Folic acid inhibits necrosis and apoptosis in ischemic and reperfusion induced injury in rat liver

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SUMMARY

Temporary clamping of the portal triad is a common strategy to minimize bleeding during liver transplantation. Increasing evidences suggests that oxygen derived free radicals and reintroduction of oxygen in ischemic tissue lead to ischemic and reperfusion injury (I/R) and lead to apoptosis and necrosis. Adult Wistar rat subjected to 60 min of partial liver ischemia followed by three hour reperfusion. Eighteen Wister rats were divided into sham-operated control group (I) (n = 6), ischemia and reperfusion group (II) (n = 6), folic acid treated group (1 mg/kg body weight/daily by oral route for 7 days before induced ischemia reperfusion maneuver) (III) (n = 6). Apoptotic and necrotic hepatocytes, mitochondrial antioxidant enzymes were measured. Liver injury was assessed by alanine transaminases (ALT), aspartate transaminases (AST), liver histopathology and electron microscopy. An ischemic and reperfusion hepatocellular injury was indicated by increased serum-ALT, AST, histopathology and electron microscopy studies. Apoptotic and necrotic cells were increased which was revealed by flow cytometry in I/R group. Pre-treatment with folic acid significantly decreased serum-ALT, AST levels, apoptotic and necrotic cells after 1 h ischemia followed by 3 h of reperfusion. Histopathology and TEM studies showed markedly diminished hepatocellular injury in folic acid pretreated rats during the hepatic I/R, which reached a level comparable to saline-treated rat of sham operated group. On the basis of our findings it may be concluded that folic acid afforded significant protection from necrosis and apoptosis in I/R injury.

Key words: Ischemia-Reperfusion; Necrosis and Apoptosis; Folic acid; Anti-oxidant enzymes

INTRODUCTION

During hepatic resection, the risk of severe intra-operative bleeding is a major risk (Delva \textit{et al.}, 1989) To avoid massive blood loss, continuous or intermittent vascular clamping of the hepatic artery and portal vein (‘Pringle manoeuvres’) is an efficient method to reduce hemorrhage (Rudiger \textit{et al.}, 2002). Unfortunately, this method resulted in hepatic ischemia-reperfusion (I/R) injury and may cause postoperative functional disorder of the liver. I/R injury is phenomenon whereby cellular damage is caused by hypoxic organ and further damage caused by resorrition of oxygen delivery. In the liver, this form of injury was recognized as a clinically important pathological disorder. I/R injury is relevant clinically in hepatic surgery,
hypovolemic shock, some types of toxic liver injury, veno-occlusive diseases and Budd-Chiari syndrome (Chattopadhyay et al., 2007).

Oxidative stress has been recognized to play a pivotal role in influencing pathophysiology of I/R leading to organ failure. The level of ROS which increases in I/R injury plays a critical role in the transition from reversible to irreversible reperfusion injury (Halestrap et al., 1998; Halestrap et al., 2004; Di Lisa, 2006). Mechanisms responsible for the increase in free radical production during ischemia/reperfusion suggested future therapeutic strategies for intervention.

Folic acid protects from DNA breakdown by improving De novo biosynthesis and folate is essential to replicate DNA and folate deficiency effects DNA synthesis and cell division, affecting the bone marrow, a site of rapid cell turnover (Zittoun et al., 1993). However role of folic acid in protection of antioxidant enzymes is not reported. Recognition of the ancillary actions of folic acid is important for understanding the mechanisms of action. Therefore, the present study has been undertaken to correlates the effects of folic acid supplementation in antioxidant levels with necrosis and apoptosis under ischemia and reperfusion induced injury.

MATERIALS AND METHODS

Chemicals
Collagenase, L-arginine was purchased from Hymedia (Mumbai, India). Alanine aminotransferase (ALT), aspartate aminotransferase (AST) kits were procured from Merck India Ltd (India). Other chemicals were obtained from Sigma (Sigma, St louis, Mo, USA).

Animals and induction of ischemia and reperfusion
Male Wistar rats weighing 200 - 250 g purchased from Laboratory Animal Resources, Indian Veterinary Research Institute, Izzatnagar, UP, India were maintained under temperature-controlled rooms at animal house, College of Pharmacy, IFTM, Moradabad, UP with 12 h alternating light and dark cycles. Adequate nutrition and water were given 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 produced as per the procedure described by Hayashi et al. (1998). Eighteen Wistar rats were divided into sham-operated control group (I) (n = 6), ischemia and reperfusion group (I/R) (II) (n = 6) and Folic acid treated group (1 mg/kg body weight/ daily by oral route for 7 days before induced ischemia reperfusion maneuver) (III) (n = 6). In all groups, rats were sacrificed after 1 h ischemia followed by 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 using sterile syringe containing 50 µl of heparin (100 USP Units/ml), and centrifuged to separate the serum. The serum samples were stored at -20 °C until used for ALT and AST assays. A portion of the ischemic and non-ischemic liver lobes were fixed in buffered 10% formalin, embedded in paraffin, and used for hematoxylin and eosin (H&E) staining. Another portion of ischemic and non-ischemic liver lobe was snap frozen in liquid nitrogen and stored at -70 °C until use for assessment of apoptosis.

Bio - Chemical Analysis
Serum AST [EC 2.6.1.2], ALT [EC 2.6.1.1] estimated by Merck kits (Merck India Ltd, Mumbai, India) according to the manufacture’s instructions.

Assay of Mitochondrial antioxidants
Mitochondria of liver were isolated by the method of Starks and Fiskum (2003). Briefly, 100 mg liver was excised with ice-cold isolation buffer containing 225 mM mannitol, 75 mM sucrose, 5 mM HEPES-KOH, pH 7.4, 1 mM EGTA, and 1 g/mL bovine serum albumin in a 15 ml Dounce homogenizer.
and homogenized manually with eight strokes of pestle A followed by eight strokes of pestle B. The homogenate was diluted with 15 ml of isolation buffer, distributed into four centrifuge tubes, and centrifuged at 3000 rpm for 4 min. The supernatant was separated and centrifuged again at 14,000 rpm for 10 min. The pellet was resuspended in 15 ml of the ice-cold isolation buffer without BSA and kept on ice, and 30 µl of digitonin (10% stock solution in DMSO) was added. After 4 min incubation with occasional stirring by slow inversion of tubes, the suspension was diluted with 15 ml of ice-cold isolation buffer containing BSA and centrifuged at 14,000 x g for 10 min. The pellet was resuspended in 8 ml of ice-cold isolation buffer containing neither BSA nor EGTA and centrifuged again at 14,000 rpm for 10 min. The final pellet containing mitochondria was resuspended in isolation buffer without EGTA or BSA to a concentration of 25 - 30 mg/ml of protein, stored in ice, and was used within 5 h.

The mitochondrial antioxidants glutathione- S – transferase (GST) measured as described by Habig et al. (1974). Briefly, 0.2 ml mitochondrial enzyme to 2 ml distilled water followed by 3.0 ml of precipitating mixture (1.67 g metaphosphoric acid, 0.2 g EDTA and 30 g NaCl) and finally distilled water was added to make up volume to 100 ml. Solution was centrifuged at 5000 rpm for 5 min. To 1 ml supernatant was added 1.5 ml of phosphate buffer pH 7.4, followed by 0.5 ml DNTB reagent. The optical density was measured at 412 nm using spectrophotometer. Absorbance of standard reduced GSH was measured and calculation was done.

Superoxide dismutase (SOD) was measured as described by Hodgson and Fridovich (1975). 50 µl of the mitochondrial enzyme was mixed with 75 mM Tris-HCl buffer (pH 8.2), 30 mM EDTA and 2 mM of pyrogallol and absorbance was recorded at 420 nm at interval of 3 min by spectrophotometer. One unit enzyme activity is equivalent to 50% inhibition of the rate of auto-oxidation of pyrogallol. The activity of SOD was expressed as units per mg of protein.

Lipid peroxides (LPO) as described by Jordan and Schenkman (1982). To 100 µl separated mitochondrial enzymes in 0.1 (M) phosphate buffer saline, 1 ml of 28% trichloroacetic acid was added and centrifuged at x 2000 g at 4 °C for 20 min. 1 ml of supernatant was separated and 900 µl of 1% thiobarbituric acid was added and volume was adjusted to 3 ml by using phosphate buffer (pH 7.0), heated in water bath for 60 min and cooled in ice bath. The absorbance was measured at 532 nm. The lipid peroxidation was calculated on the basis of the molar extinction coefficient.

Flow cytometry analysis
Hepatocytes (1 × 109/L) then washed with PBS, exposed to Propinum iodide (PI) 50 mg/L, 0.1% Triton X-100, 0.01 mM EDTA (Na) and RNase 50 mg/L at normal temperature in darkness for 12 - 24 h. Specimen were then presented to the FACS-420 Flow Cytometry Analyzer (New York, Becton Dickinson and Company, 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 hepatocytes staining with PI and cytogram (distribution).

Histopathological evaluation light by 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 x or 400 x magnification.

Transmission electron microscopy (TEM) studies
Liver tissue were fixed in Karnovsky’s solution pH 7.4 for 4 h at 4 °C and postfixed with glutaraldehyde and osmium trioxide respectively. Thin sections of 70 nm were stained with toluidine blue followed by uranyl acetate and lead citrate and viewed under Moragagni 268 D electron microscopes (Netherlands).

Statistical analysis
All values were expressed as mean ± S.D. Differences
in mean values were compared using SPSS 11.0 by one-way ANOVA and Student-Newman-Keul (SNK) test. \( P < 0.05 \) was considered as statistically significant.

**RESULTS**

**Hepatic marker enzymes**

Activities of ALT, AST were 84.72 ± 8.2 and 56.02 ± 11.92 respectively in sham operated rats which increased to 1507.36 ± 30.58 and 817.40 ± 14.52 after 1 h ischemia followed by 3 h reperfusion. Pretreatment with folic acid significantly \( (P < 0.01) \) inhibits increasing of hepatic marker enzymes in I/R group (Table 1).

Activities of anti-oxidant enzymes like GST, SOD, and LPO were 58.21 ± 4.3, 14.32 ± 2.6 and 4.10 ± 0.60 respectively in sham operated control rats which decreased to 38.51 ± 2.1, 41.54 ± 2.98 and 7.25 ± 0.33 respectively. Pretreatment with folic acid attenuated the changes of anti-oxidant enzymes and accredits towards sham operated control rats (Table 1).

Percentage of necrosis and apoptosis of primary hepatocytes of the sham operated control group were 1.02 ± 0.32 and 0.70 ± 0.08, respectively. In I/R rats, necrotic and apoptotic cells were increased to 21.54 ± 7.1 and 26.44 ± 6.0 respectively. In folic acid treated rats necrosis and apoptosis was significantly decreased as compared to I/R rats (Fig. 1 and Table 2).

After 1 h ischemia followed by 3 h reperfusion mitochondria were severely swollen and had reduction in the number of cristae as well as smooth endoplasmic reticulum increased and nucleus was not well marked whereas sham operated control rats hepatocytes observed with prominent nucleus and no abnormality was observed. Pretreatment with folic acid rats mitochondria were moderately swollen and moderately well identified nucleuses were observed as compared to I/R rats (Fig. 2).

Liver histopathology was evaluated based on sinusoidal congestion, cytoplasmic vacuolization, and necrosis and apoptosis of primary hepatocytes.

| Table 1. Shows ALT, AST, GST, SOD and LPO levels of liver in Sham operated control, I/R injury and folic acid treated I/R injury group’s rats |
|---------------------------------|-----------------|-----------------|-------------|-------------|--------|
| Group                          | ALT             | AST             | GST#         | SOD†         | LPO†‡  |
| Sham-operated control Group (I)| 84.72 ± 8.2     | 56.02 ± 11.92   | 58.21 ± 4.3  | 14.32 ± 2.6  | 4.10 ± 0.60 |
| Ischemia and reperfusion Group (II)| 1507.36 ± 30.58| 817.40 ± 14.52 | 38.51 ± 2.1  | 41.54 ± 2.98 | 7.25 ± 0.33 |
| Folic acid treated Group (III)  | 271.08 ± 5.21   | 205.46 ± 12.14  | 14.32 ± 2.6  | 5.20 ± 4.3   | 5.10 ± 0.28 |

*Mean ± S.D. n = 6. aSignificantly different from sham rat (\( P < 0.05 \)); bSignificant different from I/R injury rat (\( P < 0.05 \)); expressed as 1.U/L; cexpressed as CDNB oxidized per min per 100 mg protein for GST; dexpressed as Units per mg per 100 mg protein for SOD; eexpressed as nmol of MDA/mg of protein.

Fig. 1. Flow cytometry analysis assessing apoptosis and necrosis hepatocytes in ischemic and reperfusion injured rats after propinum iodide and annexin V staining. (a) hepatocytes of sham operated, (b) hepatocytes of I/R rats, (c) Folic acid treated rats.
Table 2. Shows Apoptotic cells and Necrotic cells (Flow cytometry) of liver in Sham operated control, I/R injury and folic acid treated I/R injury group’s rats

<table>
<thead>
<tr>
<th>Group</th>
<th>% of Necrotic Cells</th>
<th>% of Apoptotic Cells</th>
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<tbody>
<tr>
<td>Sham-operated control Group (I)</td>
<td>0.56 ± 0.21</td>
<td>0.20 ± 0.08</td>
</tr>
<tr>
<td>Ischemia and reperfusion Group (II)</td>
<td>21.54 ± 7.14*</td>
<td>26.44 ± 6.04*</td>
</tr>
<tr>
<td>Folic acid Treated Group (III)</td>
<td>15.32 ± 2.87b</td>
<td>12.10 ± 3.2b</td>
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Results are expressed as mean ± S.D. (n = 6); *Significantly different from sham rat (P < 0.05); bSignificant different from I/R injury rat (P < 0.05).

Fig. 2. TEM analysis liver sections following I/R (x8000). (a) Sham operated control rats showing short segment of smooth cisterina of the endoplasmic reticulum (ER). (b) I/R rats showing severe degeneration of normal architecture of hepatocytes. Diffuse out Lysomes (L) and were severely swelled mitochondria (M). (x 8,000). (c) Folic acid treated rats showing extended smooth endoplasmic reticulum (ER) composed of numerous profiles of smooth tubules (ser). Decreased sinusoids dilation as compared to I/R rats.

Fig. 3. Histopathology of liver stained with hematoxylin-eosin (H&E) (H&E, x 400). (a) sham operated liver section showing normal architecture of liver with central vein (c.v). (b) I/R rat liver section showing sinusoidal congestion(su), cytoplasmic vacuolization (v), hepatocellular necrosis, and neutrophil infiltration. (c) liver section showing only minor patchy spots of mild necrosis in various areas with exhibited normal structure similar to those of the sham group.

hepatocellular necrosis, and neutrophil infiltration. Histopathology examination of liver sections of sham operated control group showed normal cellular architecture with distinct hepatic cells, sinusoids spaces and central vein. Ischemic and reperfusion injury groups showed the disarrangement and degeneration of normal hepatic cells with intense centrilobular necrosis extending to mid – zone and sinusoidal hemorrhages and dilation. There was chronic inflammatory cells infiltrate in the portal tracks. There was also extensive hepatocellular necrosis, sinusoidal congestion, and neutrophil infiltration. Folic acid treated rat showed prominent kupffer cells, alisonucleosis and mild necrosis. It was observed that regions of contiguous hepatocytes with increased cytoplasmic eosinophilia and normal
nuclei. Nodular hyperplasia and atrophy were significantly reduced as compared I/R group. Folic acid treated rats showed less vacuole formation, reduced sinusoidal dilation and less disarrangement and degeneration of hepatocytes. But centrilobular necrosis and apoptosis were observed and intensity was less as compared to I/R group rats (Fig. 3).

DISCUSSION

Administration of folic acid in I/R rats showed decrease necrosis and apoptosis as compared to I/R rats. This can be explained as folate is a cofactor in 1-carbon metabolism (Bilbao et al., 1969) and prevents mitochondrial DNA damage. Folic acid deficiency causes uracil mis-incorporation into DNA and chromosomal breakage (Kruman et al., 2002; Mattson et al., 2003). Folic acid also prevents mitochondrial DNA from oxidative injury (Blount et al., 1997; Goulian et al., 1997). Thus, folic acid plays a cytoprotective role to hepatocytes, which prevents hepatocytes from I/R injury.

Present studies showed that folic acid protects antioxidant enzymes and down-regulation of apoptotic cells and necrotic cells in rat liver were found after 1 h ischemia followed by 3 h reperfusion indicating that folic acid can protect against hepatic necrosis and apoptosis by IR injury by regulating antioxidant enzymes. The present study using folic acid was aimed to identify a previously unrecognized biological effect of folate in the protection of mitochondria against oxidative insults. The molecular basis behind this increased oxidative stress in folate-depleted is not established. Reduced folate such as tetrahydrofolate and 5-methyltetrahydrofolate has been known for its antioxidant ability to scavenge free radicals in vitro (Rezk et al., 2003) and to alleviate oxidative stress in vivo (Doshi et al., 2001; Coppola et al., 2005). Folate was reported to interact with nitric oxide synthase, improve quinonoid BH4 availability and reduce superoxide production (Doshi et al., 2001; Stroes et al., 2006). The present study showed that after administration of folic acid mitochondrial antioxidant level was reduced which indicates that formation of tetrahydrofolate and 5 – methyl tetrafolate after administration of folic acid by 1st pass metabolism. Thus, our study suggests that folic acid is biologically active to protect mitochondria from oxidative injuries.

In present studies, the ultra structural changes demonstrated microscopically in I/R injured rats which showed severe degeneration of cellular architecture. Pre - treatment with folic acid showed considerable prevention in the ultra structural alternations including distribution of mitochondrial fine structure. This indicates that administration of folic acid scavenge the free radicals thereby prevent the cellular damage in the membrane. Histological examination of the liver revealed regular sinusoidal structures in folic acid treated as opposed to swollen cells with marked vacuolization seen in ischemic and reperfused rats. Thus, treatment with folic acid improved the degree hepato cellular structure.

Conclusively, the present findings suggest that treatment with folic acid improved the degree hepatocellular structure and apoptotic cells although it is difficult to analyze the pathophysiological mechanism by which folic acid inhibits from I/R injury, our finding that the folic acid attenuated antioxidant enzymes may provide a clue.

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