



## Protective Effect of Folic Acid against Apoptosis Induced by Ischemia/Reperfusion Injury in Rat Liver

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### Abstract

The anti-apoptotic gene *bcl-2* is located in mitochondria, but it is uncertain whether its expression affects hepatocyte survival in ischemia/reperfusion (I/R) injury. This experiment was designed to evaluate the role of folic acid in expression of *bcl-2* in I/R in rat liver. Eighteen Wister rats were divided into sham-operated control group (C) ( $n=6$ ), I/R group ( $n=6$ ), folic acid treated group which received 1 mg/kg/day folic acid by oral route for 7 days before induction of I/R ( $n=6$ ). Bcl-2 expression was measured by RT-PCR and western blot methods. Folic acid significantly increased Bcl-2 mRNA expression in comparison to the I/R group. Quantification of apoptotic and necrotic hepatocytes, measured by fluorescence microscopy and terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick end labeling (TUNEL) method, showed a significant decrease in apoptosis and necrosis of hepatocytes in folic acid-treated group. Histopathological examination of the liver revealed that folic acid protected from severe hepatic degeneration from I/R injury. The biochemical parameters like alanine transaminases and aspartate transaminases were significantly decreased in folic acid-treated group compared to I/R group. In conclusion, folic acid afforded significant protection against I/R injury due to its ability to inhibit I/R-induced apoptosis.

**Keywords:** Apoptosis; Bcl-2 gene; Ischemic/reperfusion injury.

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### 1. Introduction

Temporary clamping of the portal triad is a common strategy to minimize bleeding during hepatic resection. Unfortunately, this

method resulted in hepatic ischemia-reperfusion (I/R) injury and may cause postoperative functional disorder of the liver. Development of new strategies to attenuate hepatic I/R injury is important for achieving a better clinical outcome. Recently, apoptosis and cell atrophy have been indicated as an important mode of cell death during hepatic

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I/R injury [1]. Apoptosis is governed by a number of regulating genes, such as *bcl-2* whose expression determines survival or death following an apoptotic stimulus [2]. Overexpression of *bcl-2* gene by transferring this gene into hepatocytes with adenovirus increases resistance to hepatic I/R injury [3]. Folate is a cofactor in 1-carbon metabolism, during which it promotes remethylation of homocysteine. In addition to augmenting the risk of vascular events, low folate/high homocysteine may directly increase the susceptibility of neurons to brain injury [4]. Folate deficiency causes uracil misincorporation into DNA and chromosomal breakage, which has implications for neuronal damage [5-7]. In the present study, we have analyzed the affects of folic acid supplementation in sinusoidal reperfusion and apoptosis under defined surgical techniques *i.e.* portal triad clamping (Pringle manoeuvre).

## 2. Materials and methods

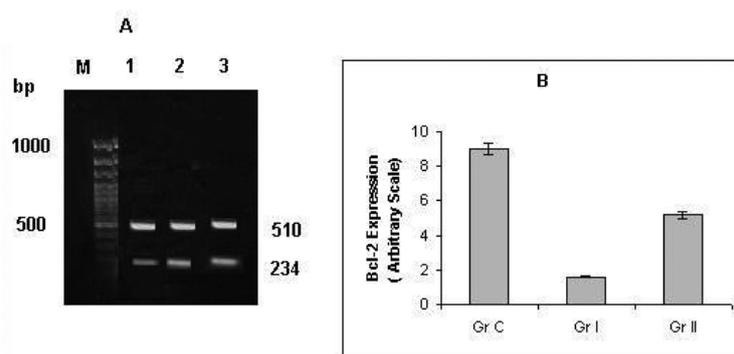
### 2.1. Animals and treatment

Male Wister rats weighing 200 to 250 g were purchased from Laboratory Animal Resources, Indian Veterinary Research Institute, Izzatnagar, UP, India, and were maintained under temperature-controlled rooms at animal house, College of Pharmacy,

IFTM, Moradabad, UP with 12 h alternating light and dark cycles. They were given nutrition and water *ad libitum*. All experimental protocols using animals were performed according to the "Principles of Laboratory Animal care" (NIH publication 85-23, revised 1985). Ischemia and reperfusion injury was induced according to the procedure described by Hayashi *et al.* [8]. The rats were divided into three groups with 6 animals in each group, and studies were performed after 3 h of reperfusion. The rats were divided into sham-operated control group (C), ischemia and reperfusion group (Group I), folic acid treated group (Group II) which received 1 mg/kg/day folic acid by oral route for 7 days before induction of I/R. After 3 h, the right atrium was punctured and blood was collected and stored with heparin at -8 °C. The blood was centrifuged and serum was separated and stored at -8 °C for biochemical analysis. The left and median lobes of the liver were removed and stored in 10% formalin for histopathological studies.

### 2.2. Biochemical analysis

Blood was collected without any anticoagulant and the serum was separated. Serum samples were used for assay of alanine aminotransferase (ALT) [EC 2. 6. 1. 2],



**Figure 1.** *bcl-2* gene expression. A: Representative photograph of the expression of *bcl-2* gene using RT-PCR analysis. *bcl-2* gene expressed at 234 bp and house keeping gene glyceraldehydes-3 phosphate dehydrogenase expressed at 510 bp. Lane M: Marker, Lane 1: sham-operated control group, Lane 2: I/R, Lane 3: Folic acid treated+I/R. B: The expression of *bcl-2* gene after 1 h of ischemia and 3 h of reperfusion. Data are expressed as the mean±SEM.

aspartate aminotransferase (AST) [EC 2. 6. 1. 1]. ALT and AST were measured by commercial Merck Kits.

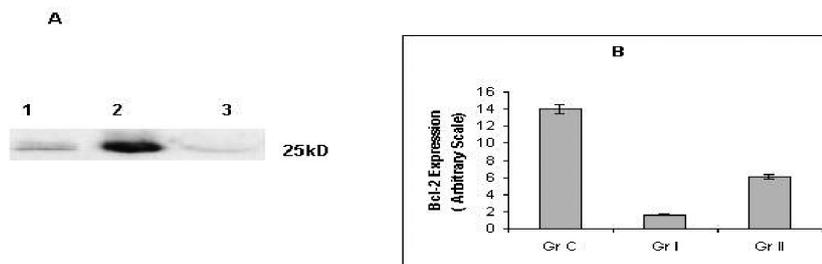
### 2.3 Reverse transcriptase polymerase chain reaction assay

Total cytoplasmic RNA of each group was isolated from liver tissue by the TRIZOL method (according to GIBCO specification). Polymerase chain reaction (PCR) primers were against rat *bcl-2* sequences 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. The product length was 234 base pairs Tm of 52.3 °C. The cDNA was then amplified by PCR amplification with Ampli Taq Polymerase. The quality of cDNA was checked by PCR amplification of the house keeping gene encoding for glyceraldehydes-3-phosphate dehydrogenase. For PCR amplification, the reaction mixture contained 100 ng (2µl) of cDNA, 5µl 10 × PCR buffer, 1 µl 30 mM dNTP mix, 1 µl forward primer (100 ng/µl) and 1 µl reverse primer (100 ng/µl) and 0.5 U Ampli Taq polymerase, and final volume was adjusted to 50 µl. After heating to 94 °C for 7 min., DNA was

subjected to 40 cycles of PCR by denaturing at 94 °C for 30s, annealing at 52.3 °C for 30s and extending at 72 °C for 1 min. The last cycle was followed by a 5 min. elongation step. Eighteen µl of amplified product was resolved by electrophoresis on 1.5% agarose gels, 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.

### 2.4. Western blot analysis

Cell lysates were prepared from liver and lysed in a buffer containing 1% Triton X-100, 10 mM Tris buffer (pH=7.4), 150 mM NaCl, 2 mg/ml aprotinin and 10 mM phenylmethyl sulfonyl fluoride (PMSF). Protein samples (50 µg) 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-



**Figure 2.** The expression of *bcl-2* protein. A: Representative photograph of the expression of *bcl-2* protein using western blot analysis. Lane 1: Folic acid-treated ischemia and reperfused liver protein (group II). Lane 2: Sham-operated control group. Lane 3: Ischemic and reperfused liver protein (group I). B: The expression of *bcl-2* protein after 1 h of ischemia and 3 h of reperfusion. Data are expressed as the mean±SEM.

mouse/rat *bcl-2* primary antibody, followed by peroxides-labeled goat anti-rabbit secondary antibody and the bound antibodies 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.

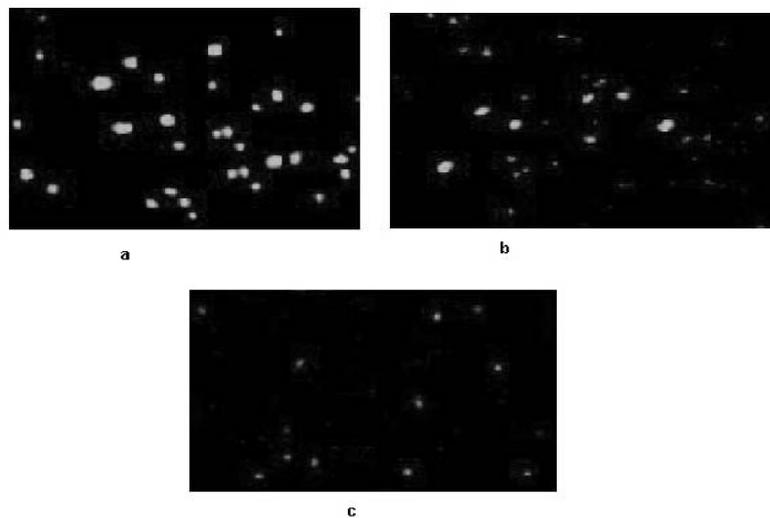
### 2.5. *In situ* detection of cell death

Liver apoptosis detected by TUNEL method. Specimens were dewaxed and immersed in phosphate-buffered saline containing 0.3% hydrogen peroxide for 10 min. at room temperature and then incubated with 20 mg/ml proteinase K for 15 min. Seventy-five  $\mu$ l of equilibration buffer were applied directly onto the specimens for 10 min. at room temperature, followed by 55 ml of TdT enzyme and then at 37 °C for 1 h. The reaction was terminated by transferring the slides to pre-warmed stop/wash buffer for 30 min. at 37 °C. The specimens were

covered with a few drops of rabbit serum and incubated for 20 min. at room temperature and then covered 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.

### 2.6. *Fluorescence microscopy*

Cells were washed with PBS and fixed with PBS-buffered (pH=7) 4% formaldehyde:1.5% methanol solution (CDH, Mumbai) at 4 °C for 15 min., then washed three times with PBS (Life Technologies, Inc.). Twenty five  $\mu$ l cells were taken in a PCR Tubes and 5  $\mu$ l ethidium bromide (100  $\mu$ l/ml) were added. Next, the cover slips were put on slides coated with buffered mounting medium consisting of 90% glycerol:10% PBS with 0.1% NaN<sub>3</sub> and 3% DABCO (triethylenedi-



**Figure 3.** Fluorescence microscopy of the liver cells. a: Nuclear morphological changes after IR injury. Heavy DNA fragmentation and condensation are seen after staining with ethidium bromide ( $\times 40$ ). b: Some DNA fragmentation and condensation is seen after staining with ethidium bromide in folic acid treated ( $\times 40$ ). c: Some apoptotic DNA shows after staining with ethidium bromide in sham operated group ( $\times 40$ ).

amine; Sigma Chemical Co., St. Louis, MO), to prevent fading. Examination was done with inverted Zeiss confocal laser scanning microscope (LSM410; Carl Zeiss, Jena, Germany). For ethidium bromide, maximum excitation was performed by a 543-nm line of the internal He-Neon laser, and fluorescence emission was observed above 570 nm with longpass barrier filter LP-570. A water immersion objective, C-Apochromat 6331.2 W corr. (Zeiss), was used. Images were converted to TIFF format, and the contrast level and brightness of the images were adjusted by using the Zeiss LSM410 program.

### 2.7. Histopathological evaluation light microscope assay

Serial slices of liver tissues were prepared from rat in each group and stained with hematoxyline-eosin (HE) and then observed under light microscope at 200× or 400× magnification.

### 2.8. Transmission electron microscopy assay

Liver tissue were fixed in Karnovsky's solution pH 7.4 for 4 h at 4 °C and post-fixed with glutaraldehyde and osmium trioxide, respectively. Thick section were cut and stained with toluidine blue. Thin sections of 70 nm were stained with uranyl acetate and lead citrate and viewed under Moragagni 268D electron microscopes (Netherlands).

### 2.9. Statistical analysis

All values were expressed as mean±SEM. Differences in mean values were compared using SPSS 11.0 by one-way ANOVA and Student-Newman-Keul (SNK) test. A  $p < 0.05$  was considered as statistically significant.

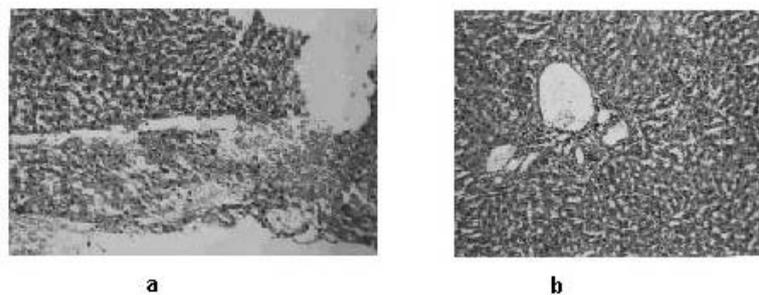
## 3. Results

### 3.1. Liver function tests

Serum ALT and AST values were markedly increased by I/R (group I) compared to the sham-operated control group (C) ( $p < 0.05$ ). Folic acid pre-treatment for 7 consecutive days (group II) markedly prevented the raise in serum ALT and AST levels induced by I/R (Table 1). Folic acid, therefore, protects liver tissue from I/R induced acute injury.

### 3.2. Semi quantitative RT-PCR analysis of apoptosis-related genes *bcl-2* mRNA

We investigated the expression of *bcl-2* gene by RT-PCR method. The length of RT-PCR products of *bcl-2* was 234 bp (Figure 1) and expression of *bcl-2* gene in group I was lower compared to the sham-operated group after I/R. After treatment with folic acid (group II), the level of *bcl-2* gene expression was significantly restored to near its level in sham-operated group after I/R (Figure 1). In group II, the amounts of *bcl-2* mRNA transcript were markedly elevated in comparison with group I at matched times



**Figure 4.** Histopathology of rat liver. a: Fatty vacuolation degeneration, individualization or small clumps of hepatocytes in the centric-lobular hepatic cord disturbed observed in group I (I/R) (H.E.× 400). b: Folic acid treated hepatocytes (group II) shows hepatic cord is well maintained and sinusoids are dilated (H.E.× 400).

**Table 1.** ALT and AST levels of liver in folic acid treated I/R injury, I/R injury and sham operated rats.

| Group                              | ALT (IU/L)                | AST (IU/L)                |
|------------------------------------|---------------------------|---------------------------|
| Sham-operated control group (C)    | 84.72±8.20                | 56.02±11.92               |
| Ischemia and reperfusion group (I) | 154.36±30.58 <sup>a</sup> | 517.40±14.52 <sup>a</sup> |
| Folic acid Treated group (II)      | 271.08±5.21 <sup>b</sup>  | 205.46±12.14 <sup>b</sup> |

Mean±SEM, n=6. <sup>a</sup>Significantly different from sham operated control group ( $p<0.05$ ). <sup>b</sup>Significant different from group I ( $p<0.05$ ).

after reperfusion ( $p<0.05$ ).

group I during I/R ( $p<0.05$ ).

### 3.3. Western blot analysis of Bcl-2 protein

*bcl-2* protein was expressed at 25 kDA (Figure 2) and expressed lower level in group I compared to the sham-operated group after 1 h of ischemia and 3 h of reperfusion. After treatment with folic acid, the amount of expression of *bcl-2* protein in group II was markedly increased in comparison to its level in group I.

### 3.5. Fluorescence microscopy

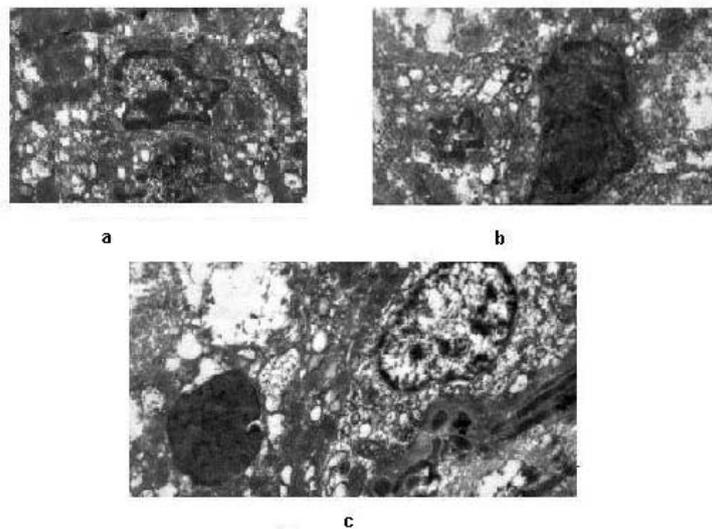
Abnormal condenses nuclear fragmentation, membrane blabbing, cell shrinkage and apoptotic body were observed in I/R treated group (group I; Figure 3a). However, little condenses and some nuclear fragments were observed in folic acid treated group (Figure 3b) and exhibited normal structure similar to those of the sham operated group (Figure 3c).

### 3.4. Change of cellular apoptotic rates

Apoptosis was measured by TUNEL assay. TUNEL-positive cells were detectable after I/R. The cellular apoptosis rate in group II was five times higher than in control group (Table 2). As shown in Table 2, administration of folic acid produced statistically significant decreases in the apoptosis rate compared to

### 3.6. Histopathological studies

After I/R, swelling and fatty vacuolation, degeneration, individualization or small clumps of hepatocytes in the centric-lobular hepatic cord with cells showing several pyknotic condensed nuclei and cellular atrophy were found in hepatocytes of group



**Figure 5.** TEM analysis of liver sections following I/R. a: Group I liver section shows hepatocellular apoptosis with mitochondria were severely swollen and had a reduction in the number of cristae (5.0 K.E.X). b: Sham operated liver section shows normal architecture of cell. Prominent nucleus with well defined cell wall and cell membrane observed (7.0 K.E.X). c: Group II liver section shows structures similar to those of the sham group. Well defined nucleus with swollen mitochondria but no sign of apoptosis ( 5.0 K.E.X).

I animals (Figure 4a). Folic acid-treated group showed normal structure of hepatocytes with well developed portal area. Hepatic cord and sinusoids were moderately conjugated. Compartment of hepatic sinus became dilated or disappeared along with little necrosis, atrophy and hemorrhage at I/R side was observed. Any pyknotic condensed nuclei were not observed, and centric lobular portion hepatic cord is well maintained (Figure 4b).

### 3.7. TEM analysis

After 1 h of ischemia and 3 h of reperfusion in group I, mitochondria were severely swollen and had a reduction in the number of cristae. Smooth endoplasmic reticulum increased, glycogen granules decreased and nucleus was not well marked, and more secondary lysosomes were observed (Figure 5a). In group C, hepatocytes were observed only with prominent nucleus, and no abnormality was observed in cell (Figure 5b). In group II, mitochondria were moderately swollen and smooth endoplasmic reticulum and well marked nucleus were observed (Figure 5c).

## 4. Discussion

In the present study, we demonstrated the beneficial effects of the folic acid on I/R injury. Treatment with folic acid significantly reduced the ALT and AST level increased by I/R and improved hepatocyte structure against I/R injury.

Some evidence have associated low blood levels of folate with a greater risk of cancer [9]. Folate is involved in the synthesis, repair, and functioning of DNA and a deficiency of folate may result in damage to DNA that may

lead to cancer [10]. Recognition of the ancillary actions of folic acid is important for understanding the agent's mechanisms of action and the pathologic mechanisms underlying I/R injury. Our previous study showed that folic acid increases DNA content and cytochrome P<sub>450</sub> levels in ischemic/reperfused liver, presumably as a result of inhibitory action of TNF- $\alpha$  by folic acid [11]. It was reported that portal triad clamping produces not only ischemic injury of the liver but also portal venous congestion [12]. Acute portal venous congestion for a long period may impair the intestinal mucosal barrier and increase intestinal permeability, causing endotoxemia, bacterial translocation, activation of reactive oxygen radicals and inflammatory cytokines, such as tumor necrosis factor- $\alpha$  [13-15]. Reperfusion of stagnant portal venous blood with deleterious chemical mediators into the ischemic liver aggravates the liver injury, leading to intra-abdominal sepsis and abscess formation, which is the major cause of postoperative septic complications induced by hepatic I/R injury [16, 17]. However, attempts to protect against hepatic I/R injury by alleviating the possible detrimental effects of portal venous congestion have not achieved satisfactory results. Hepatic injury caused by ischemia can be described as necrosis. It was reported that apoptosis of hepatocytes and sinusoidal endothelial cells is a critical mechanism contributing to hepatic I/R injury [8]. A number of genes regulate the apoptotic process. The family of bcl-2-related proteins plays a key role in the regulation of apoptosis. The *bcl-2* gene, can promote cell survival through protein-protein interactions with other *bcl-2*-related protein family members. Recent

**Table 2.** Effect of folic acid treatment on the apoptosis rates in liver cells after 1 h of ischemia and 3 h of reperfusion (TUNNEL assay).

| Group                                      | % of Apoptotic cells    |
|--|-------------------------|
| Sham-operated control treated group (C)    | 0.45±0.021              |
| Ischemia and reperfusion treated group (I) | 24.11±1.26 <sup>a</sup> |
| Folic acid treated group (II)              | 10.19±1.54 <sup>b</sup> |

Mean±SEM, n=6. <sup>a</sup>Significantly different from Group C ( $p<0.05$ ).

<sup>b</sup>Significant different from group I ( $p<0.05$ ).

studies indicated that overexpression of *bcl-2* gene could reduce hepatocellular apoptosis after reperfusion and protect against hepatic I/R injury [18, 3]. *Bcl-2* protein is a homodimer which inhibits cell death. Therefore, increased expression of *bcl-2* may determine survival following apoptotic stimuli and attenuate the anti-apoptotic effect of *bcl-2* gene by reducing post-ischemic apoptosis [19, 20]. In this study, upregulation of *bcl-2* gene expression in rat liver were found after 1 h of ischemia and 3 h of reperfusion, indicating that folic acid can protect against hepatic apoptosis induced by I/R injury by regulating the expression of *bcl-2* gene. In the present study, treatment with folic acid improved the degree of hepatocellular structure. Although it is difficult to analyze the pathophysiological mechanism by which folic acid inhibits from I/R injury, our finding that the folic acid altered the expression levels of *bcl-2* gene may provide a clue.

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