitochondria and decreased in the cytoplasm from the 12 to 72 h in the BDL group, which was significantly different from that in the control and sham groups at the corresponding time points. Compared with the BDL group, Cytc expression levels in the cytoplasm for the BDL + Cytc group tended to gradually and significantly increase. Morphological changes in PMN mitochondria were marginal in BDL rats and marked in the BDL + Cytc group. In the BDL rats, PMN apoptosis was inhibited, a process induced by the mitochondrial apoptotic signaling pathway in which Cytc has an important role. High $\Delta\Psi$m in the mitochondria and decreased Cytc expression levels in the cytoplasm result in PMN apoptosis inhibition. Intravenous injection of Cytc may help compensate for the lack of Cytc proteins in the cytoplasm, inducing PMN apoptosis following BDL.

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Introduction

Polymorphonuclear neutrophils (PMN) have an important role in host defense and various inflammatory diseases (1). Increased number, enhanced chemotaxis and superoxide anion generation of PMN have been observed in animal and clinical research with obstructive jaundice (OJ), which is closely associated with exaggerated systemic inflammation (2-6). Several recent reports have focused on the fate of peripheral PMN and their significance in inflammatory diseases or surgical stress (7-11). Inhibited peripheral PMN apoptosis has been demonstrated in burns (7), severe trauma (8), systemic inflammatory response syndrome (SIRS) (9) and acute pancreatitis (10). The inhibition of PMN apoptosis may result in prolonged PMN life span in the circulation, leading to excessive inflammation (12). If the PMN function is infinitely exaggerated, it may lead to SIRS or even progress to multiple organ dysfunction syndrome (13).

The precise mechanisms underlying PMN apoptosis during OJ are poorly understood. Peripheral PMN have the shortest half-life among leukocytes (14). Senescent PMN undergo apoptosis, which is the principal signaling pathway responsible for cell destruction (15). It is thought that PMN apoptosis

involves three signaling pathways (16): The endogenous pathway, the exogenous pathway and the endocytoplasmic reticulum pathway. These three signaling pathways communicate and interact with one another in physiological conditions, which has an important role in regulating PMN function and cell dissolution (17). Among the three signaling pathways, the passage from cytochrome *c* (Cytc) to caspase cascade activation in the mitochondria is an important step that regulates cell death (18). Only 10 min are required to complete this process, which is termed the key '10 min event' (18). Therefore, the mitochondrial signaling pathway has an important role in controlling PMN apoptosis.

Recent studies reported that the outcome of acute inflammation in OJ is closely associated with the function of peripheral PMN (2-6). Previous studies demonstrated that delayed PMN apoptosis can cause excessive inflammation, and that the potential PMN apoptosis suppression mechanism may result in a decrease in caspase-3 activity (19,20). It has been reported that caspase cascade activation is involved in the cell apoptotic signaling pathway, which has an impact on the initiation and outcome of inflammation (20). However, the endogenous mitochondrial apoptosis signaling pathway regulating cellular life-span and affecting PMN apoptosis in OJ has yet to be elucidated.

The present study used bile duct-ligated (BDL) rats to mimic OJ in order to investigate the changes in peripheral PMN apoptosis, the regulatory mechanism of the endogenous apoptotic signaling pathway, and the effect on PMN apoptosis following treatment with Cytc, as well as to attempt to elucidate the mechanisms underlying PMN apoptosis dysfunction in OJ.

Materials and methods

Animals. A total of 110 male Sprague-Dawley rats (weight, 200-250 g; age, 6-8 weeks) were purchased from the Medical Experimental Animal Center of Guangdong Province (Guangzhou, China), of which 104 survived. Food and water were provided *ad libitum*. The animals were housed in a laminar-flow specific pathogen-free atmosphere. A temperature of 22±1˚C and 12 h light/dark cycle were maintained. All the experimental protocols were approved by the Animal Ethics Committee of the First Affiliated Hospital of Shenzhen University (Shenzhen, China).

Experimental design. The rats were randomly divided into four groups, of which the following numbers of rats survived: A control group (n=8), a sham group (n=32), a BDL group (n=32), and a BDL + Cytc group (n=32). The control group represented untreated rats at the beginning of the experiment. The remaining three groups were divided into rats at four time points: 12, 24, 48 and 72 h following the BDL surgical procedure, with 8 rats at each time point group.

Prior to undergoing surgery the rats were subjected to a fast for 12 h with *ad libitum* access to water. All surgical procedures were performed under 10% chloral hydrate (0.4-0.5 ml/100 g; Sigma-Aldrich, St. Louis, MO, USA) intraperitoneal anesthesia with aseptic surgical techniques. Briefly, the common bile duct of the rats in the sham group was dissected away from the surrounding tissue but not ligated. In the BDL group and BDL + Cytc group, the surgical and postoperative care were the same as for the sham group. The rats in these two groups underwent BDL using double 4-0 silk sutures. Rats in the BDL + Cytc group were also injected intravenously with 20 mg/kg Cytc (Sigma-Aldrich) following BDL. The rats in the control group received no surgical procedure or treatment.

Sample collection. At the various time points prior to (in control rats) or following surgery (in experimental animals), all surviving animals were anesthetized with 10% chloral hydrate (0.4-0.5 ml/100 g), and the abdomen was opened prior to collection of blood samples from the inferior vein cava for biochemical analysis and PMN isolation. The rats were then sacrificed using cervical dislocation. The blood samples from each rat were sent to the Central Laboratory at the First Affiliated Hospital of Shenzhen University for analysis.

Liver function and blood routine examination. A total of 1 ml of blood sample for each rat was centrifuged (600 x g, 10 min, 4˚C). The upper plasma was collected and assayed for total bilirubin (TBIL) and alanine aminotransferase (ALT) levels using a multi-channel automatic biochemical analyzer (Hitachi 7600; Hitachi, Ltd., Tokyo, Japan). A further 0.5 ml of blood from each rat was used to count the number of blood cells using a three classification blood cell analyzer (ABX Hemastar-3; Horiba ABX SAS, Montpellier, France).

Isolation of peripheral PMN. In order to isolate peripheral PMN, 5 ml blood samples were obtained from each rat. PMN were harvested using gradient centrifugation and hypotonic erythrocyte lysis, as described previously (21). The cells were centrifuged at 900 x g for 10 min with cold RPMI-1640 medium (GE Healthcare Life Sciences, Logan, UT, USA) supplemented with 1% fetal bovine serum (Zhejiang Tianhang Biotechnology Co., Ltd., Huzhou, China). Giemsa staining (Amresco, LLC, Solon, OH, USA) and trypan blue (Sigma-Aldrich) exclusion test determined a purity of >95% and a viability of >98% of the PMN, respectively, in each sample.

PMN apoptosis. An Annexin V-fluorescein isothiocyanate (FITC) Apoptosis Detection kit (cat. no. FAK011.100; Neobioscience, Shenzhen, China) was used to detect early and late apoptotic activity following PMN isolation. According to the standard protocol, the binding buffer was diluted at 1:4 with sterile deionized water. A total of 50 μl PMN suspension was resuspended with 150 μl binding buffer, and 5 μl Annexin V-FITC was added and gently mixed. Following an interval of 3 min, 10 μl propidium iodide (PI; 20 μg/ml) was added, incubated in the dark for 10 min following gentle mixing at room temperature, and then added to 250 μl binding buffer prior to further mixing. The cells were detected and analyzed using a Fluorescence Activated Cell Sorter (FACS; Beckman Coulter Epics XL; Beckman Coulter, Inc., Brea, CA, USA). A total of $5x10^3$ cells were counted, and Annexin-V^+/PI^- cells were indicative of apoptosis. The percentage of Annexin-V^+/PI^- cells was used to determine the apoptotic rate.

Measurement of PMN mitochondrial membrane potential ($\Delta\Psi m$). A total of 10 μl PMN suspension was washed twice with phosphate-buffered saline (PBS). The supernatant

Table I. Serum ALT and TBIL biochemical levels at each time point.

Plasma component	Groups	0	12 h	24 h	48 h	72 h
ALT, U/l	Control	31.05±8.66	-	-	-	-
	Sham	-	35.10±8.65	42.19±7.21	24.64±6.36	25.65±6.61
	BDL	-	62.75±10.82[a]	57.25±6.67[a]	52.38±10.56[a]	49.95±8.08[a]
	BDL + Cytc	-	67.90±19.00[a]	60.13±11.46[a]	54.25±6.32[a]	51.75±7.74[a]
TBIL, mg/dl	Control	0.14±0.06	-	-	-	-
	Sham	-	0.10±0.05	0.08±0.05	0.08±0.03	0.09±0.04
	BDL	-	1.28±0.16[a]	4.04±0.88[a]	5.42±0.94[a]	7.14±1.60[a]
	BDL + Cytc	-	1.24±0.15[a]	3.61±0.99[a]	5.03±0.90[a]	6.69±1.48[a]

Values are presented as means ± standard deviation. [a]P<0.01 vs. the control group and the sham group. ALT, alanine aminotransferase; TBIL, total bilirubin; BDL, bile duct-ligated; Cytc, cytochrome *c*.

was discarded and 20 μl rhodamine-123 added (Rho-123; 10 μg/ml; Sigma-Aldrich), incubated for 20 min at 37˚C, then washed twice in PBS for 5 min. The cells were detected and analyzed using the FACS. A total of $5x10^3$ cells were counted and the above procedures were repeated three times. Finally, the fluorescence intensity of the cells was detected by FACS using Rho-123, and the mean fluorescence intensity (MFI) was calculated.

Western blot analysis. Mitochondrial or cytosolic proteins from the PMN cells were extracted using a Cell Mitochondrial Isolation kit (Beyotime Institute of Biotechnology, Haimen, China), according to the manufacturer's protocol. They were then denatured in 5X sodium dodecyl sulfate (SDS)-loading buffer (1,000 mmol/l Tris-HCL, 200 mmol/l dithiothreitol, 10% SDS, 1% bromophenol blue and 50% glycerol; all components sourced from Sigma-Aldrich) at 100°C for 10 min. Equal protein concentrations, determined using a bicinchoninic acid assay (Beyotime Institute of Biotechnology), were separated on 12% SDS-polyacrylamide gels (40 μg/lane) to detect Cytc in the subcellular fractions. Proteins were transferred to nitrocellulose membranes (Pierce Biotechnology, Inc., Rockford, IL, USA) and these were blocked in 5% skim milk solution for 1 h at room temperature. Membranes were then incubated with rabbit primary antibodies at a dilution of 1:1,000, as follows, overnight at 4˚C: Anti-Cytc (cat. no. 4272; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-Cytc oxidase IV (a mitochondrial marker; cat. no. 4844; Cell Signaling Technology, Inc.) and tubulin (cat. no. 2128; Cell Signaling Technology, Inc.), to control for equal sample loading in each subcellular fraction. Membranes were washed in Tris-buffered saline with Tween 20, then incubated at room temperature for 1 h with horseradish peroxidase-conjugated goat anti-rabbit IgG (dilution, 1:2,000; BioVision, Inc., Milpitas, CA, USA). The membranes were then subjected to enhanced chemiluminescence (ECL) using an ECL detection kit (Pierce Biotechnology, Inc.), and quantified using Gel Pro Analyzer v. 4.0 software (Media Cybernetics, Inc., Rockville, MD, USA).

Transmission electron microscopy. Following PMN suspension centrifugation (600 x g, 10 min, 4˚C), 2.5% glutaraldehyde (Ted Pella, Inc., Redding, CA, USA) was added to the precipitate and stored at 4°C overnight. The PMN were then dehydrated, saturated, embedded in Epon-812 epoxy resin (Ted Pella, Inc.), fixed using 1% osmic acid (Ted Pella, Inc.), dehydrated using an ethanol gradient (50, 70, 90 and 100%), polymerized, sectioned with an EM UC7 ultramicrotome (Leica Microsystems GmbH, Wetzlar, Germany) and dyed using uranium acetate-lead citrate (Ted Pella, Inc.). Subsequently the sections were dried and images were captured using a transmission electron microscope (TEM; JEM-1400; JEOL, Ltd., Tokyo, Japan).

Statistical analysis. The data are presented as means ± standard deviation. SPSS 17.0 (SPSS, Inc, Chicago, IL, USA) was used for to carry out the statistical analyses. Multiple comparisons were performed using One-way analysis of variance with Bonferonni's post-hoc test. P<0.05 was considered to indicate a statistically significant value.

Results

Characteristics of the experimental models. The serum biochemical measurement values for the various groups are shown in Table I. ALT and TBIL levels in both the BDL and BDL + Cytc groups were significantly higher compared with those in the control group and the corresponding time point for the sham group (P<0.01). TBIL levels in the BDL and in the BDL + Cytc groups were significantly increased 12 h following the surgical procedure and remained ≥1.24 mg/dl throughout the subsequent experimental time points. The sham surgical procedure had no significant effect on TBIL levels. ALT levels in the BDL and in the BDL + Cytc groups were significantly increased compared with the control and sham groups 12 h after the surgical procedure, but decreased gradually in a time-dependent manner, reaching a minimum of 49.95 U/l throughout the subsequently observed time points.

Time-dependent changes in the number of white blood cells (WBC) and PMN following treatment with BDL and the Cytc. The WBC and PMN counts in each group, determined from blood sample collection, are presented in Table II. The WBC

Table II. Time-dependent changes in the number of WBC and PMN at each time point.

Blood cells	Groups	0	12 h	24 h	48 h	72 h
WBC, n/μl	Control	5,081±2,150	-	-	-	-
	Sham	-	5,250±2,162	5,811±1,799	5,679±1,996	5,838±832
	BDL	-	9,661±2,871[a]	9,411±2,549[a]	9,876±1,517[a]	10,949±3,288[a]
	BDL + Cytc	-	9,098±2,713[a,b]	8,986±1,250[a,b]	9,045±1,452[a,b]	9,481±2,706[a,b]
PMN, n/μl	Control	488±258	-	-	-	-
	Sham	-	570±88	683±176	673±212	688±207
	BDL	-	2,220±709[a]	2,590±692[a]	2,353±556[a]	2,761±911[a]
	BDL + Cytc	-	2,151±793[a,b]	2,300±817[a,b]	2,208±799[a,b]	2,460±420[a,b]
PMN/WBC, %	Control	9.55±2.75	-	-	-	-
	Sham	-	13.51±7.51	12.14±2.21	13.88±9.00	12.20±4.74
	BDL	-	23.31±4.14[a]	27.67±3.51[a]	23.90±4.60[a]	26.00±6.37[b]
	BDL + Cytc		23.80±4.46[a,b]	25.07±6.91[a,b]	24.63±8.58[a,b]	27.61±8.82[a,b]

Values are presented as means ± standard deviation [a]P<0.01 vs. control group and the sham group; [b]P>0.05 vs. BDL group. WBC, white blood cell; PMN, polymorphonuclear neutrophils; BDL, bile duct-ligated; Cytc, cytochrome *c*.

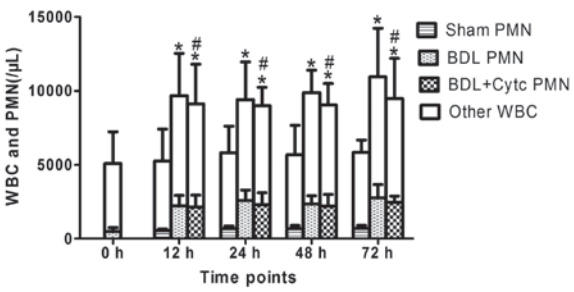

Figure 1. Time-dependent changes to PMN and WBC levels prior to and following the sham or BDL surgical procedure. PMN and WBC levels in the sham group remained low, displaying no significant difference compared with the control group. PMN and WBC levels in the BDL group increased gradually following the BDL surgical procedure. The BDL + Cytc group also displayed a similar increase in PMN and WBC levels, although this increase was not significant compared with the BDL group (#P>0.05). The error bars represent the mean ± standard deviation. *P<0.01 vs. the control group and the corresponding time point for the sham group. WBC, white blood cell; PMN, polymorphonuclear neutrophils; BDL, bile duct-ligated; Cytc, cytochrome *c*.

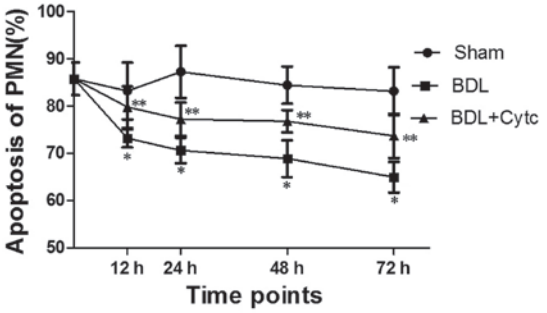

Figure 2. Time-dependent changes to PMN apoptosis levels, displayed as mean ± standard deviation. *P<0.01 vs. the control group and sham group. **P<0.01, vs. the BDL group at the corresponding time point. PMN, polymorphonuclear neutrophils; BDL, bile duct-ligated.

and PMN counts increased significantly 12 h following BDL, and continued to increase at 24, 48 and 72 h following BDL. The WBC and PMN counts revealed similar changes in the BDL + Cytc group, which were observed to be non-significantly different compared with the BDL rats at each time point (P>0.05). The sham surgical procedure had no significant effect on WBC and PMN count (Fig. 1).

Time-dependent changes in PMN apoptosis following BDL and treatment with Cytc. Sequential changes in PMN apoptosis at each time point are shown in Fig. 2. Significantly decreased PMN apoptotic levels were observed as early as 12 h following BDL, and these levels decreased further from 73.13±1.89% at 12 h to 64.93±3.24% at 72 h (Fig. 2). The values then remained unchanged until the end of the observation period. Compared with control group rats (85.70±3.43%) and the sham rats at the respective time points, the PMN apoptosis rate of the BDL group was significantly decreased (P<0.01). Compared with the corresponding time points for the BDL group, the BDL + Cytc group showed a significantly increased PMN apoptosis rate (P<0.01).

Time-dependent changes in PMN ΔΨm following BDL and the Cytc administration on the mitochondrial apoptotic signaling pathway. Mitochondria have life-supporting functions and are able to regulate apoptosis (22-24). Cytc has a key role in the mitochondria, and participates in the assembly of a multimolecular complex known as the apoptosome, which is a core element of the apoptotic signaling pathway (22,23,25). ΔΨm, which prevents apoptosis proteins (Cytc and so on) from being released from the mitochondria into the cytoplasm or nucleus, was detected using Rho-123 staining and FACS, and expressed as the MFI (22,23). When the Rho-123 MFI value is lower, the ΔΨm level is higher (26).

As shown in Fig. 3, Rho-123 MFI in PMN mitochondria was markedly decreased as early as 12 h following BDL, from

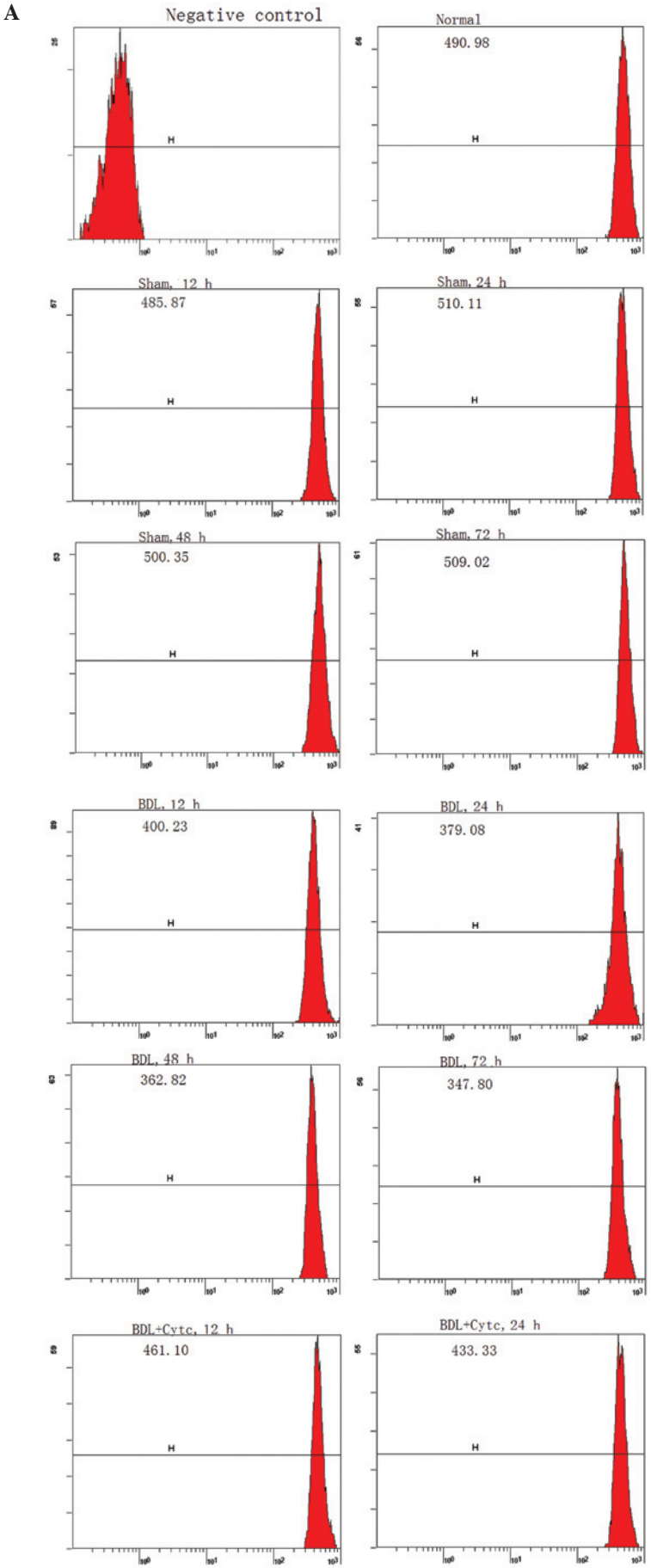

Figure 3. Time-dependent changes in PMN MFI. (A) PMN MFI was detected using Rho-123 staining on fluorescence-activated cell sorting. When the Rho-123 MFI value is lower, the ΔΨm level is higher. Rho-123 MFI decreases in a time-dependent manner in the BDL group. However, the BDL + Cytc group displayed a gradual increase in MFI compared with the corresponding time points in the BDL group.

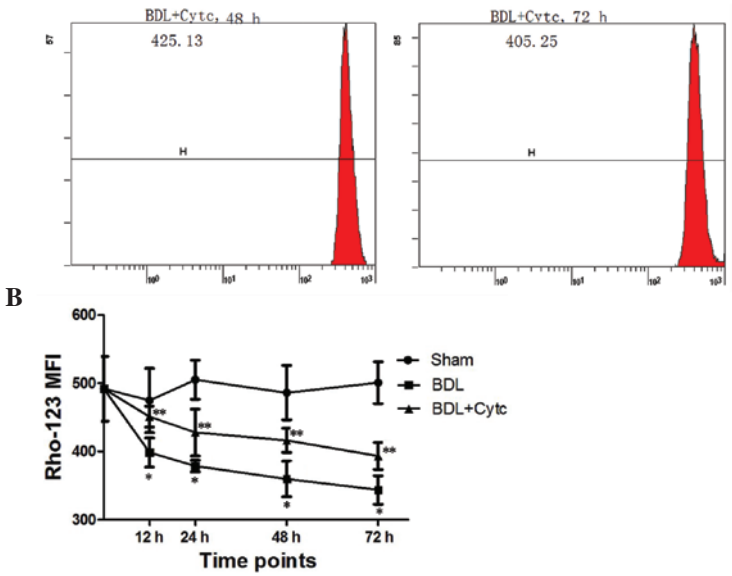

Figure 3. Continued. (B) The values are presented as the mean ± standard deviation. *P<0.01 and **P<0.01, vs. the control group and the corresponding time point of the sham group; **P<0.01, vs. the corresponding time point of the BDL group. PMN, polymorphonuclear neutrophils; MFI, mean fluorescence intensity; Rho-123, rhodamine-123; ΔΨm, mitochondrial membrane potential; BDL, bile duct-ligation; Cytc, cytochrome *c*.

398.51±21.26 at 12 h to 343.60±22.23 at 72 h, which then remained unchanged until the end of the observation period. Compared with the control group rats (492.07±47.21) and the respective sham rat time points, the BDL group exhibited significant changes in MFI (P<0.01). Compared with the corresponding time point for the BDL group, the MFI in the BDL + Cytc group gradually increased (P<0.01).

Time-dependent changes in Cytc expression levels in the mitochondria and cytoplasm. Cytc expression levels in both the mitochondria and cytoplasm of the PMN are shown in Fig. 4. Cytc expression levels in the mitochondria increased in a time-dependent manner 12-72 h following BDL, and were markedly higher compared with the control group and the corresponding time point for the sham group. There were no differences in Cytc expression levels between the BDL and BDL + Cytc groups in the mitochondria (Fig. 4A,C). Cytc expression levels in the cytoplasm gradually decreased 12-72 h following BDL, and were markedly lower compared with that of the control group and the sham group at the corresponding time points. Cytc expression levels in the cytoplasm for the BDL + Cytc group gradually exceeded that of the BDL group at each time point (Fig. 4B and D).

Morphological changes in PMN mitochondria following BDL and Cytc administration. The morphological changes in the PMN mitochondria were observed under TEM. As shown in Fig. 5, typical apoptotic changes in PMN, characterized by cell and nucleus shrinkage, retraction of pseudopodes, plasma membrane blebbing, chromatin condensation and nuclear fragmentation, were found in BDL rats, and markedly in BDL + Cytc rats. Furthermore, damaged structures, cracked cristae and vacuolization phenomena in the mitochondria were marginal in the BDL group, although more extensive in the BDL + Cytc group 12-72 h following the surgical procedure.

Discussion

Peripheral PMN is a type of acute inflammatory cell, the number and function of which impact inflammatory processes and their reversal, during the development of inflammatory diseases (27). Senescent PMN in the peripheral blood or infiltrating tissue undergo apoptosis. Apoptotic PMN are phagocytosed by macrophages without the release of proinflammatory mediators, leading to limited tissue injury and the end of inflammatory processes (12,16). Inhibited peripheral PMN apoptosis has been demonstrated in burns (7), severe trauma (8), SIRS (9) and acute pancreatitis (10). The present study demonstrated that peripheral PMN apoptosis in rats was markedly inhibited 12 h following BDL and decreased in a time-dependent manner. The results also indicated that the number of PMN and WBC in BDL rats increased significantly, as compared with the control group rats and sham rats at each corresponding time point. Inhibited PMN apoptosis may result in increased numbers of PMN in the blood circulation, which benefits the host defense against systemic bacterial invasion, causing the uncontrolled release of toxic metabolites and leading to amplified systemic inflammation and organ injury (10,28). It has been reported that cytoplasmic microinjection of Cytc promotes cell apoptosis activation (29). Accordingly, the present study used Cytc injection (20 mg/kg) in the tail vein of rats subjected to BDL to investigate the activating effect of Cytc on PMN apoptosis. The results demonstrated that the number of PMN decreased and PMN apoptosis increased. Compared with the corresponding time point for the BDL group, rats intravenously treated with Cytc following BDL exhibited a significant increase in the PMN apoptosis rate at 12, 24, 48 and 72 h. It was therefore hypothesized that intravenous Cytc administration in rats subjected to BDL has an important role in inducing PMN apoptosis. The precise mechanism underlying this process requires further clarification.

Cell apoptosis is a complex process that has been the subject of numerous studies (12,16,17). Apoptosis is a form of programmed

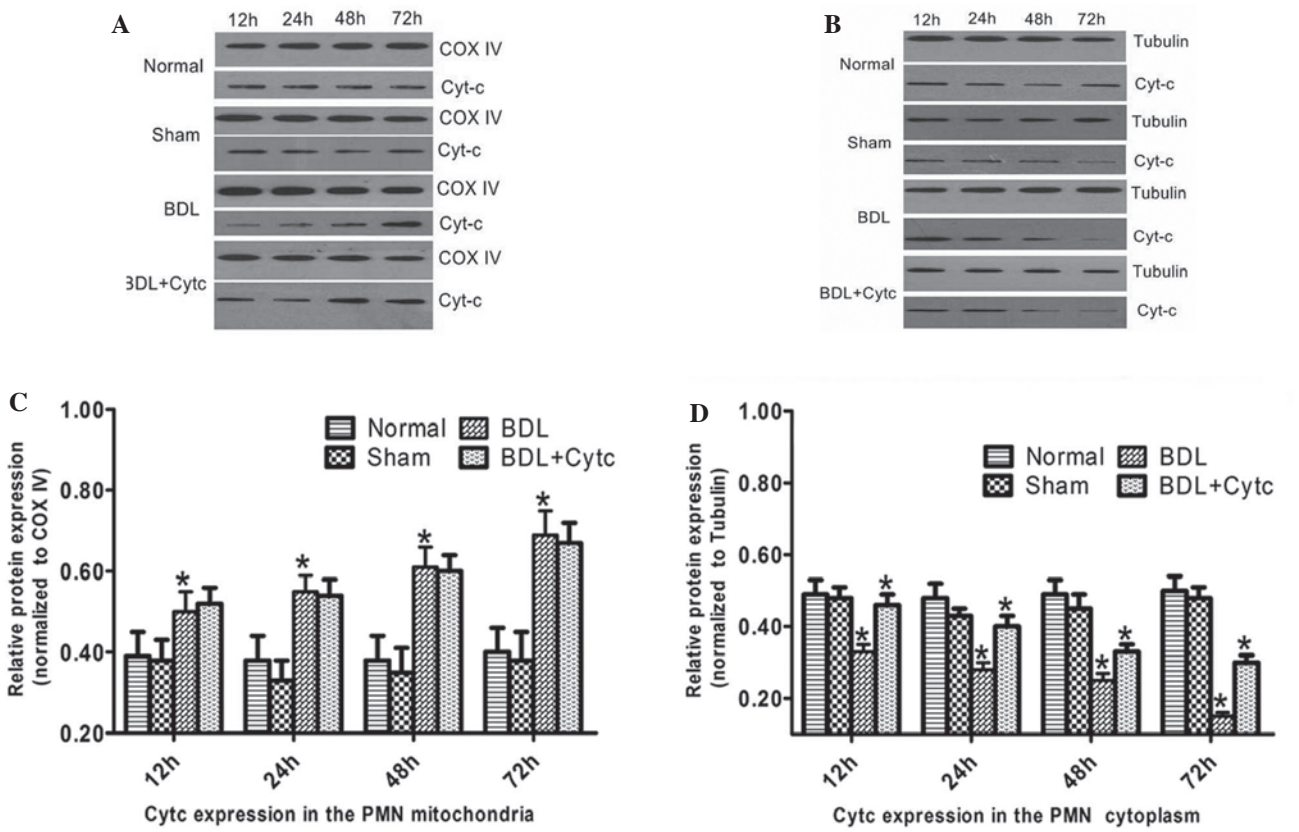

Figure 4. Western blots of Cytc expression in the PMN mitochondria and cytoplasm and graphs to reflect these data. Mitochondrial and cytosolic proteins were purified from the PMN at each time point and subjected to western blot analysis. (A) Cytc expression was increased in the mitochondria and (B) decreased in the cytoplasm from 12 to 72 h in the BDL group, which was significantly different from that in the control (normal) group and the sham group at the corresponding time points. Cytc expression in the cytoplasm for the BDL + Cytc group exceeded that of the BDL group at each time point. Control western blots demonstrate that the purified mitochondrial and cytosolic protein fractions were not contaminated with the mitochondrial membrane-bound protein COX-IV or the cytosolic protein β-tubulin. (C and D) Graphs to reflect these western blot analysis data. *P<0.01 vs. control and sham groups. PMN, polymorphonuclear neutrophils; Cytc, cytochrome *c*; BDL, bile duct-ligation; COX-IV, Cytc oxidase-IV.

cell death, characterized by cell and nucleus shrinkage, retraction of pseudopodes, plasma membrane blebbing, chromatin condensation and nuclear fragmentation (30). This process is tightly controlled by gene regulation, receptor recognition and signal transduction (16). However, the potential mechanisms underlying PMN apoptosis have been extensively debated. It is thought that several factors have roles in this process and affect apoptosis development (22,31-34). Matsuda *et al* (11) showed that cytokine-modulated inhibition of PMN apoptosis at a local site augments PMN functions and induces excessive inflammatory response. The anti-apoptotic B cell lymphoma-2 (Bcl-2) family members Bcl-X-linked, A1 and myeloid cell leukemia-1, have been demonstrated to inhibit PMN apoptosis and promote survival by antagonizing the pro-apoptotic proteins (35). However, Fas ligation or ligation of the tumor necrosis-related apoptosis-inducing ligand receptor on PMN may also trigger apoptosis (36-38). Involved in either apoptosis or inflammation, caspases in PMN can be activated by intrinsically regulated apoptosis as well as death receptor-mediated apoptosis (22,24). Despite a limited role of PMN mitochondria in cellular metabolism, certain investigations have suggested that these organelles may be involved in PMN cell death (39,40). The results of the present study demonstrated that the mitochondria, although limited in number, do participate in PMN apoptosis in BDL rats. These organelles exhibit low mitochondrial enzymatic activity and do not synthesize a large amount of ATP, but preserve their ΔΨm and contain proapoptotic proteins, which trigger PMN apoptosis if released into the cytosol (41). The mitochondria of the PMN were observed using TEM, demonstrating the presence of marginal morphological characteristic changes in BDL rats, and marked changes in BDL + Cytc rats, which exhibited damaged structures, cracked cristae and vacuolization phenomenon in the mitochondria.

Cytc is thought to be one of the most important pro-apoptotic proteins, and Cytc levels are markedly reduced in normal cell cytoplasm and markedly increased in apoptotic cells (22). ΔΨm was detected using Rho-123 staining and FACS, and expressed as MFI to investigate the effect of Cytc on the mitochondrial apoptotic signaling pathway following BDL. The MFI value in BDL rats decreased from 415.66±30.77 at 12 h to 388.51±31.66 at 72 h, a value that was significantly different compared with that of control group rats and sham rats at the corresponding time points. These results suggest that the ΔΨm levels in PMN mitochondria increase gradually following BDL. Therefore,

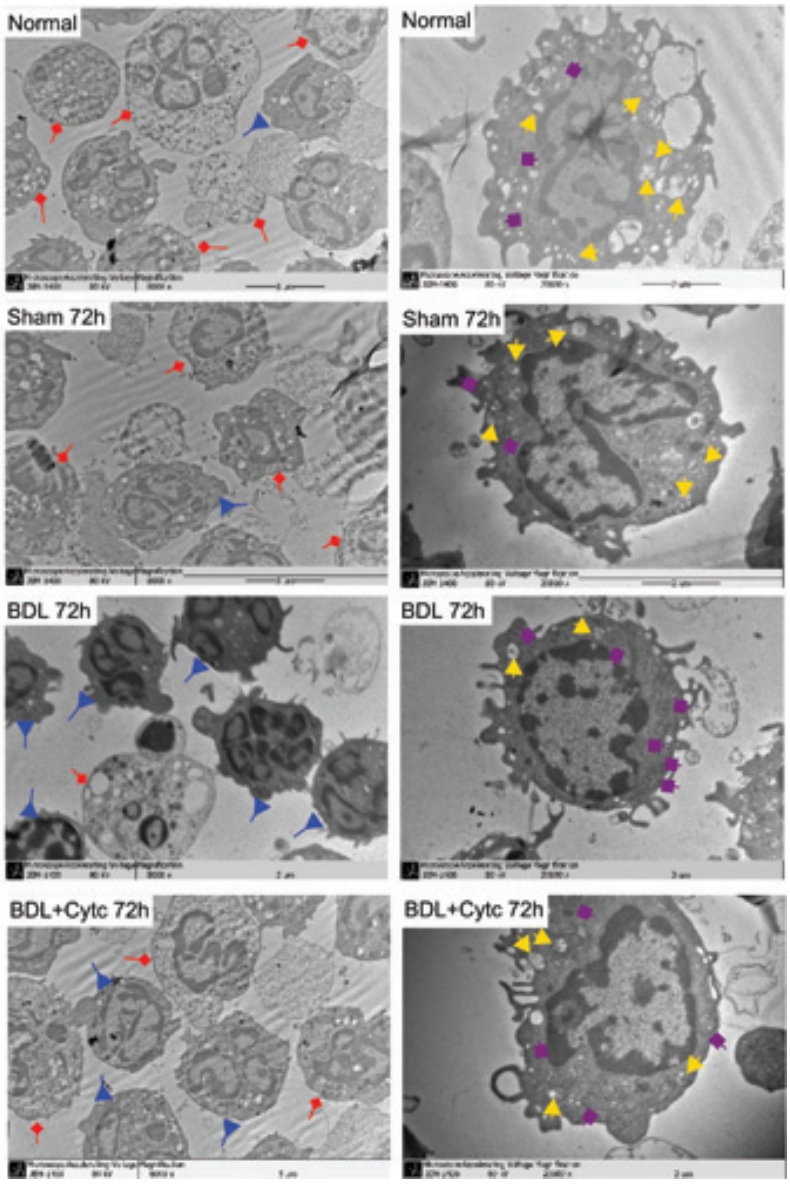

Figure 5. Morphological changes of mitochondrial PMN. Left panel, morphological changes of the PMN as determined by TEM (magnification, x8,000). Fewer apoptotic PMN and more normal PMN were observed at 72 h in the BDL group, but differing results were found in the control group and sham and BDL + Cytc groups at the corresponding time points, which displayed more apoptotic PMN and fewer normal PMN. Right panel, morphological changes of the PMN as determined by TEM (magnification, x20,000). Fewer damaged mitochondria and more normal mitochondria can be observed in the PMN of the BDL group at 72 h, but differing results were found in the control group and sham and BDL + Cytc groups at the corresponding time points, which displayed more extensively damaged structures, cracked cristae and vacuolization of the mitochondria. Apoptotic PMN are represented in red; normal PMN are represented in blue; damaged mitochondria are represented in yellow; and normal mitochondria are represented in purple. PMN, polymorphonuclear neutrophils; TEM, transmission electron microscopy; BDL, bile duct ligation; Cytc, cytochrome *c*.

Cytc stored in the mitochondria did not move into the cytosol, inhibiting PMN apoptosis.

In an attempt to clarify the role of mitochondria in apoptosis, Cytc expression was investigated using western blotting. Cytc expression gradually increased in the mitochondria and decreased in the cytoplasm from 12 to 72 h in the BDL group, and these expression levels were significantly different from those of the control group and corresponding time points of the sham group. In the BDL + Cytc group Cytc was intravenously administered to the rats following BDL, demonstrating that the ΔΨm of the PMN mitochondria gradually decreased and the Cytc expression levels in the cytoplasm increased. These results were significantly different from those of the BDL group at the corresponding time points. However, Cytc expression levels in the mitochondria remained elevated in the BDL + Cytc group from 12 to 72 h, and these expression levels were not significantly different from those of the BDL group at the corresponding time points. The possible mechanisms underlying this phenomenon are as follows: i) The PMN may have spontaneously lost some of the Cytc proteins during the BDL process. ii) The ΔΨm in the mitochondria is thought to be the conductor of the mitochondrial permeability transition pore. When the ΔΨm decreases the pores open and Cytc proteins are released from the mitochondria into the cytoplasm (41). This suggests that Cytc expression levels gradually increase in the cytoplasm of the BDL + Cytc group from 12 to 72 h. iii) Intravenous Cytc administration may directly infiltrate into the PMN cytoplasm, and has no

significant effect on Cytc expression in the mitochondria. It is difficult to determine whether intravenous Cytc administration affects ΔΨm in the mitochondria, and if so the exact amount of Cytc proteins that infiltrate into the cytoplasm via the intravenous route. Further studies are required in order to elucidate this mechanism.

In summary, the results of the present study suggested that PMN apoptosis is inhibited in BDL rats and the mitochondrial apoptotic signaling pathway participates in the apoptosis process. PMN mitochondria usually maintain their ΔΨm within normal range. High ΔΨm in the mitochondria and decreased Cytc expression levels in the cytoplasm may result in PMN apoptosis inhibition in BDL rats. Intravenous Cytc administration may help to compensate for the lack of Cytc proteins in the cytoplasm, inducing PMN apoptosis and reversal of inflammatory processes following BDL. These results provide an important theoretical basis for inflammatory complications during OJ.

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