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Ca^{+2} Concentrations are Key Determinants of Ischemia–Reperfusion-Induced Apoptosis: Significance for the Molecular Mechanism of Bcl-2 Action

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Abstract The mechanism of action of the anti-apoptotic oncogene Bcl-2 and Ca^{+2} regulation in ischemia–reperfusion injury is still obscure. In this present study, we investigated mitochondrial Ca^{+2} overloads and mechanism of action of Bcl-2. Eighteen Wistar rats were divided into sham-operated control group (I) (n=6), ischemia and reperfusion group (II) (n=6), and amlodipine-treated group (1 mg kg^{-1} body weight/daily by oral route for 7 days before inducing ischemia–reperfusion maneuver) (III) (n=6). Rats were subjected to 1 h of hepatic ischemia followed by 3-h reperfusion. Mitochondrial Ca^{2+} content was determined and damage was confirmed by transmission electron microscopy. Decrease of mitochondrial Ca^{+2} level is related to reduction of apoptosis and cellular changes, viz. increased Bcl-2 expression followed by reduction in secondary endoplasmic reticulum, whereas ischemia/reperfusion group shows overloading Ca^{+2} ions and decrease in Bcl-2 expression as compared to sham-operated rats. Thus, Bcl-2-dependent reduction of Ca^{+2} is an important component of the anti-apoptotic program in ischemia–reperfusion injury.

Keywords Ca^{+2} · Bcl-2 · Apoptosis · Ischemia–reperfusion

Introduction

The Bcl-2 gene was first identified at the chromosomal breakpoint of t (14:18)-bearing B cell lymphomas [1] and was found to act as a new class of oncogenes that functions to prevent apoptosis instead of directly promoting cellular proliferation [2]. Regulation of mitochondrial membrane permeabilization is the major mechanism by which Bcl-2-like

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proteins exert their regulatory effect on apoptosis. As a site of convergence for multiple death-inducing stimuli, the mitochondria are a pivotal decision center which control life and death by releasing apoptogenic factors in the cytosol.

Ischemia–reperfusion (I/R) injury is a pathophysiological process whereby hypoxic organ damage is caused due to deprivation of oxygen. In the liver, this form of injury was recognized as a clinically important pathological disorder during studies of experimental liver transplantation [3]. Calcium within the cell exhibits a highly compartmentalized distribution [4]. Free calcium in the cytoplasm is maintained at approximately 100 nM, whereas compartments such as the endoplasmic reticulum exhibit a several-fold higher concentration of calcium [5, 6]. Earlier work showed that ischemia is associated with impaired calcium homeostasis. Ischemic tissue injury causes an increase in free intracellular calcium that leads to diminished recovery of dilation function after ischemia (vasoconstriction), compromised membrane integrity, and decrease in reserves of cellular adenosine triphosphate [7]. The results of the experiments described here demonstrate that Bcl-2 overexpression is associated with enhanced Ca^{+2} overloading, which further demonstrate the importance of Ca^{+2} to maintain cell viability and providing insight into the apoptotic effect of Bcl-2.

Materials and Methods

Chemicals and Reagents

If not mentioned otherwise, all reagents were obtained from Sigma (Sigma, St. Louis, MO, USA). Bcl-2, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary monoclonal antibody was purchased from BioSource International, USA.

Animal Model

Male Wistar rats (200–250 g) were purchased from Laboratory Animal Resources, Indian Veterinary Research Institute, Izzatnagar, UP, India, maintained in a temperature-controlled room in the animal house with 12 h alternating light and dark cycles, and were given adequate nutrition and water ad libitum. All animals received humane care in compliance as per guide for the care and use of laboratory animals. Experimental protocols were reviewed and approved by institutional ethical committee. Ischemia and reperfusion injury was produced as per the procedure described by Chattopadhyay et al. [8, 9]. Eighteen Wistar rats were divided into sham-operated control group (I) (n=6), ischemia and reperfusion group (II) given 0.9% saline (5 mL kg^{-1}, p.o.) for 7 days (n=6), and amlodipine-treated group (1 mg kg^{-1} body weight/daily by oral route for 7 days before inducing ischemia–reperfusion maneuver) (III) (n=6). In all groups, rats were sacrificed after 1-h ischemia followed by 3-h reperfusion. Ischemic lobe was snap frozen in liquid nitrogen and stored at −70°C for isolation of mitochondria, Bcl-2 gene expression, and transmission electron microscopy (TEM) studies.

Assay of Mitochondrial Ca^{+2} Effluxes

The mitochondria of liver were isolated by the method of Starkov and Fiskum [10]. Cystolic mitochondrial calcium was estimated using diagnostic kit (Qualigens Diagnostics, India) as per manufacturer's guidelines. The increase in absorbance was measured at 570 nm using a beam spectrophotometer (ECL, Hyderabad, India) and the results were expressed as nanomoles of calcium per milligram of protein in all the samples.

Assay of Cytochrome *c* Oxidase Activity (Ferro-Cytochrome *c*; Oxygen Oxidoreductase, EC 1.9.3.1)

Cytochrome *c* was measured as per the procedure described by Pearl et al. [12]. Results are expressed as micromoles of oxidized cytochrome *c* per minute per milligram of mitochondrial protein.

Flow Cytometry Analysis

Flow cytometry was used to determine the sub-G_1–G_o fraction in fixed cells stained with propidium iodide (PI) and ethidium bromide. Hepatocytes ($1\times10^9 L^{-1}$) were washed with phosphate-buffered saline (PBS), exposed to a mixture of PI and ethidium bromide 50 mg L^{-1}, 0.1% Triton X-100, 0.01 mmol L^{-1} EDTA $(Na)_2$, and RNase 50 mg L^{-1} at normal temperature in darkness for 18 h. Specimens were then presented to the FACS-420 flow cytometry analyzer (Becton Dickinson Co., USA) to evaluate apoptosis levels. The apoptotic and necrotic cells were finally analyzed with the Modfit 3.0 DNA software on the basis of percentage of hepatocyte staining with PI and ethidium bromide.

Reverse Transcription Polymerase Chain Reaction

Total RNAs were isolated from samples of frozen liver (1 g) by using 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 cycler (Eppendorf, Germany). Polymerase chain reaction primers against rat Bcl-2 sequences were obtained from gene bank (Fastaf). The sense primer was a 21-mer with a sequence of CGT-CAT-AAC-TAA-AGA-CAC-CCC and the reverse primer was also a 21-mer with a sequence of TTC-ATC-TCC-AGT-ATC-CGA-CTC, purchased from Integrated DNA Technologies, Inc. Milpitas, CA 95035, USA. The product length was 234 kb and the polymerase chain reaction (PCR) profile was set for denaturation 1 min at 94°C, annealing 90 s at 56°C, and extension 2 min at 72°C; semi-quantization was optimized to 35 cycles. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript abundance was used as an endogenous control. The cDNA was amplified by PCR amplification with Ampli Taq Polymerase (Bangalore Genni, India). Amplified product was resolved by electrophoresis on 1.5% agarose gels (Sigma), stained with ethidium bromide, and visualized under ultraviolet light. A 1-kb DNA ladder molecular weight marker (Life Technologies) 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 and lysed in a buffer containing 1% Triton X-100, 10 mM Tris (pH7.4), 150 mM NaCl, 2 mg mL^{-1} aprotinin, and 10 mM phenyl methyl sulfonyl fluoride. Protein samples (50 μg) were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing condition and transferred overnight to a

nylon membrane. Both were incubated with rabbit anti-mouse/rat Bcl-2 and GAPDH primary antibody (BioSource International) followed by peroxide-labeled goat anti-rabbit secondary antibody and bound antibody were detected by enhanced chemiluminescences. Bands were quantitatively measured by densitometry analysis system. Image Analysis System Version 1.5 was used and the data are expressed in relative optical density units.

Fluorescence Microscopy

Viable hepatocytes were prepared by the collagenase perfusion modified method [9]. Cell morphology of I/R-induced apoptosis was studied by staining the cells with a combination of fluorescent DNA binding dyes, acridine orange, and ethidium bromide. Cells were harvested and washed with PBS after isolation of hepatocytes from different groups. After staining with a mixture of 100 μg mL^{-1} acridine orange and ethidium bromide for 5 min, the cells were observed under a fluorescence microscope (Olympus, Japan).

Transmission Electron Microscopy of Liver Tissue

Liver tissues were fixed in Karnovsky's solution pH7.4 for 4 h at 4 °C and post-fixed with glutaraldehyde and osmium trioxide, respectively. Ultra-thin sections (70 nm) were stained with uranyl acetate followed by lead citrate and viewed under Moragagni 268D electron microscopes. The experiments were carried out in blinded experimental conditions and the liver tissue sections were examined.

Statistics

All values are expressed as mean±SD. Differences in mean values were compared using Statistical Package for Social Sciences (SPSS South Asia, Bangalore, India, version 11.0) by one-way analysis of variance and Student–Newman–Keuls test. $P < 0.05$ was considered as statistically significant.

Results

Ca^{+2} and Cytochrome *c* Levels

The activity of mitochondrial Ca^{+2} levels was 7.11±0.39 in sham-operated group, which increased to 12.36±1.54 after 1-h ischemia followed by 3-h reperfusion. Pretreatment with amlodipine reverted the alerted activities of Ca^{+2} levels to near control levels when compared to the sham-operated control rats.

Levels of cytochrome *c* were 0.32 which decreased to 0.25 in the I/R group. Pretreatment with calcium antagonist amlodipine reverted the altered activities of cytochrome *c* levels to near those of sham-operated control rats (Table 1).

Flow Cytometry Analysis of Necrotic and Apoptotic Hepatocytes

The necrotic and apoptotic cells were 1.02±0.32 and 0.70±0.08, respectively, in the sham-operated control rats and increased to 21.54 ±7.1 and 26.44±6.0, respectively, after 1-h ischemia followed by 3-h reperfusion. Amlodipine (1 mg kg^{-1}) attenuated increased necrosis and apoptosis caused by I/R-induced toxicity (Table 1 and Fig. 1)

Table 1 Effects of Ca^{+2} efflux and Ca^{+2} channel inhibitor on percent apoptosis, percent necrosis, and cytochrome *c* level after 1-h ischemia followed by 3-h reperfusion injury in rat liver.

Group	Percentage of necrotic cells	Percent of apoptotic cells	Ca^{+2} effluxes[a]	Cytochrome[a]
Sham-operated (I)	1.02±0.32	0.70±0.08	7.11±0.39	0.32±0.07
Ischemia and reperfusion (II)	21.54±7.1*	26.44±6.01*	12.36±1.54**	0.25±0.05*
Amlodipine treated (III)	8.21±0.22**,****	14.65±2.36**,****	8.70±1.22*,***	0.27±0.04*,***

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

*P<0.05, **P<0.01—significantly different from sham-operated group; ***P<0.05, ****P<0.01—significantly different from I/R injury group

[a] Expressed as nM L^{-1} mg^{-1} of protein

RT-PCR Analysis of Bcl-2m RNA Expression

Anti-apoptotic Bcl-2 gene and housekeeping glyceraldehyde-3 phosphate dehydrogenase (GAPDH) gene amplified by reverse transcription polymerase chain reaction (RT-PCR) and separated by electrophoresis by staining with ethidium bromide. PCR products of Bcl-2 and GAPDH were expressed at 234 and 510 bp, respectively (Fig. 2). Expression of Bcl-2 gene in I/R group was negligible in comparison to sham-operated group after 1-h ischemia

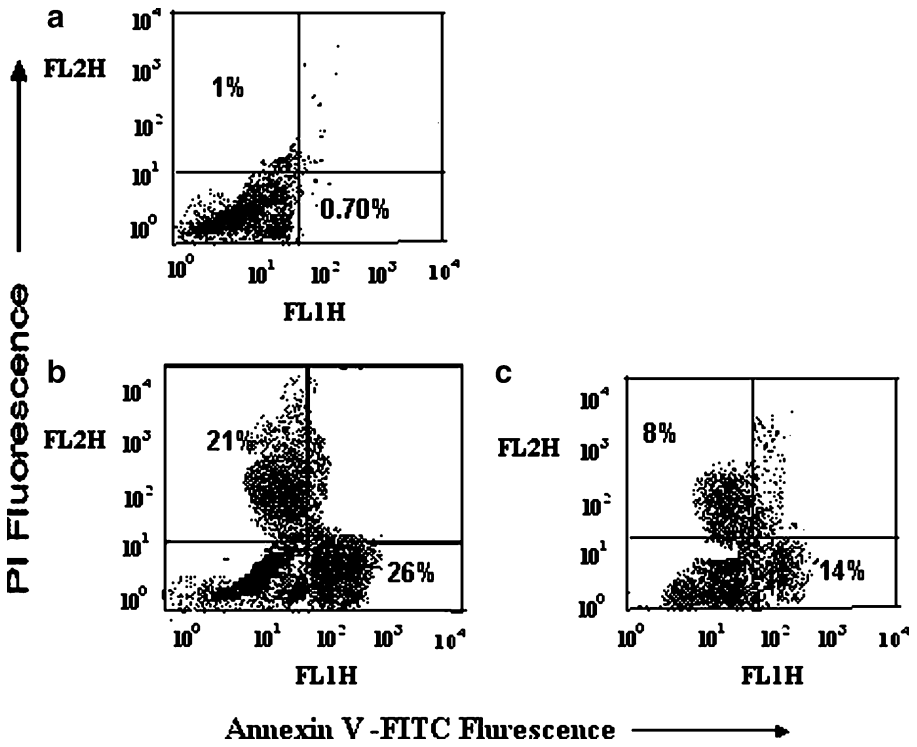

Fig. 1 I/R-induced necrosis in hepatocytes. Flow cytometry analysis assessing apoptosis after propidium iodide and ethidium bromide staining. **a** Flow cytogram of sham-operated control rats (I). **b** Flow cytogram of ischemic and reperfused rats (II). **c** Flow cytogram of Ca^{+2} channel inhibitor (amlodipine) treated rats (III)

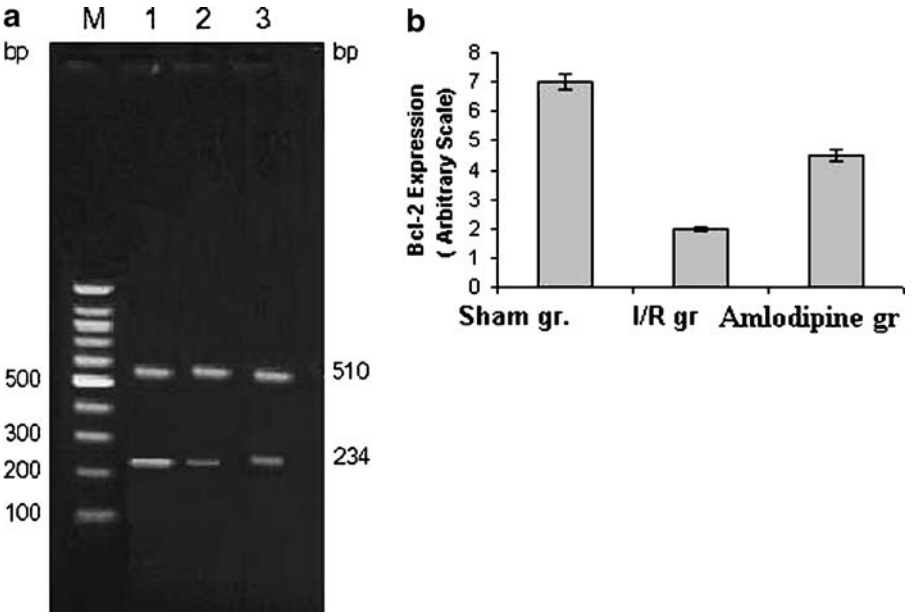

Fig. 2 Bcl-2 gene expression. **a** Expression of Bcl-2 gene by RT-PCR analysis. *Lane M* marker, *lane 1* sham-operated control rats (I), *lane 2* I/R rats (II), *lane 3* Ca^{+2} channel inhibitor (amlodipine) treated (III) rats. **b** Expression of Bcl-2 gene. Data are expressed as mean±SD

followed by 3-h reperfusion. Pretreatment with amlodipine significantly increased Bcl-2 gene expression as compared to I/R group.

Western Blot Analysis of Bcl-2 Protein

Bcl-2 protein was expressed at 25 kDa and control protein level GAPDH was expressed at 37 kDa, respectively. Bcl-2 expression was decreased in I/R group rats as compared to sham-operated control rats after 1 h of hepatic ischemia followed by 3-h reperfusion. In pretreatment with amlodipine, amounts of expression Bcl-2 protein markedly increased as compared to I/R rats, whereas expression of control protein, GAPDH, was constant in all experimental groups (Fig. 3).

Fluorescence Microscopy for Cell Characterization

Apoptosis was evaluated based on distinct morphological features, viz. cell shrinkage, chromatin condensation, oligonucleosomal DNA fragmentation, and breakdown of the cell into smaller units (apoptotic bodies). Reperfusion of the ischemic liver caused severe hepatocellular apoptosis (Fig. 4b), whereas in sham-operated rats there were no signs of apoptosis (Fig. 4a). Pretreatment with amlodipine prevented necrosis and apoptosis (Fig. 4c).

Ultrastructure Analysis by Transmission Electron Microscopy

Mitochondria of hepatocytes of I/R group swelled, cristae were cleared out (Fig. 4b), whereas pretreatment with amlodipine group (Fig. 4c) showed improved cytoplasmic changes as well as retained the fine ultrastructure of the mitochondria (Fig. 4a and 5).

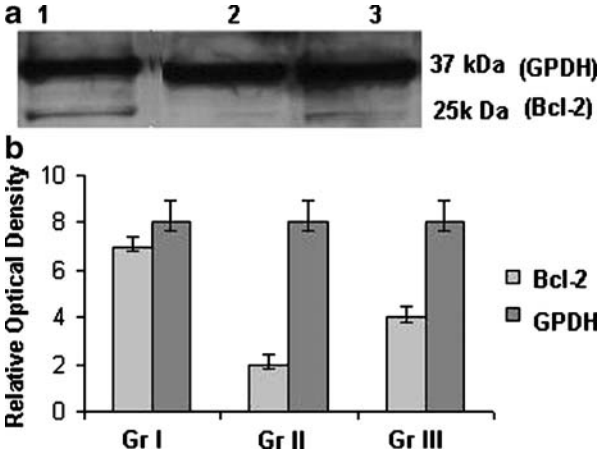

Fig. 3 Expression of Bcl-2 protein. **a** Photograph showing expression of Bcl-2 protein by western blot analysis. *Lane 1* sham-operated control rats (group I), *lane 2* I/R rats (group II), *lane 3* Ca^{+2} channel inhibitor (amlodipine) treated rats (group III). Bcl-2 protein of sham-operated control rats was strongly expressed as compared to I/R rats' liver tissues. **b** Graphical expression of Bcl-2 protein after 1 h of hepatic ischemia followed by 3-h reperfusion. Data are expressed as mean±SD

Discussion

The present study showed that, after 1-h ischemia followed by 3-h reperfusion, mitochondrial cystolic Ca^{2+} ions increased and expression of Bcl-2 gene was suppressed. Pretreatment with amlodipine significantly attenuated efflux of Ca^{2+} into mitochondria and increased Bcl-2 expression which resulted in diminished hepatic necrosis and apoptosis caused by liver I/R in rats.

Earlier studies suggest that Bcl-2 may have a more general role in regulating mitochondrial metabolism and function [11, 13] apart from its anti-apoptotic role. Further, Bcl-2 opposes release of cytochrome *c* either by binding and sequestering pro-apoptotic members, or by binding to proteins such as voltage-dependent anion channel (VDAC) and blocking the formation or opening of a cytochrome *c* release pathway. Bcl-2 is localized to the outer mitochondrial membrane and Bcl-2 family proteins have been reported to interact with VDAC [14], and Bcl-2 promotes VDAC closure [15]. Ca^{2+} is also one of the major ions in regulating VDAC opening and closing. It is likely that in I/R injury overloading of Ca^{2+} in mitochondria leads to opening of VDAC, which inhibits the Bcl-2 gene function. Also, vasodilatation of blood vessels inhibits VDAC opening as well as folic acid inhibits opening of VDAC by DNA stabilization from apoptosis [16].

Further, the most important finding of our study is that, after I/R injury, Bcl-2 expression and cytochrome *c* levels decreased as compared to sham-operated control rats. This result can be explained on the basis of findings of Chen et al. [17], which showed that inhibition of Bcl-2 gene expression results in Bax proteins to migrate and bind to the "permeability transition pore" of mitochondrial membrane inducing loss of selective ion permeability. This results in release of intermembrane space contents including cytochrome *c* and apoptosis-inducing factor (AIF) into the cytosol. AIF moves directly to the nucleus, where it produces chromatin condensation and nuclear fragmentation, while cytosolic cytochrome *c* sets in motion the terminal events of apoptosis of necrosis and apoptosis.

Further, our observation showed that, after 1-h ischemia followed by 3-h reperfusion, accumulation of Ca^{2+} increases which leads to autophagosomes, the reduction in the number of mitochondria, and nuclear condensation. Pretreatment with amlodipine showed considerable prevention in the ultrastructural alteration including disruption of

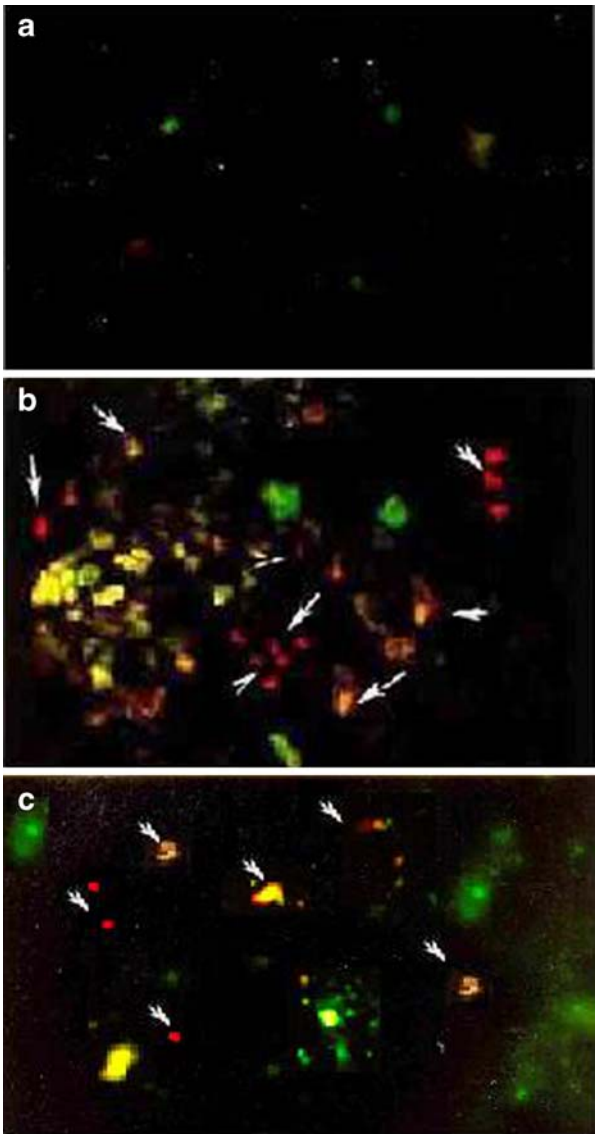

Fig. 4 Fluorescence microscopy of hepatocytes stained with a mixture of ethidium bromide and acridine orange (100 mg mL^{-1}). Viable cells are observed with green nuclear fluorescence and apoptotic cells containing an orange nucleus (*marked arrow*) and exhibiting chromatin condensation. Magnification ×200. **a** Sham-operated group (I). **b** Ischemia followed by reperfusion group (II). **c** Ca^{+2} channel inhibitor (amlodipine) treated group (III)

mitochondrial and nuclear fine structure, and restored numbers of E R as compared to sham-operated control rats.

The present study demonstrated that, after 1-h ischemia followed by 3-h reperfusion, Bcl-2 expression was inhibited and numbers of necrotic cells and apoptotic cells increased which indicated that I/R injury caused necrosis and apoptosis in the rat liver. Studies in the present work showed that pretreatment with amlodipine significantly upregulated Bcl-2 expression, which resulted in diminished hepatic necrosis and apoptosis caused by liver I/R in rats. This observation can be explained as amlodipine specifically blocks L-type Ca^{2+} channels that are exclusively localized to the plasma membrane along with Bcl-2 where it functions to maintain low Ca^{+2} levels [18].

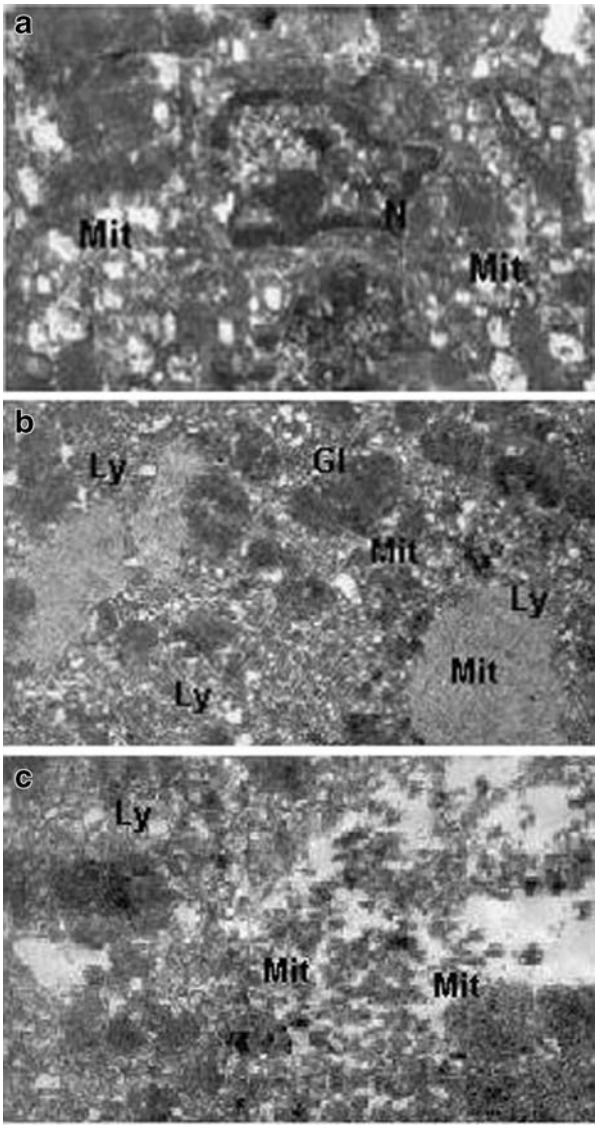

Fig. 5 Transmission electron microscope analysis of liver sections following I/R. **a** Sham-operated control rat (I). **b** I/R-induced group (II). **c** Ca^{+2} channel inhibitor (amlodipine) treated group (III). *Er* endoplasmic reticulum, *Mit* mitochondria, *G* glycogen, *N* nucleus. Uranyl acetate and lead citrate stain ×8,000

Therefore, the present studies support the finding that treatment with amlodipine restored Bcl-2 function after I/R injury by maintaining low Ca^{+2}. Also, this can be explained in the work by Gibson et al. [19] that amlodipine indirectly restored Bcl-2 expression by inhibiting Ca^{2+} efflux into mitochondria and prevention of activation of calcineurin which reduces phosphorylation of Bad, a Bcl-2 family pro-apoptotic protein, and therefore reduced apoptosis in I/R rat. However, this present study provides new insight into the mechanism of calcium channel blocker by which it mediates protection from apoptosis by indirect Bcl-2 upregulation.

The present investigation thus shows that Ca^{+2} concentrations regulate Bcl-2 expression and are key determinants of ischemia–reperfusion-induced apoptosis.

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