Bcl-2 Expression Alters the Threshold for Apoptosis in Hepatic Ischemic and Reperfusion Injury

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Summary

Twelve wistar rats were divided into sham-operated control group (1) (n = 6), ischemia - reperfusion group (II) (n = 6). Bcl-2 expression was measured by RT-PCR and western blot methods. Apoptosis measured by flow cytometry and terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick end labeling (TUNEL) methods. Changes in biochemical parameters viz. Alanine Transaminase (ALT), Aspartate Transaminase (AST) in blood serum was measured in all groups. After 1 h ischemia followed by 3 h reperfusion, the levels of liver enzymes in group II were significantly ($P<0.05$) as compared to group I. further, Bcl-2 expression and apoptotic hepatocyte were increased in group II as compared to group I ($P<0.05$). Bcl-2 expression alters the threshold for apoptosis in hepatic ischemia followed by reperfusion injury.

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Introduction

Ischemia and reperfusion (I/R) injury is a phenomenon whereby cellular damage is causd by hepatic oxygen (ischemic phase) and followed by hyper oxygen (reperfusion phase) delivery in the liver. I/R injury this form of injury was recognized as a clinically important pathological disorder by Toledo –Pereyra *et al.,* [1] and is relevant clinically in hepatic surgery, hypovolemic shock, some types of toxic liver injury, veno-occlusive diseases and Budd-Chiari syndrome [2] . Cell atrophy and auto -phagocytes of organelles observed by ischemia [3] which leads to apoptosis. Recently, apoptosis has been indicated as an important mode of cell death during hepatic I/R injury [4]. Apoptosis is governed by a number of regulating genes, such as Bcl-*2* and expression of Bcl-2 determines survival or death following an apoptotic stimulus [5]. There has been increasing evidences that liver ischemia and reperfusion caused postoperative complications such as initial graft dysfunction, endo-toxemia, and peritonitis and increased in risk for development of acute and chronic rejection. Role of Bcl-2 gene in liver transplantation followed by hepatic ischemia and reperfusion injury has not yet been completely elucidated. Therefore, this study was designed to investigate the involvement of Bcl-2 gene in survival of hepatocytes after ischemia and reperfusion induced injury. To determine apoptosis and involvement of Bcl-2 gene in survival of hepatocytes and metabolic function we used ischemia followed reperfused rat liver transplantation model and focused insight into the underlying mechanisms of graft failure.

Materials and Methods

Materials

If not mentioned otherwise all reagents were obtained from Sigma (Sigma, St louis, Mo, USA). Bcl-2 primary monoclonal antibody was purchased from Bio source international, USA).

Treatment of animals

Twelve wistar rats were divided into sham-operated control group (I) (n = 6) and ischemia and reperfusion group (II) (n = 6)). All rats were treated in accordance with the guideline for the Care and Use of Laboratory Animals (NIH Publication No.86-23, revised 1985) with the permission Institutional ethical committee. The hepatic I/R protocol were

performed as described our previous study [2] with some modification. In brief, after the induction of anesthesia (urethane 10 mg/kg i.p), the liver of each was exposed through a midline laparotomy. Complete ischemia of the median and left hepatic lobes was produced by clamping the left branches of the portal vein and the hepatic artery for 60 min. The right hepatic lobe was perfused to prevent intestinal congestion. After the period of ischemia, the ligatures around the left branches of the portal vein and hepatic artery were removed. To accurately evaluate blood flow of the median and left hepatic lobes after ischemia, the right branches of the portal vein and the hepatic artery were ligated to prevent shunting to the right lobe after reperfusion and perfused for 3 h. The wound was closed with 3-0 silk. Sham-operated animals were similarly prepared except that no ligature was placed to obstruct the blood flow to the left and median hepatic lobes. Instead, the blood flow to the right lobe of the liver was occluded. In all groups rats were sacrificed after 1 h ischemia and 3 h reperfusion.

Peripheral blood and tissue procurement

Blood samples were obtained from the right ventricle via a left anterior thoracotomy at the time of sacrifice. The blood was collected in a sterile syringe containing 50 µl of heparin (100 USP Units/ml), and centrifuged to separate the serum. The serum samples were stored at -70°C until use for ALT and AST assays. A portion of ischemic and non-ischemic liver lobe was snap frozen in liquid nitrogen and stored at -70°C for Reverse- Transcription Polymerase Chain Reaction (RTPCR). Further, an additional section of liver was frozen in optimal cutting temperature compound (OTC media) and stored at -70°C until use for assessment of apoptosis.

Reverse transcriptase polymerase chain reaction assay

Total RNAs were isolated from samples of frozen liver (1g) using by the Trizol method (Life Technologies , Rockville, MD, USA). RNA quality and integrity was assured by spectrophotometric analysis (OD_{260}nm) .Total RNAs (5 µg) were first reverse-transcribed into cDNA using oligo (dT) 12-18 as primer and AMV reverse transcriptase (Boehringer, Mannheim, Germany). Reverse transcripts (equivalent to125 ng of total RNA) were used directly for each amplification reaction. Experiments were performed

using a light cycle rapid thermal cycle (Eppendorf, Germany). Polymerase chain reaction primer were against rat Bcl-2 sequences obtained from gene bank (Fastaf).The sense primer was a 21 –mer with a sequences of CGT-CAT-AAC-TAA-AGA-CAC-CCC and the reverse primer was also a 21 mer with a sequences of TTC-ATC-TCC-AGT-ATC-CGA-CTC and purchased from Integrated DNA technologies, Inc. Milpitas, CA 95035, USA. The product length was 234 and the PCR profile was set for denaturation 1 min at 94^{o}C, annealing 90 s at 56^{o}C and extension 2 min at 72^{o}C , and semi- quantization was optimized to 35 cycles. GAPDH (glyceraldehyde– 3 – phosphate dehydrogenase) transcript abundance was used as an endogenous control. The cDNA was amplified by PCR amplification with Ampli Taq Polymerase (Bangolore Genni,India). Amplified product was resolved by electrophoresis on 1.5% agarose gels (sigma, St Louis USA), stained with ethidium bromide and visualized under ultraviolet light.A 1 kbp DNA ladder molecular weight marker (Life Technologies, Rockville, MD, USA) was run on every gel to confirm expected molecular weight of the amplification product. Bands were quantitatively measured by densitometry analysis system (Molecular Analyst/PC, Windows software for Bio-Rad's (Hercules CA). Image Analysis System Version 1.5, and the data are expressed in relative optical density (OD) units.

Western Blot analysis

Cell lysates were prepared from liver & lysed in a buffer containing 1% Tritron X-100 10 mM Tris (PH 7.4), 150 mM NaCl, 2 mg / ml aprotinin & 10 mM Phenylmethyl Sulfonyl fluoride (PMSF) .Protein samples (50 mg) were analyzed by sodium dodecyl sulphate (SDS) poly acrylamide gel electrophoresis (PAGE) under reducing condition transferred overnight to nylon membrane. Both were incubated with rabbit anti-mouse/rat Bcl-2 primary antibody, followed by peroxides - labeled goat anti- rabbit secondary antibody (Bio source international, USA) and bound antibody were detected by enhanced chemiluminescences. Bands were quantitatively measured by densitometry analysis system (Molecular Analyst/PC, Windows software for Bio-Rad's (Hercules CA). Image Analysis System Version 1.5, and the data are expressed in relative optical density (OD) units.

Scanning electron Microscopy

Serial slices of liver tissues were prepared from rat in each group and were fixed in Karnovsky's solution pH 7.4 for 4 h at 4h at 4^{o}C .After processing and hardening the tissue cell surface examined under scanning electron microscope (Leo, 435 VP, Cambridge, U.K.)

Terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick end labeling (TUNEL) assay:

Formalin fixed, paraffin embedded liver tissue was sectioned at 6 μm, deparaffinizied in xylene and rehydrated through a graded series of alcohol ending with in PBS and 0.3% hydrogen peroxide for 10 min at room temperature and then incubated with 20 mg/mL proteinase K for 15 min. Seventy-five microliters of equilibration buffer were applied directly to the specimens for 10 min at room temperature, followed by 55 μL of TdT enzyme and then incubated at 37 ^{0}C for 1 h. The reaction was terminated by transferring the slides to prewarmed stop/wash buffer and incubated for 30 min at 37^{o}C. The specimens were covered with a few drops of rabbit serum and incubated for 20 min at room temperature and then washed with 55 mL of anti-digoxigenin peroxidase and incubated for 30 min at room temperature. Specimens were then soaked in Tris buffer containing 0.02% diaminobenzidine and 0.02% hydrogen peroxide for 1 min to achieve color development. Finally, the specimens were counterstained by immersion in hematoxylin. The cells with clear nuclear labeling were defined as TUNEL-positive cells represented apoptotic cells.

Statistical analysis

Data are expressed as mean (SD).The significance of difference was analyzed by one-way ANOVA followed by Tukey's post –hoc test. $p< 0.05$ was considered significant.

Results

Bio - Chemical Analysis

Table 1 represents the activities of liver function marker enzymes (ALT, AST) in the liver of sham operated group and experimental group rats. Activities of these enzymes were significantly ($p<0.05$) higher in group II (I/R induced injury) as compared with group I (sham operated rats).

Table 1. **ALT and AST level after 1h ischemia followed by 3h reperfusion injury rat liver .**

Groups	ALT [a]	AST[a]
Group I .	84 .7 2 ± 8.2	56.02 ±11.92
Group II.	507.36 ±30.58*	817.40 ± 14.52*

Results are expressed as mean ± SD (n=6).

Significantly (* $P<0.05$) different from control group.
[a] Expressed as IU/L

Semi quantitative RT-PCR analysis of apoptosis-related genes Bcl-2 mRNA

We investigated expression of Bcl-2 gene in differentially treated group through RT-PCR analysis. The length of RT-PCR products of Bcl-2 was 234 bp (Figure 1) and expression of Bcl-2 gene in group II was lower comparison with sham-operated group I after 1 h ischemia and 3h reperfusion. ($P<0.05$)

Western Blot analysis of Bcl-2 protein

Bcl-2 protein expressed at 25 KDa. Group I expression was significantly (P, 0.05) higher as compared to group II.

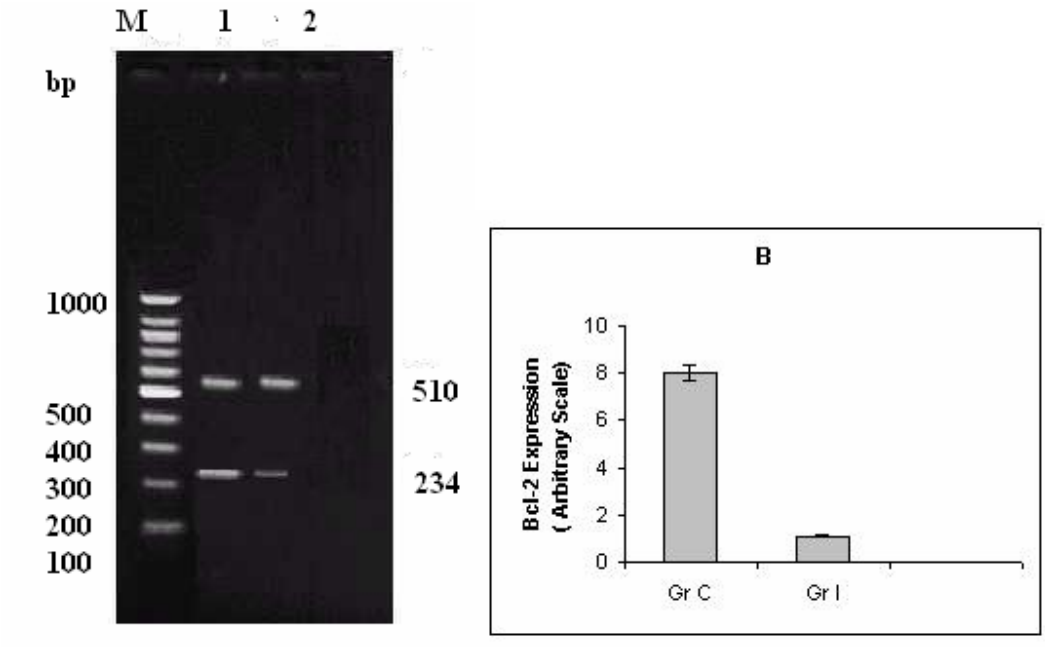

Figure 1: Bcl-2 gene expression. **A:** Representative photograph of the expression of Bcl-2 gene using RTPCR analysis. Bcl-2 gene expressed at 234 bp and house keeping gene was glyceraldehydes-3 phosphate dehydrogenase expressed at 510 bp .Lane M : Marker, Lane 1: sham-operated control group (I) , Lane 2: I/R rat (II), **B:** The expression of Bcl-2 gene after 1 h ischemia and 3 h reperfusion . Data are expressed as the mean ±S.D

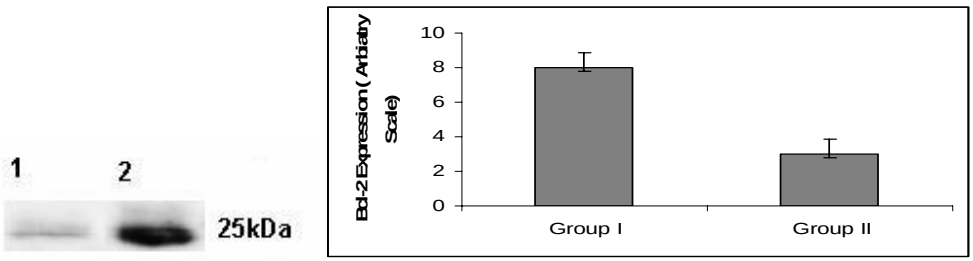

Figure. 2: Expression of Bcl-2 protein .**A:** Representative photograph of the expression of Bcl-2 protein using western blot analysis. Lane 1: Sham-operated control group, Bcl-2 was strongly expressed in the sham-operated liver. Lane 2: Ischemic and reperfused liver (Group II), Expression level was decreased after 1 h ischemia and 3 h reperfusion in group I.**B**: The expression of Bcl-2 protein after 1 h ischemia followed by 3 h reperfusion. Data are expressed as the mean ±S.D.

Scanning Electron Microscopy

Ultra structural alterations such as formation of apoptotic bodies and budding were evident on surface of group II (Figure 3B) when comparison with sham operated group I (Figure 3A)

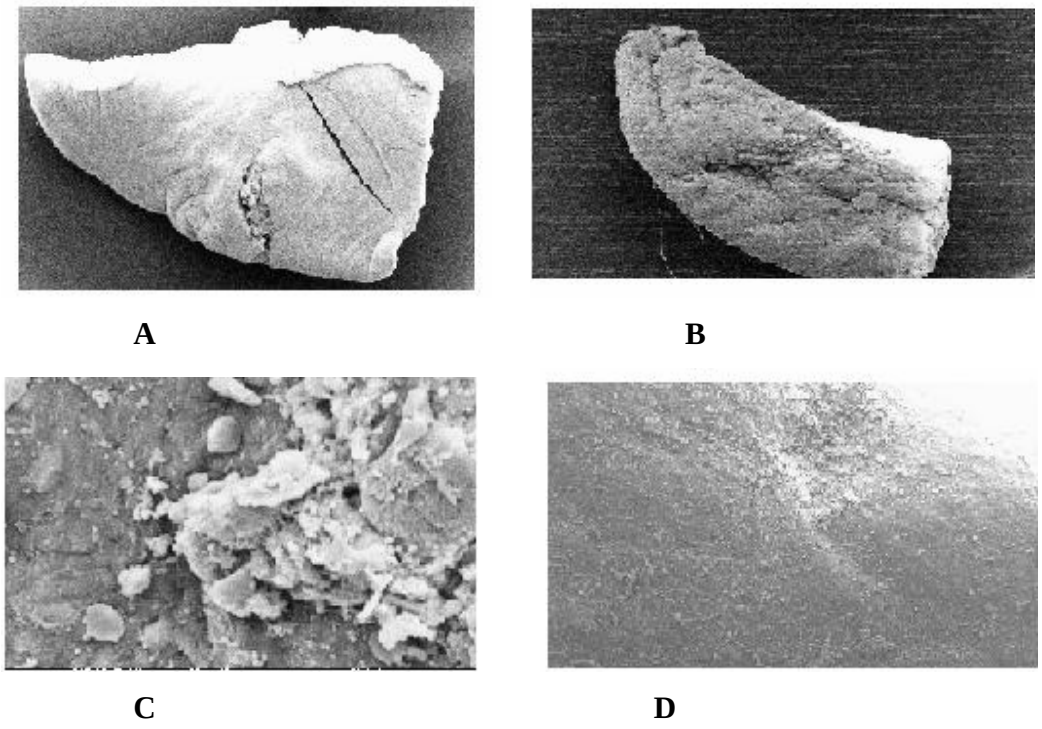

Figure 3: SEM Photomicrograph of Liver surface. A: Left section of liver of group II animals. (12X).B: Left section rat liver of group I (12X). C: Severe apoptotic bodies and budding in tissue surface of I/R group (1.5 K. E. X).D: Some budding in tissue surface but no apoptotic bodies observed in group I animals (Magnification: 1.5 K.E. X).

Change of cellular apoptotic rates

Apoptosis was measured by TUNEL assay. TUNEL-positive cells were detectable after 1 h ischemia and 3 h reperfusion .The cellular apoptotic rate in group II was five times greater than group I. ($P<0.05$). (Table 2)

Table 2: Percentage of Apoptotic and necrotic cells in liver after 1h ischemia and 3 h reperfusion measured by TUNNEL assay.

Group	% of Apoptotic cells
Group I .	1.30±2.5
Group II.	14.44±6.0*

* Mean ± SD, n=6.

Significantly (* P<0.05) different from control group

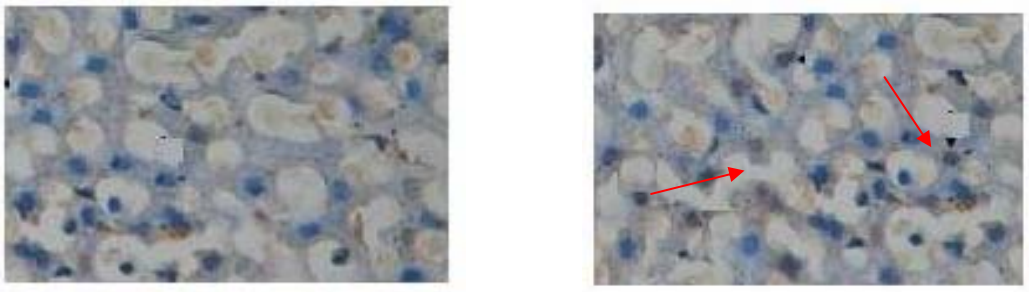

Figure 4: TUNEL Photomicrograph of Liver**. A:** Sham operated control rats; B: I/R groups showing number of apoptotic cells.

Discussion

In our present study toxic effect of ischemia and reperfusion has been investigated in hepatocytes which related to expression Bcl-2 gene and apoptosis. The results of our study showed that after 1 h ischemia followed by 3 h reperfusion Bcl-2 expression was significantly reduced and apoptosis rate was increased significantly (P. 0.05) as compared to sham operated control rats. Bcl-2, a member of the Bcl-2 -related gene family, can promote cell survival through protein-protein interactions with other Bcl-2 related gene family members. Bcl-2 was originally located at the breakpoint site of the t (14:18) chromosomal translocation in B-cell lymphomas, contributing to neoplastic B –cell

expression by preventing B-cell death [4]. Apoptosis is governed by the Bcl-2 family of proteins, further as a central checkpoint. The Bcl-2 family consists of both cell death promoters and cell death preventers and Bcl-2 is known as one of anti-apoptotic proteins. The pro-apoptotic molecules such as Bcl-2 determine the response to a death signal [6, 7]. Bcl-2 seems to prevent apoptosis induced by many stimuli, for example, irradiation, FASL [7] TRAIL [8] and deprivation of growth factor. Further, it has been shown to suppress cytochrome c (cyt.c) efflux from mitochondria, inhibit calcium release from the endoplasmic reticulum [9, 10]. I/R injured hepatocytes was not significantly expressed m RNA of Bcl-2, it is the possible reason after 1 h ischemia and 3h reperfusion hepatocytes was turn to apoptosis, which confirmed by TUNEL assay and SEM. Our previous study showed that apoptosis caused by I/R injury may be due to action endonuclease or by acting on cell organelles, alternating signal transduction pathways or affecting the intracellular enzymes responsible for proper functioning and survival of the cell [11]. Also we demonstrated in our previous study that DNA content and decrease cytochrome P 450 in ischemia and reperfusion injury, presumably as a result of increase activity of TNF – α by toxic effect of ischemia and reperfusion. TNF – α may mediated direct toxicity to mitochondria and induce apoptosis or cell death [2]. During reperfusion, cells are killed by a combination of several mechanisms including intracellular oxidant stress, exposure to external cytotoxic mediators, and prolonged ischemia. Cell death of hepatocytes and endothelial cells during reperfusion is characterized by swelling of cells and their organelles, release of cell contents, eosinophilia, karyolysis, and induction of inflammation [12]. These morphological features are characteristic for oncotic necrosis. However, in recent years it was postulated that most liver cells actually die by apoptosis [13, 14], which is morphologically characterized by cell shrinkage, formation of apoptotic bodies with intact cell organelles, and the absence of inflammation [15]. The present studies showed by SEM studies shrinkage of cell surface, formation of apoptotic bodies and budding with intact cell organelles after ischemia and reperfusion

In conclusion, the experimental data demonstrated ischemia and reperfusion injury exerts its apoptotic effect via regulating gene expression of Bcl-2. Ischemia-reperfusion injury is a complex pathophysiology with a number of contributing factors. The ischemic insult can lead to sublethal cell injury, which is aggravated by the formation of reactive oxygen from various intracellular sources during reperfusion. In addition, formation of proinflammatory mediators and the recruitment and activation of macrophages, neutrophils, and lymphocytes can further enhance the injury. Microcirculatory disturbances lead to under perfused areas in the liver and may cause ischemic injury. All mechanisms contribute to various degrees in the overall pathophysiology. Therefore, it is difficult to achieve effective protection by targeting individual mediators or mechanisms. In contrast, the most promising protective strategy against ischemia-reperfusion injury explored during the last few years is preconditioning, which appears to increase the resistance of liver cells to ischemia and reperfusion events. Preconditioning or pharmacological interventions that mimic these effects have the greatest potential to improve clinical outcome in liver transplantation and liver surgery with vascular exclusion.

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