PHARMACOLOGICAL, MOLECULAR AND BIOCHEMICAL ASPECTS IN ATTENUATION OF ISCHEMIC & REPERFUSION INJURY BY FOLIC ACID, AMLODIPINE AND L- ARGININE IN RAT LIVER TRANSPLANTATION MODEL

THESIS

Submitted in partial fulfillment
of the requirements for the degree of
DOCTOR OF PHILOSOPHY

By
PRONOBESH CHATTOPADHYAY

Under the Supervision of
Prof. Arun Kumar Wahi

BIRLA INSTITUTE OF TECHNOLOGY AND SCIENCE
PILANI (RAJASTHAN) INDIA

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CERTIFICATE

This is to certify that the thesis entitled "Pharmacological, Molecular and Biochemical Aspects in Attenuation of Ischemic & Reperfusion Injury by Folic acid, Amlodipine and L- Arginine in Rat Liver Transplantation Model" submitted by Pronobesh Chattoapdhyay, ID. No. 2005PHXF407 for award of Ph.D. Degree of the Institute embodies original work done by him under my supervision.

Signature (Supervisor) :

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MORADABAD

Date: 29/12/2008

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Place: Moradabad

Pronobesh Chattopadhyay

Orthotopic liver transplantation is now well-established treatment for chronic end-stage liver disease. With improved supportive care, the mortality of 97% in patients who were managed without transplantation has decreased to 57%.

During liver transplantation, the risk of severe intra - operative bleeding is a major risk. To prevent massive blood loss, continuous or intermittent vascular clamping of the hepatic artery and portal vein ligation is known as "Pringle manoeuvere", is an efficient method to reduce hemorrhage, which leads to ischemic, and reperfusion injury. Liver dysfunction or failure after transplantation is still a significant clinical problem after transplantation surgery. The organ shortage for transplantation forces consideration of cadaveric or steatotic grafts, though ischemia-reperfusion injury is higher as compared to fresh graft, therefore, for further improvement, to reduce ischemia- reperfusion, the present study was undertaken.

L- arginine, amlodipine and folic acid were selected for studies on I/R rat liver as well as direct and indirect effects related to hepatocellular injury to understand the biological mechanisms of the chemopreventive potential of L- arginine, amlodipine and folic acid in I/R injury at cellular and molecular level .

The present study deals with the changes in hepatic marker enzymes (AST and ALT), microsomal cytochrome P_{450} , DNA, RNA, mitochondrial respiratory marker enzymes and tri carboxyl acid marker enzymes as well as ATP production, Ca^{+2} overloading in 1h ischemia followed by 3 h reperfusion (I/R) as compared to sham operated control rats. Further, it deals with studies on the alternation of cellular changes in I/R using histopathology, TEM and SEM. Fluorescence studies after staining with DAPI and acridine orange & ethidium bromide was done for assessment of hepatocytes viability after I/R injury. TUNEL assay and flow cytometry techniques were used to determine necrosis and apoptosis levels and Hoechst 33258 staining was used for chromatin aberration after I/R injury. Apoptotic marker gene Bcl-2 amplified by RTPCR reaction and western blot methods after I/R injury were used to study the level of apoptosis.

Administration of folic acid in I/R rats improved liver function, antioxidant enzymes and liver cytomorphology as well as reduced serum TNF-α level, necrotic and

apoptotic hepatocytes and improved mitochondrial enzymes function as compared to I/R rats which indicates that attenuation of TNF-α by folic acid is possible mechanism of cytoprotection in I/R injury.

Pretreatment with amlodipine significantly attenuated efflux of Ca^{2+} into mitochondria and Bcl-2 expression, prevented hepatocellular damage, improved mitochondrial enzymes in I/R rats which indicated that increased Bcl-2 expression on pretreatment with amlodipine in I/R rat inhibits Ca^{2+} efflux into mitochondria which resulted in prevention of hepatocellular damage and apoptosis.

Administration of L- arginine significantly decreased plasma-ALT, AST and ischemic zone after 1 h ischemia followed by 3 h of reperfusion besides nitric oxide production in hepatocytes was increased by 2 fold as compared to I/R group rats. Histopathology and TEM studies showed marked decrease in hepatocellular injury in L- arginine pretreated rats during hepatic I/R, which was comparable to saline-treated rat of sham operated group.

Cell apoptosis and necrosis decreased in L- arginine treated group of I/R rats as compared to folic acid and amlodipine treated I/R rats which indicates that L- arginine is most potent showing significant protection from I/R injury by up regulating the nitric oxide production.

Contents

List of Abbreviations

A	Adenine
ACD	Acid Citrate Dextrose
ADP	Adenosine - Di – Phosphate
AIF	Apoptosis-inducing factor
ALF	Acute liver failure
ALP	Alkaline Phosphatase
ALT	Alanine Transaminase
ANT	Adenine nucleotide Transaminase
Ant	Adenine nucleotide translocase
A-SMase	Acidic sphingomyelinases
AST	Aspartate Transaminase
ATP	Adenosine- Tri –Phosphate
BSA	Bovine Serum Albumin
C	Cytosine
$Ca\ Cl_2$	Calcium Chloride
Ca^{+2}	Divalent calcium
CAT	Catalase
CCD	Computer Captured Display
CC	Calcium channel currents
Cyt –c	Cytochrome –c
DAPI	4,6-diamidino-2-phenylindole
DCIP	2,6- Dichlorophenol Indophenol sodium salt dehydrade
DMSO	Dimethyl Sufoxide
DNA	Deoxy ribonucleic acid
DNTB	5,5 , dithio bis 2 nitro benzoic acid
DRT	Deoxy ribonucleoside triphosphates
EB	Ethidium bromide
EBSS	Earle Balanced Salt Solution
ECM	Endothelium collagen matrix

EDTA	Disodium ethldiamane tetra acetic acid
EGTA	Ethylene glycol-o,o'bis (2- amino ethyl)-NNN'N' tetraacetic acid
ER	Endoplasmic reticulum
ESLD	End stage Liver Disease
FBS	Fetus Bovine Serum
$FeCl_3$	Ferric chloride
FHF	Fulminant hepatic failure
FITC	Fluorescine isothiocynate
g	Gram
G	Guanine
G_1	Gap 1
G_2	Gap 2
GFP	Green Fluorescence Protein
GPx	Glutathione Peroxidase
GSH	Reduced Glutathione
H& E	Hematoxylin and Eosin
H_2O_2	Hydrogen peroxide
HBSS	Hank balance Salt Solution
HCl	Hydrochloric Acid
HEPES	N-2,Hydroxyethyl piperazine N-2 ethanosulphonic acid
h	Hour
IAEC	Institutional Animal Ethics Committee
I.U	International unit
I/R	Ischemia followed by reperfusion
IL	Interlukin
IPTG	Isopropyl thio galactoside
IR	Inverted Repeat
i.v	Intravenous
KC's	Kupffer Cells

KCN	Potassium cynide
KGDH	α-ketoglutarate dehydrogenase
KH_2PO4	Potassium dihydrogen phosphate
L	Litre
L- NAME	N^G-nitro-L-arginine methyl ester
LPO	Lipid peroxide
LSU	Large Subunit
M	Molar
MAPKs	Mitogen-activated protein kinases
MDA	Malonyldehyde
MDH	Malate Dehydrogenase
MFO	Mixed function Oxidase
MOPS	3-(N-Morpholino) propanesulphonic acid
Mit	Mitosis
mg	Milligram
mm	Millimeter
min	Minutes
ml	Milliliter
mM	Milimolar
μM	Micromole
Mrp2	Multi-drug resistance-associated protein
MTT	3- (4- 5) dimethyl thiazolyl -2 – yl) 2,5 diphenyl tetrazolium bromide
$Na_2HPO4, 2H_2O$	Di- sodium hydrogen phosphate dihydrade
NaCl	Sodium Chloride
NADH	Nicotinamide adenine dinuclotide phosphate
$NaHCO_3$	Sodium bicarbonate
NaOH	Sodium hydroxide

NCB	Nitrocellulose Blotting membrane
NO	Nitric Oxide
NOS	Nitric Oxidase Synthase
N-SMase	Neutral sphingomyelinases
nt	Nucleotides
O. D	Optical Density
O_2^{-2}	Oxygen Radical
O_2	Oxygen Molecules
OH	Hydroxyl
OLT	Ortho Liver Transplantation
ORF	Open Reading Frame
PAGE	Poly Acrylamide Gel Electrophoresis
PBS	Phosphate Buffered Saline
PI	Propidium iodide
PMN	Polymorph -nuclear
pmp	People per year
PMS	Phenazine methosulphate
p.o	Preferably orally
PPD	p – Phenylene Diamine
q. s	Quantity sufficient
r.p.m.	Revolution per minutes
RNA	Ribonucleic acid
RNOS	Reactive Nitrogen-Oxygen Species
RNS	Reactive Nitrogen Species
ROI	Reactive oxygen Ions
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute
Rpo	RNA Polymerase
rRNA	Ribosomal RNA

SDH	Succinate Dehydrogenase
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SOD	Super oxide dismutase
SSU	Small Subunit
T	Thymine
T_3	Tri-iodo Thyroxin
TBDE	Trypan Blue Exclusion Test
TCA	Tri Carboxylic acid
TNF-α	Tumor Necrosis Factor alpha
TNFR1	Tumor Necrosis Factor Receptor 1
Tris	Tris (Hydroxy methyl) Amino methane
tRNA	Transfer RNA
TTC	2, 3, 5 –triphenyltetrazolium chloride
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling
U	Uracil
Un	Unit
XD	Xanthine Dehydrogenase
XO	Xanthine Oxidase
µl	Micro liter

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Chapter - 1

Introduction and Literature Survey

1.1. Introduction

Orthotopic liver transplantation (OLT) is known as removal of liver from donor and re-implantion in recipient's body by surgical procedure. Orthotopic liver transplantation remains the only definitive therapy for patients who are unable to achieve regeneration of sufficient hepatocyte mass to sustain life following injury and is now well-established treatment for chonic end-stage liver disease (ESLD) (Ostapowicz et al., 2002). Acute liver failure (ALF) is a broad term that encompasses both fulminant hepatic failure (FHF) and sub-fulminant hepatic failure (or late-onset hepatic failure, sub-acute hepatic necrosis) that can result from various causes (Dessinger et al., 2000).

Liver transplantation has enjoyed increasing success during the last decade. It has emerged from an experimental stage to become the mainstay of treatment for end-stage liver diseases with more than 4000 transplants performed annually. Despite the success of liver transplantation, rejection, infection, donor availability, and poor immediate graft functions present persistent problem and contribute to mortality rates in the range of 15-25 % within 1-2 years of surgery.

Liver transplantation technique was clinically first utilized around 15 years ago (Tuttle-Newhall et al., 2005). In Latin America, approximately 470 million people are affected with chonic liver, bile duct and gall bladder disease. In 2002, approximately 1100 liver transplantations were performed in Latin America and statistically 2.5 livers transplantations per million people per year (pmp) were done. In Korea, 2,435 OLT livers were transplanted (Czerwinski et al., 2005). In year, the maximum transplantation rate was in Argentina i.e. 4.5 pmp followed by Brazil 3.9 pmp and Chile 3.6.pmp respectively. Living donor liver transplantation procedures were followed in Latin American Countries but this procedure faciliated the organ shortage for pediatric patients (Hepp and Innocenti, 2004).

In India a total 343 liver were transplanted up to year 2007, which included deceased donor liver transplant 95 cases and live donor liver transplant 247 cases (Kakodkar et al., 2007) but the cost of transplantation is very high. The cost has varied between Rs1.5 and 2 million rupees in hospitals which have a successful ongoing Programme. Transplant professionals must research to decrease the mortality rate associated with waiting on liver transplant list. Every liver is damaged to some extent during the

transplantation process, but some livers are so severely injured that they are unable to sustain life (primary nonfunction) and, often, must be immediately retransplanted. Others show borderline function (primary dysfunction), for which patients often require prolonged treatment in the intensive care unit. In order to reduce the rate of primary nonfunction and dysfunction, it will be important to understand the mechanisms underlying liver injury during storage and reperfusion.

Potential complications of surgery and life long immunosuppression need to be considered for accurate evaluation of ALF. The prognosis of FHF varies with the underlying etiology and other factors. Prognostic scoring systems, although derived from data on relatively large numbers of patients, still has shortcomings in accurate predictions for ALF due to the variable etiology and thus variable potential for recovery (Polson and Lee 2005).

There is no single prognostic indicator or even scoring system that can determine outcome with reliable accuracy. Thus, decision to list a patient for transplantation is difficult and is based on overall acuity and progression, taking into consideration many clinical and biochemical parameters. However, ischemia followed by reperfusion (I/R) injury is major cause for death during liver transplantation and considered as main clinical parameters in liver transplantation.

1.2. Ischemia and reperfusion injury

I/R injury is phenomenon whereby cellular damage in a hypoxic organ is accumulated following the restoration of oxygen delivery. In the liver, this form of injury was recognized as a clinically important pathological disorder during studies of experimental liver transplantation (Toledo –Pereyra et al., 1975). I/R injury is relevant clinically in hepatic surgery, hypovolemic shock, some types of toxic liver injury, veno-occlusive diseases and Budd-Chiari syndrome. There is considerable evidence demonstrating that mitochondria are the principal targets in the development of I/R induced hepatic injury (Elimadi et al., 2001). I/R injury, particularly in the intestine, have been the subject of numerous studies. However, much remains to be learnt and treatments being tried require further improvement. In the years since McCord's first report on the involvement of oxygen free radicals in the pathophysiology process (McCord and Roy, 1982), new mediators of intestinal cell damage have been discovered (Kubes et al., 1990). It is now generally accepted that polymorphonuclear

(PMN) leukocytes are the main agents of reperfusion injury, due to lesions they cause in the vascular endothelium (Granger et al., 1989; Park, 1991; Carey et al., 1992; Williams et al., 1999). The endothelial lesion worsens histological injury in reperfused tissue and extends damage to other organs like kidney, liver and lung. Since oxygen free radicals appear to be the initiators of this pathological process, the capacity of different antioxidant drugs to reduce the damage caused by reperfusion have been studied (Kubes et al., 1990).

1.3. Preservation-reperfusion injury

Recent advances in the understanding of the preservation-reperfusion injury as well as the sinusoids suggest that the hepatic preservation-reperfusion injury involves specific changes in liver. Transplanted livers undergo preservation-reperfusion injury, which results mainly by hypothermia. In orders to develop preventive modalities, the effects of hypothermia have been studied. In addition to the classic effects of hypothermia, patient's etiologies of liver disease also provoke cellular injury. These specific cellular changes may be best understood in the framework as injury to the hepatosinusoidal cell and to the extra cellular connective tissue of the sinusoids (Holloway et al., 1989).

Hypothermia decreases metabolic rate and the rate at which intracellular enzymes degrade cellular components but does not completely inhibit metabolism. Most enzyme systems in normothermic animals show a 1.5-2.0 – fold decrease in activity for every 10°C decrease in temperature. It has been calculated that when the temperature is reduced from 37°C to 0°C metabolic rate diminishes. Previous reports on glycogen metabolism in the hypothermic pig liver and in rats and rabbits have shown considerable metabolic activity at 1°C. As the liver tolerates at least 1 h. of warm ischemia, cooling the liver to 0°C should theoretically extend preservation for 12-13 h (Kubes et al., 1990).

Cooling of solid organs is considered a fundamental requirement for prolonged storage, but causes un-physiological energy and substrate deficits. Cooling results in rapid reduction in Adenosine- tri- phosphate (ATP) and adenosine- di -phosphate (ADP), presumably because residual energy requirements exceed the capacity of the cell to generate ATP by anaerobic glycolysis. The drop in concentrations of ATP has been observed in several models. In the rabbit liver, ATP levels decreased from 1640

to 270 nmol $L^{-1}g^{-1}$ tissues after 6 h. and to 90 nmol $L^{-1}g^{-1}$ tissues after 24 h of cold storage in University of Wisconsin (UW) solution. In a model of isolated hepatocytes, cellular ATP content decreased by about 50% and 70% after 24h and 48h preservation respectively in UW solution at 4°C. Although a direct correlation between ATP content during cold preservation and outcome of human transplantation has been reported but ATP was not a viability marker. It may be an important marker of viability in livers from fed animals or in the human in which the organ undergoes warm ischemia (rewarming) during transplantation, which often exceeds 1h. In humans total adenine nucleotides decrease by 87% in over 120 min of warm ischemia and the level of recovery of ATP is inversely related to the duration of warm ischemia (Granger et al., 1989).

Hypothermia also results in intracellular acidosis, mainly due to anaerobic glycolysis and lactate accumulation. The consequences of intracellular acidosis are unclear. In OLT model in rats, an alkaline preservation solution showed improved outcome. Hypothermia also results in injury by reactive oxygen ions (ROI). ATP degradation results in the accumulation of hypoxanthine with concomitant conversion of xanthine dehydrogenase (XD) to xanthine oxidase (XO), probably associated with elevated intra-cytosolic calcium and protease activation (Belzer and Southard, 1989). On oxygenated reperfusion, XO further catalyzes the degradation of hypoxanthine to xanthenes and uric acid, which in turn produces ROI. Other sources and mechanisms of production of ROI, including those related to leukocytes and macrophages are still not known. Some ROI production may occur during hypoxanthine but this is not substantial.

The hepatic preservation –reperfusion injury leads to hepatosinusoidal injury. All cells including liver cells are damaged by the classic effects of hypothermia, but there is evidence that a specific injury may occur to the cells of the hepatic sinusoids. This type of injury to the microcirculation has not been described as a major feature of hypothermic injury in other organs.

Another line of evidence supporting a liver specific component of hepatic hypothermia injury arises from the similarity of preservation injury. The hypothermic liver injury involves damage to the epithelial cells resulting in white cell platelet adhesion in hepatocytes on reperfusion and, in some cases intravascular coagulation.

There is evidence that these events are mediated by activation of kupffer and perhaps endothelial cells, with release of potent inflammatory mediators. These aspects of preservation –reperfusion injury are similar to the effects of other disease processes affecting the liver initiated by bacteria and bacterial products, viruses and xenobiotics. In experimental viral hepatitis, the lesions observed by light microscopy is an endothelial injury that is followed by increased adherence of white cells and platelets (Linge and Pichlmay, 2006) Subsequent local release of ROI and protease from liver and adherent white cells may contribute to significant micro-circularly damage. In xenotransplanataion models, natural antibodies cause acute rejection. It has been proposed that these antibodies bind to the endothelium of the donor organ, triggering the complement cascade resulting in endothelial activation (Volk and Marrero, 2006). Such activation induces loss of vascular integrity, development of a pro-coagulant environment and increase in neutrophil adhesion, the last being mediated atleast in part, by complement receptor 3. These are very similar to events observed in liver transplantation, suggesting common pathogenic mechanisms. Recently, similar characteristics have been described in a murine model of alcohol –induced injury.

1.4. Significance of ischemia and reperfusion

Thomas Starzl in 1963 performed the first human liver transplantation (Starzl et al., 1989), which has evolved an established therapy for end stage liver disease or acute liver non-function. Due to the increasing experience and optimization of surgical technique and immunosuppression, survival rates were improved significantly, which are about 85%-90% after one year of transplantation (Otte, 1989). Superior organ preservation also accounts for this development. The commonly used solution of UW facilitates preservation times for up to 20 h (Bismuth et al., 1987) and has replaced the former, less effective Euro-Collins (EC) solution despite improved preservation techniques. The success of liver transplantations is still influenced by I/R. Long preservation times are in direct proportion to the incidence of re-transplantation, indicating that preservation injury is a major cause for graft loss. In 5 to 15% of transplanted livers a primary non-function occurs that can be defined by transaminase increase, loss of bile production and disturbances in clotting. The events highly correlate with preservation time and strongly increase after more than 12 h of preservation. 30% of graft recipients suffer from primary dysfunction which is

characterized by a post-surgical increase of liver enzymes, accompanied by a retarded regeneration of hepatic synthesis (Belzer and Southard, 1998) resulting in a three times higher risk of graft loss. Non-anastomotic biliary structures also show a correlation with the preservation time (Holloway et al., 1989). These ischemic bile duct structures emerge during the first three months after transplantation and are difficult to cure. Thus, improved protection against I/R could decrease the rate of preservation related complications and, moreover, should increase the number of organs available for liver transplantation. Elucidation of the involved patho-mechanisms and developing protective strategies are therefore important objectives. Therefore, the present studies were undertaken to see the liver protection effects of folic acid, L- arginine and amlodipine in I/R injury.

1.5. Literature survey

The temporary disconnection of blood flow is a necessity in a number of surgical procedures on the liver. This event, also referred to as ischemic period, can last for a short time as in liver resection at maintained organ temperature or can last up to several hours in the cold as it is the case in liver transplantation. Ischemias are detrimental cellular mechanisms that are even augmented by the subsequent restoration of blood flow after surgery or insertion of an organ, culminating in the manifestation of I/R injury.

In the field of hepatic transplantation, the preservation injury, a severe form of I/R, contributes to serious complications. Primary non-function, dysfunction, and nonanastomotic biliary strictures are major causes of retransplantation and mortality. Because of the increasing numbers of OLT and the concomitant lack of suitable donor organs, there is a great demand for better protection against I/R to decrease the rate of preservation– related complications. Cytoprotection is mediated via the guanylyl cyclase-coupled A receptor.

The literature survey shows that: -

Verapamil restored mitochondrial function from I/R injury (Shinohara et al., 1997).

Amlodipine treatment reduced infarct size of liver cholesterol-fed rabbits (Hohida et al., 1998).

Ca^{+2} channel blocker, Benidipine, limits infarct size via bradykinin- and NO-dependent mechanisms (Asanuma et al., 2001).

Propofol is effective in attenuation of lipid peroxidaton by with minimal hepatocellular protection from I/R injury (Kim et al., 2007). Ischemic preconditioning and intermittent clamping increase the tolerance of fatty liver in hepatic ischemia-reperfusion injury in male zucker rat (Saidi et al., 2007).

Lipoic acid administration prior to 90 min of hepatic ischemia improved tolerance in ischemia, by down regulation of pro-apoptotic Bax (Duenschede et al., 2007).

Glycine administration increased animal survival in I/R liver injury by reducing tumour necrosis factor - α (TNF-α) content. Administration of high-dose of lipoic acid on the other hand led to a significant reduction in necrosis- and apoptosis-related cell death in I/R of the liver without a reduction in liver TNF- α (Duenschede et al., 2007). Glycine has a protective effect against inflammatory reactions, and reduces hepatocellular injury induced by hepatic warm I/R in rats (Yamanouchi et al, 2007).

Nitrite dynamically modulates mitochondrial resilience to reperfusion injury and may represent effectors of the cell-survival program of ischemic preconditioning (Shiva et al., 2007).

Carnosine useful as a prophylactic treatment to protect the liver against hypoxia-reoxygenation damage (Foud et al., 2007).

Rutin treatment could be useful for preventing oxidative damage associated with hepatic post-ischemic reperfusion injury (Lanteri et al., 2007).

Normothermic conditions, following 60-minute liver ischemia period Somatostatin and Ursodeoxycholic acid decreased the effects of cholestatic hepatic injury especially and improve the condition (Pergel et al., 2007).

Apoptosis rates were reduced in animals pretreated with Erythopoietin by inhibiting mRNA expression of tumor TNF- α and STAT-3 and transaminases were significantly reduced among the erythopoietin -treated animals after 6 h and 12 h of I/R injury (Schmeding et al., 2007).

Administration of Diethyldithiocarbamate resulted in a significant decrease in the I/R injury to the liver (Maio et al., 2007).

T_3 administration involving transient oxidative stress in the liver exerts significant protection against I/R injury and T_3 preconditioning in I/R injury associated with NF-kappaB and STAT3 activation and acute-phase response (Fernadez et al., 2007).

Cardiotrophin-1 is an essential endogenous cytokines in defense of the liver against I/R and is a key mediator of the protective effect induced by ischemic preconditioning (Lniguez et al., 2007).

2-aminoethoxydiphenyl borate protects from I/R injury by inhabiting cellular Ca^{2+} uptake and subsequent inhibits mitochondrial Ca^{2+}overload in I/R injury (Nicoud et al., 2007).

Prophylactic effect of L-arginine in experimentally induced intestinal ischemia and L-arginine attenuates the degree of tissue damage in intestinal ischemia and promotes healing of intestinal mucosa (Fotiadis et al., 2007).

Hepatic ischemia-reperfusion injury during liver transplantation can lead to cholestasis and remote organ dysfunction and also showed that increase hepatic multi-drug resistance-associated protein (Mrp2) mRNA expression may contribute to cholestasis during I/R injury (Tanaka et al., 2008).

Amlodipine is effective in reduced ovaries tissue damage induced by ischemia (Halici et al., 2008).

1.6. Therapeutic strategies

Livers from donors with prolonged intensive care or prolonged ischemia time being rejected are more vulnerable to I/R and graft as well as patient survival is diminished after use of such organs, therefore, better protection against I/R and strategies to counteract the pathomechanisms are of great interest.

1.7. Established therapies

1.7.1. Storage and rinse solutions

Mostly, liver is preserved in the UW-solution, which has superior properties as compared to older EC preservation solution as its ingredients, lactobionate and glutathione, was identified as protective agents for liver preservation. Lactobionate has strong osmotic properties and was shown to reduce hypothermic cell swelling (Southard et al., 1995) whereas glutathione seems to be beneficial because of its antioxidant properties. To prevent reperfusion injury, flushing of the graft with Carolina rinse solution containing antioxidants before transplantation proved to be superior to Ringer's lactate and albumin solution besides after preservation in Carolina rinse solution Kupffer cell (K.C) activation and neutrophil adherence were

inhibited was observed as well as hepatic microcirculation and survival were improved (Gao et al., 1991; Post et al., 1993).

1.7.2. Ischemic preconditioning

Ischemic preconditioning refers to a phenomenon in which tissues are rendered resistant to the deleterious effects of I/R by previous exposure to brief periods of vascular occlusion. The protective effects were first shown in the cardiac cells (Murry et al., 1986) and have also been demonstrated in the liver (Moncada et al., 1991). In rats, ischemic preconditioning prior to harvesting of the liver for transplantation resulted in improved survival and decreased serum transaminases and TNF-α (Tumor necrosis factor). The protective effect is thought to result, at least in part, from the release of adenosine by the ischemic tissue (Yin et al., 1998). Moreover, attenuation of post ischemic generation of reactive oxygen species (ROS) seems to mediate protection (Peralta et al., 2002).

1.7.3. Antioxidative strategies

Generation of ROS is a central event in the reperfusion period. K.C and activation of neutrophil and XO are the major sources of these detrimental mediators. Extracellular fluids, such as blood plasma, contain little antioxidant capacity as compared to hepatocytes (Hallowell et al., 1990).

Therefore, administration of antioxidants during the early phase of reperfusion was suggested to have beneficial effects. Various antioxidants demonstrated their protective potential viz. glutathione (Bilzer et al., 1999), superoxide dismutase (Mizoe et al., 1997), allopurinol (Kusumoto et al., 1995), N-acetylcysteine (Koeppel et al., 1996), and α-tocopherol (Marubayashi et al., 1986) and shown to attenuate hepatic I/R.

1.7.4. Future therapies

The strict regulation of apoptotic cell death and survival pathways allows the development of therapeutic intervention strategies. Hepatocytes and stellate cells contain different protective mechanisms against cytotoxic cytokines, bile acids and reactive oxygen intermediates. Stellate cells may proliferate in response to these factors (Schoemaker and Moshage, 2004). Thus, both prevention of cell death in hepatocytes and induction of apoptosis in activated stellate cells may constitute relevant therapeutic strategies.

1.7.5. Hepatocyte-directed therapy

In acute liver injury, inhibition of apoptosis of hepatocytes may be beneficial. Targets for anti-apoptotic interventions include caspases, though endogenous or exogenous pathway and preservation of mitochondrial integrity via anti-apoptotic Bcl-2 family members. Anti-inflammatory agents are often considered to decrease liver damage during acute liver injury. However, whether this strategy is suitable for all pathological conditions remains to be seen. Anti-TNF (Tumor necrosis factor) therapy in bacterial infection-induced acute liver disease prevented liver injury but resulted in decreased bacterial clearance and decreased overall survival (Moore et al., 2003). Some anti-inflammatory strategies may attenuate cytokine production and NF-kB activation and thus sensitize hepatocytes to apoptosis (Schoemaker, 2004). Patients who suffer from cholestatic liver injury are often treated with ursodeoxycholic acid, which normally constitutes 3% of total human bile acids (Paumgartner and Beuers, 2004). It has been demonstrated that the taurine conjugate of ursodeoxycholic acid protects against bile acid induced apoptosis via direct effect on the mitochondrial membrane and activation of survival pathways such as mitogen-activated protein kinases (MAPKs) (Wright, 2001).

1.7.6. Therapeutic targeting of stellate cells

Acute and chronic liver injury may induce repair mechanisms, which lead to the excessive deposition of scar matrix (liver fibrosis) leading to liver cirrhosis. This is a process in which activated stellate cells are the central players. Induction of apoptotic cell death may be a promising therapeutic approach because apoptosis of activated stellate cells decreases liver fibrosis (Beljaars et al., 2003).

1.7.7. Anti-apoptotic Bcl-2-family genes as a promising strategy for liver therapy

Anti-apoptotic Bcl-2 family members like Bcl-2, Bcl-XL or A1/Bfl -1 prevent the activation of the mitochondrial/apoptosome death pathway, which is activated in hepatocytes by many noxious stimuli. Among the members of the Bcl-2 family, A1/Bfl -1, and NF-kB-regulated gene appear to be important for hepatocyte survival. It blocks hepatocyte cell death by inhibiting the mitochondrial/apoptosome death pathway and thus the activation of the caspase cascade (Schoemaker, 2004). During chronic liver injury, the expression of Bcl-2 is induced only in cholangiocytes which implies that Bcl-2 is involved in the protection of hepatocytes against bile-acid-

induced liver injury. Although Bcl-2 transgenic hepatocytes are protected against Fas-induced apoptosis (Mignon, 1998), it is not clear whether over expression of Bcl-2 in hepatocytes prevent necrotic cell damage resulting from chronic liver injury (Kane et al., 1995).

1.8. Amlodipine in ischemia and reperfusion injury

Amlodipine is a second-generation dihydropyridine calcium channel blocker (CCB) shown to have a gradual onset and a sustained duration of action because of the selectivity of the dihydropyridines for vascular smooth muscle and their lack of significant side effects (Stepien et al., 2002). 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 decreasing reserves of cellular adenosine triphosphate. The pharmacokinetic profile of amlodipine may prove valuable in protection of the ischemic and reperfused injury. A single intravenous (i.v) dose of 10 mg of amlodipine results in bio- availability of 64% and a calculated half-life of 34 hours but i.v dose is not recommended for tachycardia in ongoing surgical subjects (Burges et al., 1994).

1.9. L- Arginine in ischemia and reperfusion injury

Nitric Oxide (NO) is an important modulator of tissue blood flow, arterial pressure, neurotransmission, and immune cell function (Ming et al., 1999). There is evidence that implicates NO as a modulator of the adhesive interactions among leukocytes, platelets, and endothelial cells (Wiest and Groszmann, 2002). L- arginine at a dose level of 100mg $^{-1}$k.g $^{-1}$ p.o daily showed significant protection from alcoholic injury (Squadrito and Pryor, 1998). L- arginine is the precursor of NO in vivo. It has been shown that NO-donating compounds provide significant protection against the micro vascular dysfunction which is normally associated with I/R (Vallance, 2003). It has been confirmed that sustained NO production via L arginine administration ameliorated effects of intestinal I/R injury (Moncada et al., 1991)

1.10. Folic acid in ischemia and reperfusion injury

Folate is a cofactor in 1-carbon metabolism, during which it promotes the remethylation of homocysteine. In addition to augmenting the risk for vascular events, low folate/high homocysteine may directly increase the susceptibility of neurons to

brain injury. Folate deficiency causes uracil misincorporation into DNA and chromosomal breakage, which has implications for neuronal damage (Goulian and Bleile, 1980; Blount et al., 1997; Kruman et al., 2002). Folic acid protects from DNA breakdown by improving De novo biosynthesis, so it is predicted that in I/R injury folic acid may have role to prevent I/R injury.

1.11. References

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Chapter - 2

Objectives

2.1. Gap in existing research

The temporary disconnection of blood flow is a necessity in number of surgical procedures on the liver, which is also referred to as ischemic period. It can last for a short time as in liver resection at maintained organ temperature or can last up to several hours in the cold as in liver transplantation. In both case, initiation of detrimental cellular mechanisms that are augmented by the subsequent restoration of blood flow after surgery or insertion of an organ occurs, culminating in the manifestation of I/R.

In the field of hepatic transplantation, the preservation injury, a severe form of I/R, contributes to serious complications. I/R injury is a phenomenon whereby cellular damage occurs due to less oxygen delivery into the liver tissue. In the liver, this form of injury was recognized as a clinically important pathological disorder (Carden et al., 1993). Deprivation of oxygen, nutrients, or growth factor may be important to cause I/R injury resulting in cell atrophy and apoptosis. The acute injury phase, which is characterized by liver injury occurring within 1–6 h after reperfusion, is associated with kupffer cells activation, release of the pro-inflammatory cytokines, and generation of reactive oxygen species (ROS) (Carden et al., 1993). Apoptosis and cell atrophy have been indicated as an important mode of cell death during hepatic I/R injury (Cursio et al., 1999; Corry et al., 2003). The maximum apoptosis and necrosis was reported after 1h ischemia and followed by 30 min reperfusion and then gradual decline of necrosis and apoptosis (Delva et al., 1989).

Nitric oxide (NO) is a potent vasodilator synthesized from L-arginine by NO, which diffuses freely across cell membranes and acts intracellularly by activation of guanylate cyclase. NO is inducer of vasodilatation at the level of the sinusoid as well as at presinusoidal sites (Curran et al., 1989; Curran et al., 2003). It is also reported that that L- arginine attenuated from I/R injury by blocking activity of arginase in C57BL/6 mice prevents warm I/R injury and inhibition of arginase also resulted in protection from hepatic I/R-induced damage in association with markedly lower hepatic TNF, IL-6, and inducible NOS mRNA levels compared to I/R injury (Jeyabalan et al., 2008). Supply of L-arginine increased tissue levels of NO and reduced morphologic intestinal injury among mice undergoing I/R (Cintra et al., 2008). Sodium nitroprusside, a potential NO donor, significantly decrease catalase

activity and increase serum NO level in I/R rats as compared to vehicle treated I/R rats. Even in the presence of NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L- NAME) in I/R rats the effects L-NAME and synthesized NO is nullify by sodium nitroprusside (Emre et al., 2008).

Further, it is reported that in vitro I/R caused dissociation between cytochrome c and mitochondria in Hep G2 cells was prevented by administration of Ca^{2+} channel inhibitor, 2-aminoethoxydiphenyl borate (2-APB), which also protected I/R injury (Nicoud et al., 2007). Antagonist of L-type calcium channels inhibits neuronal death after ischemic insult suggested a novel therapeutic approach (Li et al., 2007).

Studies on the effect of calcium channel currents (COC) on freshly isolated rat Kupffer cells and role of Ca^{2+} channel blockers, 2-aminoethoxydiphenyl borate (2-APB), SK and F96365, econazole and miconazole in reducing COC as well as attenuated I/R injury (Jiang et al., 2006).

It is reported that Diltiazem improved ATP-generating capacity during reperfusion by improving liver tissue blood flow and prevented I/R injury in rat liver (Chin et al., 2005).

Folate is essential to replicate DNA and folate deficiency efefcts DNA synthesis and cell division, affecting the bone marrow, a site of rapid cell turnover (Zittoun et al., 1993).

The studies indicated that folic acid pretreatment blunts myocardial dysfunction during ischemia and ameliorates post-reperfusion injury due to preservation of high-energy phosphates, reducing subsequent reactive oxygen species generation, eNOS-uncoupling, and post-reperfusion cell death (Mones et al., 2008). Low folate levels are found to increase the risk of benzene-induced chromosomal damage in apoptosis (Endosh et al., 2007).

So far no work has been reported on attenuation of necrosis and apoptosis in hepatic ischemia followed by reperfusion injury therefore, it was decided to select active molecules, which could be useful with survival of hepatocytes after ischemia and reperfusion injury.

2.2. Rationality of studies

Primary non-function, dysfunction, and nonanastomotic biliary strictures frequently question the outcome of liver transplantation, which are major causes of

retransplantation and mortality. Because of the increasing numbers of OLT and suitable donor organs, there is a great demand for better protection against I/R to decrease the rate of preservation– related complications. Several approaches have been made in the recent time in order to protect from hepatic I/R. Necrosis and apoptosis, which is associated with I/R injury during transplantation, is the main cause of failure of liver transplantation.

Previous study showed that apoptosis and necrotic cells initial peak was after 1h ischemia and followed by 30 min reperfusion and second peak was after 1 h ischemia and 3 h reperfusion followed by gradual decline of necrosis and apoptosis but maximum necrosis and apoptosis found after 1 h ischemia followed by 3 h reperfusion (Delva et al., 1989;Lee and Clemens, 1992), therefore the present study was undertaken at single point time level i.e after 1 h ischemia followed by 3 h reperfusion injury.

During reperfusion, hepatocytes undergo necrosis and apoptosis 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 (Horie et al., 1999; Weiss, 1989). These morphological features are characteristic for oncotic necrosis. Previous studies postulated that most liver cells actually die by apoptosis (Gujral et al., 2001), which is morphologically characterized by cell shrinkage, formation of apoptotic bodies with intact cell organelles, and the absence of inflammation (Kohli et al., 1999). Apoptosis during I/R injury is a highly coordinated way of cell death, which is reflected by the complexity of apoptotic signal transduction. Apoptotic stimuli generate signals that are either transmitted across the plasma membrane to intracellular regulatory molecules or address directly to targets present within the cell. Ischemia-reperfusion syndrome, particularly in the liver, has been the subject of numerous studies in recent decades. Therefore, for the present study three molecules were selected to prevent attenuated necrosis and apoptosis. The reasons for their selection are given below.

- To study whether amlodipine can play protective role in I/R injury by Ca^{+2} overloading. Amlodipine is well known second-generation dihydropyridine

calcium channel blocker that inhibits Ca^{+2} overload in mitochondria during I/R. Further, amlodipine is known to preserve membrane integrity and decreasing reserves of cellular adenosine triphosphate.

- To observe whether vasodilatary effects of L- arginine could be useful in I/R injury as L-arginine is precursor of NO, NO is a potent vasodilator and vasodilatation prevents ischemic injury, in general.
- To observe whether folic acid, due to its DNA protective effects, could be useful in I/R injury. Folate is a cofactor in 1-carbon metabolism, during which it promotes the remethylation of homocysteine and prevents DNA damage. This DNA damage prevention may be useful in I/R injury.

2.3. Objectives

Taking into account the gap in present research and mechanism of action of selected molecules, the study was designed with following objectives:

- To study the effect of Folic acid on ischemia and reoxygenation injury in rat liver.
- To study the role of amlodipine therapy in hepatic I/R injury in rats.
- To study the role of L- arginine in sinusoidal vasodilatation by NO in I/R with regard to reduction of necrosis and apoptosis in the hepatocytes following transplantation in rat liver model.
- To study the role of the different mitochondrial enzymes in ischemia and reoxygenation injury in rat liver and the attenuation of mitochondrial enzymes by Folic acid, amlodipine and L- Arginine.
- To study the role of apoptotic marker gene Bcl-2 in ischemia and reperfusion injury in rat liver and effects of folic acid, amlodipine and L-arginine in expressing Bcl-2 gene and protein in I/R injury.

2.4. References

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Chapter - 3

Folic acid, Amlodipine and L- Arginine Inhibits Hepatic Infraction in Ischemia followed by Reperfusion in Rat Liver

3.1. Introduction

Ischemia and subsequent reperfusion (I/R) of the liver may occur under different clinical conditions such as the complete temporary occlusion of the supplying blood vessels during liver surgery especially when dealing with extensive hepatic trauma or resection of large intrahepatic lesions such as cysts or tumors (Pringle, 1908). The use of extracorporeal circulation in cardiac or vascular surgery is associated with low-flow I/R of the liver. Hepatic low flows I/R often occur during hemorrhagic, cardiogenic, or septic shock states followed by resuscitation. Besides, in different types of warm I/R, cold I/R is an important step of the liver transplantation procedure. There is ample evidence that the sequence of hepatic I/R may cause liver injury. In addition to postoperative bleeding, hepatic failure is the main causes of death in hospitals after warm I/R of the liver (Nagao et al., 1987; Delva et al., 1989). In patients undergoing hepatectomy, the degree of the resulting impairment of hepatic perfusion and oxygenation determines the extent of liver injury, as well as the incidence of liver failure and mortality (Kainuma et al., 1992; Margarit et al., 1999). Similar results have also been reported in patients after cold I/R associated with liver transplantation (Klar et al., 1997). The degree of impairment of the hepatic mitochondrial redox state determines the survival rate of patients after hemorrhagic shock and resuscitation (Nakatani et al., 1995).

The extent of liver injury caused by I/R depends primarily on the presence of pre - existing liver diseases, such as cirrhosis (Kurokawa et al., 1996; Gujral et al., 2001) and duration of ischemia (Maynard et al., 1997). If the injury is severe enough to cause liver dysfunction, it is associated with a profound deterioration of the prognosis (Pannen and Robotham, 1995). The liver plays a central role in the metabolic and immunologic response to stress (Jarrar et al., 1999), which increases mortality most likely due to the subsequent progression to multiple organ failure (Jaeschke et al., 1994; Moore et al., 1996; Allen et al., 2001).

Accumulating evidence suggests that alterations of pathogenesis of I/R-mediated liver injury are caused by degree of blood flow through portal artery. For example, hepatosplanchnic blood flow was reduced in humans at 60 min of reperfusion after 55 min of hypovolemia, even though when arterial blood pressure, cardiac output, and blood flow to the muscles, skin, and kidneys were already fully restored (Jaeschke et

al., 1998). Perturbations of the hepatic microcirculation have been observed in different animal models after I/R, including decrease in sinusoidal diameters and flow, increases in the heterogeneity of hepatic microvascular perfusion, and even complete cessation of blood flow within individual sinusoids (Menger et al., 1993). In ischemia followed by reperfusion, hepatic circulation is prevented causing oxidative injury, which is common pathogenesis in I/R injury. Infarct size also indicates the degree of pathogenesis in I/R injury.

Therefore, identification of the underlying pathophysiological mechanisms is of major importance, because this may form the basis for development of new strategies aimed at hepatic perfusion and may also play a major role in limiting I/R-induced liver injury.

3.2 Materials and Methods

3.2. 1. Materials

Amlodipine was obtained as gift sample from Dr. Reddy's Laboratories Ltd., Hyderabad, India. L- Arginine and Folic acid (analytical grade) were purchased from Central Drug House, Mumbai and calcium chloride dihyrate ($CaCl_2.2H_2O$), citric acid, dextrose, D-glucose, di-potassium hydrogen phosphate (K_2HPO_4), di-sodium hydrogen phosphate dihyrate ($Na_2HPO_4.2H_2O$), glacial acetic acid, hydrochloric acid (HCl), dihydrogen phosphate (KH_2PO_4), Disodium ethyl di amino tetra acetic acid (EDTA $(Na)_2$ sodium chloride (NaCl), sodium citrate trihydrate, sodium dihydrogen phosphate dihyrate ($NaH_2PO4.2H_2O$), sodium dodecyl sulphate (SDS), sodium hydrogen carbonate ($NaHCO_3$), diethyl ether, sodium hydroxide (NaOH), starch, sulphuric acid, tri-sodium acetate, were purchased either from BDH, Himedia, Quilagens, SD-fine chemicals, India. Urethane, heparin and 2, 3, 5 – triphenyltetrazolium chloride (TTC) used were procured from Sigma, St Lois, MO, USA. Trypan blue and bovine serum albumin was purchased from SRL, Mumbai and suture 3-0 silk (Ethicon) was purchased from Jonson and Jonsosn Limited, Mumbai. Buffer used were prepared using triple distilled water.

3.2.2. Animals

Mature Wistar rats weighing 150-250 g were procured from Laboratory Animal Resource, Division of Animal Genetics, IVRI, Izatnagar (Reg. No CPC – 196) and acclimatized to laboratory condition at Animal House of IFTM.

All animals received human care in compliance as per Guide for the care and use of Laboratory Animals. Experimental protocols were reviewed and approved by Institutional Animals Ethics Committee (Protocol no.11/837/ac/CPCSEA/2005). The animals were kept in polypropylene cages and maintained on balanced ration provided by Feed Technology Unit, Division of Animal Nutrition, IVRI, of following composition:

Ingredients	%
Wheat (crude)	60
Maize (crushed)	30
Wheat bran	07
Mineral mixture	02
Sodium Chloride	01

Milk 10 ml $^{-1}$ rat $^{-1}$day^{-1} or 5% skimmed milk powder was added to mixture. Twenty g/rat/day feed was given. The animals had free access to clean drinking water.

3.2.3. Animals groups

Thirty wistar rats were divided into sham-operated control group (I) (n = 6), ischemia and reperfusion group (II) (n = 6), were given 0.9 % saline (5ml kg^{-1}, p.o) for 7 days; amlodipine treated group (given amlpdipine 1 mg kg^{-1} body mass daily by oral route for 7 days before induced ischemia reperfusion maneuver) group III (n=6), Folic acid treated group (given folic acid 1 mg kg^{-1} body mass daily by oral route for 7 days before inducing ischemia reperfusion maneuver) (IV) (n = 6) and L-Arginine treated group (given L-arginine 100 mg kg^{-1} body mass daily by oral route for 7 days before inducing ischemia reperfusion maneuver) (V) (n = 6).

3.2.3. Ischemia and reperfusion injury

Ischemia and reperfusion injury was produced as per the procedure described by Hayashi et al., 1986. After the induction of anesthesia (urethane 10 mg kg^{-1} body mass daily by i.p), the liver was exposed through a midline laparotomy and the left branches of the portal vein and the hepatic artery were clamped for 60 min to produce complete ischemia of the median and left hepatic lobes. 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. The wound was closed with 3-0 silk sutures. Sham-operated animals were similarly

operated except that no ligature was placed to obstruct the blood flow to the left and median hepatic lobes, and, the blood flow to the right lobe of the liver was occluded. After 3h reperfusion, rats were euthanized by diethyl ether and blood of left and median lobes were collected.

3.2.4. Peripheral blood and tissue sampling

Blood samples were collected from the right ventricle via a left anterior thoracotomy at the time of sacrifice using sterile syringe without any coagulant and centrifuged to separate the serum. The serum samples were stored at -70°C until used for cytokine, ALT and AST assays. A portion of the ischemic and non-ischemic liver lobe was fixed in buffered 10% formalin, embedded in paraffin, and stained using hematoxylin and eosin (H&E). Other portions of ischemic and non-ischemic liver lobes were snap frozen in liquid nitrogen and stored at -70°C for Reverse-Transcription Polymerase Chain Reaction (RT-PCR) and isolation of mitochondria. An additional section of liver was frozen in optimal cutting temperature compound (OTC media) and stored at -70°C for assessment of apoptosis.

3.2.5. Isolation of hepatocytes (Kreamer et al., 1986)

After sacrificing rats, the left lobes were cannulated and perfused with 6 to 8 ml/min to Earle' balanced salt solution (EBSS) containing 0.25 mM of di-sodium EDTA for 4 min. The di-sodium EDTA was flushed out with EBSS for 4 min followed by perfusion with EBSS containing 1 mM calcium chloride, 0.68 mg/ml collagenase H, and 0.07 mg/ml trypsin inhibitor for 8 to 12 min until the cell matrix was ruptured. The cells were gently teased out of the liver capsule into Williams medium E (WME), pH 7.4, containing 1% (w/v) BSA, and the cell suspension was filtered through nylon mesh and centrifuged for 3 min at 100g. The supernatant was discarded and the cell pellet was resuspended in WME containing 1% BSA (w/v), and centrifuged twice. The final cell pellet was resuspended in 5 ml of WME containing 0.1% BSA, and the viability was observed using trypan blue exclusion method. All incubations were performed in microcentrifuge tubes in a Thermomixer (Eppendorf AG, Hamburg Germany) set at 37°C and 900 rpm. The incubation volume was 1 ml for all compounds. Incubations were initiated by the addition of dimethyl formamide to 5 µl of substrate to bring concentration of 0.5%v/v after 5-min pre-incubation of the cell suspension. Termination of incubations was by snap freezing in liquid nitrogen.

3.2.6. Liver infarcts size determination (Joshi and Jain, 2004)

At the end of 1 h ischemia followed by 3 h reperfusion, the liver was incubated at 37°C for 30 minutes in 0.08% solution of 2,3,5 –triphenyltetrazolium chloride (TTC) prepared in Kerbs- Henseleit buffer. The liver was then fixed in formalin. The area of necrosis was quantitated by measuring the stained area versus the unstained area (necrotic) using computer-assisted plainimetry.

3.2.7. Cell viability assay (Baltrop et al., 1991)

The hepatocytes viability was measured by trypan blue 0.5% exclusion test (TBDE). Heapatocytes were washed using HBSS (Hank Balance salt solution) followed by equal volumes trypan blue (6.2 mM L^{-1} and NaCl 0.8 mM L^{-1}) and gently mixed. After 2 minutes cells were counted under inverted microscope using hemocytometer. After cell counts the cell suspension was diluted to a final density of 1X 10^6 cells/ml of HBSS, containing 15mM HEPES, 5% FBS (Fetus Bovine Serum) Penicillin (100 U/ml) and Streptomycin (100 µg/ml). Aseptic techniques were used throughout the preparation of the lymphocytes. Positive control of cytotoxicity was measured of all groups in comparison to I/R group after incubation for 6h at 37°C in humidified atmosphere rich in 5% CO_2 +95% air in Carbon Dioxide incubator (Flow, Germany). The cell viability was measured by TBDE methods after 6h incubation and all group cells viability were compared with I/R group.

3. 3. Results

3.3.1. Liver morphology

Changes in physical appearance of liver were observed at different phases of ischemia followed by reperfusion. After 1h complete ischemia, left liver turned blackish due to prevention of blood circulation by portal ligation. After reperfusion (Opening ligation) blood flow to hepatic circulation showed discoloration of left liver but after 3 h reperfusion epical portion of liver showed permanent ischemic zone (black coloration). L- Arginine treated rats showed minimum ischemic zone as compared to all other groups (Figure 3.1).

3.3.2. Infarct size

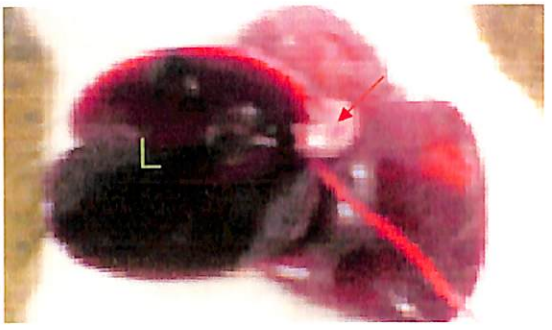
Infarct size was measured by TTC staining and by computer-assisted planarimetry. Normal areas of liver were stained deep red with TTC but infarcted tissue did not take any stain. Infarct size of I/R rats was 26 times greater than sham operated control rat.

Administration of L- arginine reduced 45% (P< 0.01) of infarct size in I/R rat as compared to vehicle treated I/R rat. Folic acid and amlodipine also significantly (P< 0.01) reduced infarct size as compared to I/R rats (Table.3.1., Figure3.2 & 3.3).

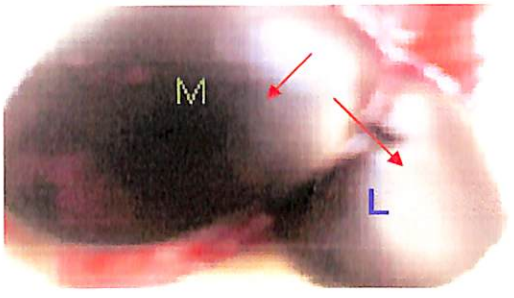
3.3. 3. Cell viability Assay

Viable hepatocytes in the sham-operated rats were 90.78 ± 4.20 %. After 1h ischemia followed by 3h reperfusion viable hepatocytes decreased significantly (P< 0.01) as compared to sham-operated control rats whereas in Folic acid treated group viable hepatocytes increased significantly (P< 0.01) as compared to I/R group rats. Amlodipine also significantly (P< 0.01) increased viable hepatocytes as compared to folic acid treated group and I/R group rats. Viable hepatocytes in the L- arginine treated group were 82.15± 2.22 % which showed significant increase (P< 0.01) as compared to the entire group. (Table 3.2, Figure 3.4 & 3.5). All the treatment groups were found to increase the viable cell count and the versus were statistically significantly as compared to the I/R group.

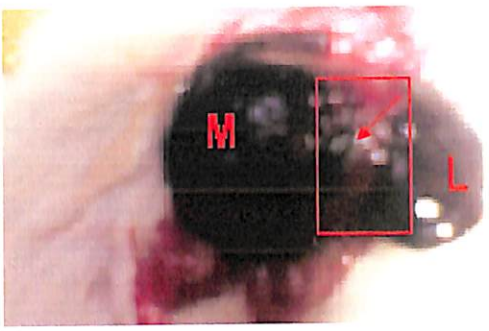
A: Ischemia followed by reperfusion in Wistar rats.



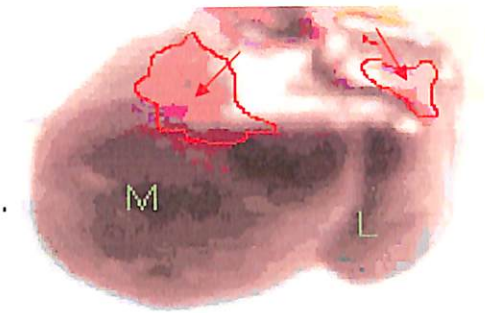
B: Ligation of left lobe (L) to produce ischemia.



C: Ischemic median lobe (M) and left lobes (L) becomes complete ischemic after 1h.

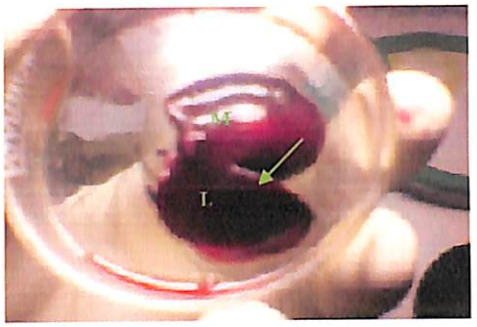


D: Ischemic left (L) and median lobe (M) of I/R liver after 1 h ischemia followed by 2h reperfusion. Some portion of liver shows permanent injury.

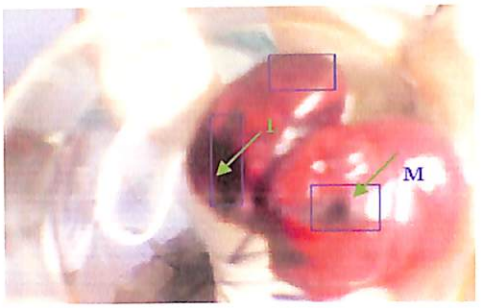


E: After 1 h ischemia followed by 3 h reperfusion median (M) and left (L) lobe becomes completely injured. Red marked area is un-ischemic zone.

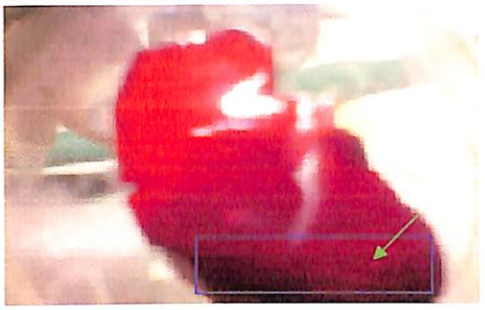
Figure: 3.1 (A-E). Photomicrograph of the effect of folic acid, amlodipine and L-arginine on liver morphology after I/R in rat liver.



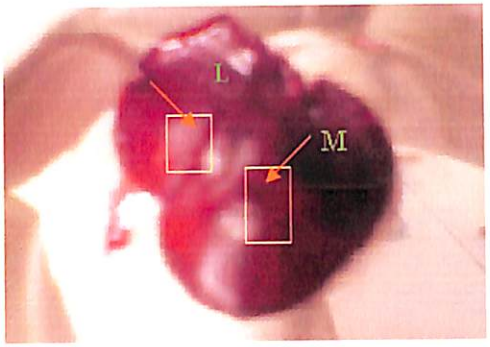
A: Sham operated control rats shows unstained median (M) and left (L) lobe after TTC staining.



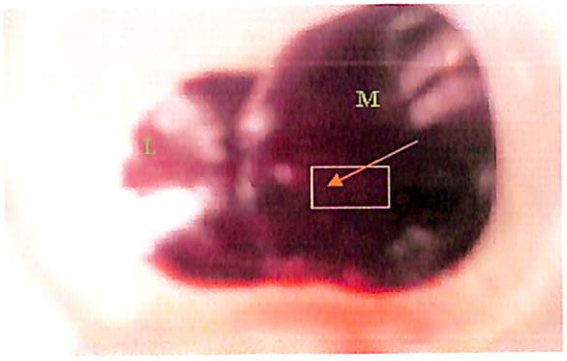
B: I/R rats show stained median (M) and left (L) lobe after TTC staining. Maximum area of liver show ischemic zone (stained area) as compared to unstained area (blue square, green mark) which correspond to I/R caused injury in liver.



C: Folic acid treated rats show decreased infarct zone (stained area, green arrow) as compared to I/R rats. Epical portion of liver recovered from I/R injury.



D: Amlodipine treated rats show reduced infarct zone to folic acid treated and I/R rats. Infarct zone is marked as yellow square.



E: L- arginine treated rats show reduced infarct zone which is marked as yellow Square. Epical portion liver shows non-ischemic zone and median portion of liver shows infarct area.

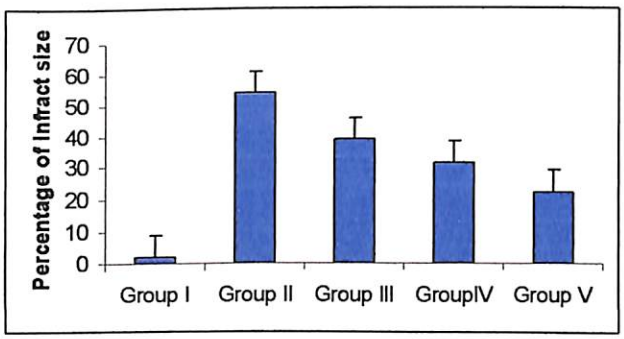
Figure: 3.2. (A-E). Photomicrograph of the effect of folic acid, amlodipine and L-arginine on infarct size after I/R in rat liver.

Table: 3. 1. The effect of folic acid, amlodipine and L- arginine on infarct size after I/R in rat's liver.

Groups		Percentage Infarct Size
Sham-operated	(Group I)	2.34 ± 0.54
I/R Injury	(Group II)	54.31 ± 6.76*
Folic acid treated	(Group III)	38.91± 3.22*#
Amlodipine treated	(Group IV)	31.43± 2.91*#
L -Arginine treated	(Group V)	22.34± 3.56*#

Results are expressed as mean ± SD (n=6). * Statistically significant difference ($p< 0.01$) from sham operated rats.

Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.



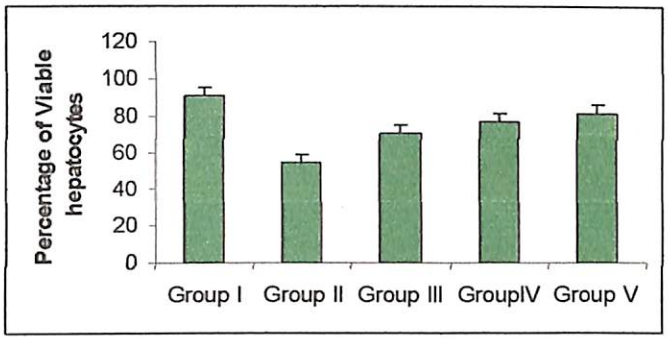
[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

Figure: 3.3. Graphical representation of the effect of folic acid, amlodipine and L-arginine on infarct size after I/R in rat liver.

Table: 3.2. The effect of folic acid, amlodipine and L- arginine on cell viability after I/R in rat's liver.

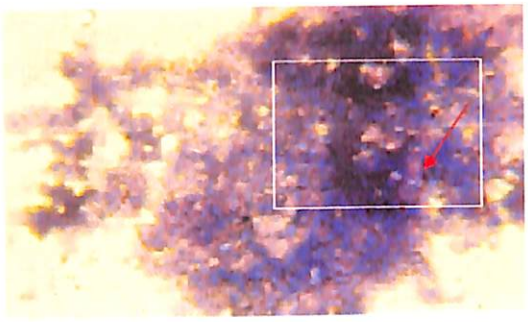
Groups		Percentage of Viable hepatocytes
Sham-operated	(Group I)	90.78 ± 4.20
I/R Injury	(Group II)	54.11 ± 3.19*
Folic acid treated	(Group III)	70.91± 3.22*#
Amlodipine treated	(Group IV)	77.02± 4.55*#
L -Arginine treated	(Group III)	82.15± 2.22*#

Results are expressed as mean ± SD (n=6). * Statically difference (P< 0.01) from sham operated rats. # Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.

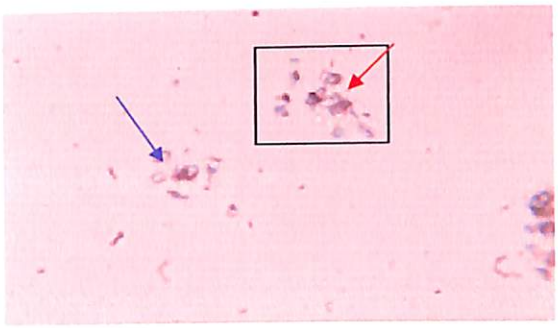


[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

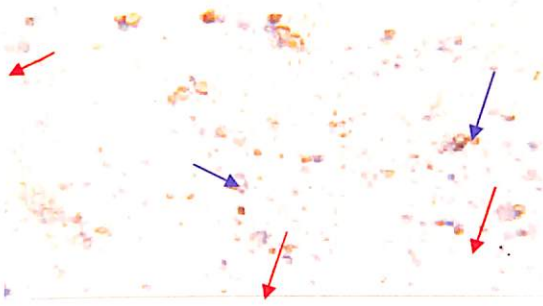
Figure: 3.4. Graphical representation of the effect of folic acid, amlodipine and L-arginine on cell viability.



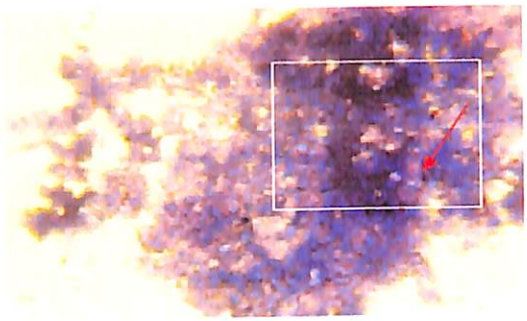
A: Sham operated control group viable hepatocytes. Isolated hepatocytes were dense and no individualization. (x 400)



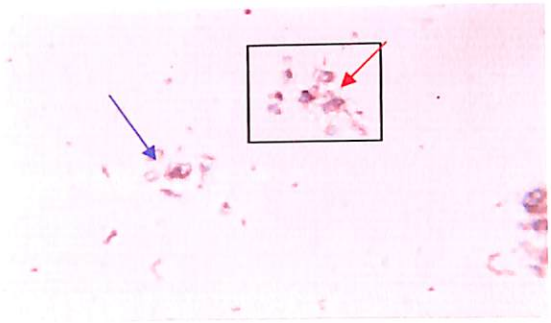
B: I/R rats hepatocytes. Viability of hepatocytes (red arrow) was decreased and hepatocytes were rarified (blue arrow) (x 400).



C: Folic acid treated hepatocytes. Viable (red arrow) and non- viable (blue arrow) hepatocytes were observed (x 400).

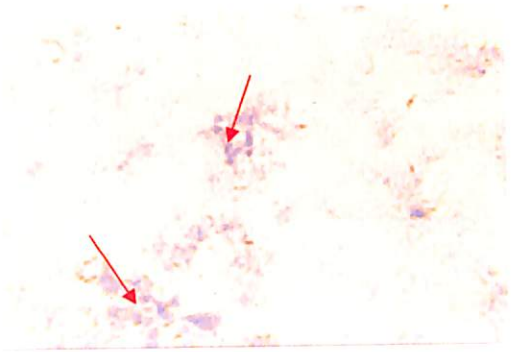


A: Sham operated control group viable hepatocytes. Isolated hepatocytes were dense and no individualization. (x 400)

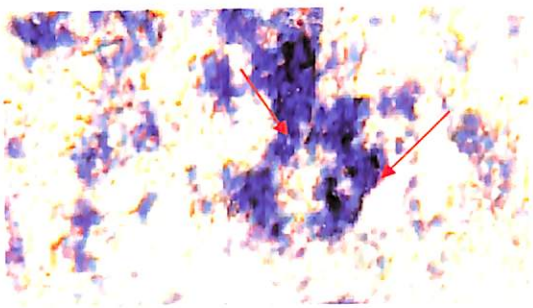


B: I/R rats hepatocytes. Viability of hepatocytes (red arrow) was decreased and hepatocytes were rarified (blue arrow) (x 400).

C: Folic acid treated hepatocytes. Viable (red arrow) and non- viable (blue arrow) hepatocytes were observed (x 400).



D: Amlodipine treated hepatocytes showing viable hepatocytes (x 400).



E: L- arginine treated hepatocytes showing viable hepatocytes clump mass (x 400).

Figure: 3.5 (A-E). Photomicrograph of folic acid, amlodipine and L- arginine on cell viability after I/R in rat liver.

3.4. Discussion

The present study showed that after 1 h ischemia in I/R group rats, left and median lobes were darkened (Figure 3.1.B) due to accumulation of blood and after 3 h reperfusion complete darkening was apparent in apical portion of liver which correlates with the extent of liver injury after hemorrhagic shock and resuscitation and during reperfusion after portal triad cross-clamping as reported by Ezzat and Lautt (1987) and Chun et al. (1994) respectively.

The portal vein passively drains most of the venous blood from the splanchnic compartment. Therefore, the arterial inflow resistance to liver primarily determines

portal venous flow. Consequently, in such a "portal-splanchnic response," alterations in portal flow, which causes systemic and regional macro hemodynamic changes, and obstructions of the sinusoidal lumen by swollen presinusoidal cells, trapped blood cells, or thrombotic material. The present studies also showed that interruption of hepatic micro vascular blood flow during clamping of portal vein in ischemic phase changes hepatic morphology, which is in agreement with earlier findings of Takala (1996) and Kainuma et al. (1996) respectively.Endothelium – derived NO is considered to be primarily an important determinant of vascular tone and platelet activity. Nitric oxide regulates resting hepatic blood flow and in I/R condition NO is one of the important a determinant factor to differentiate infarct and normal tissue. L-arginine is precursor of NO. Present study showed minimal infarct area in L- arginine treated group as compared to all other groups, therefore as expected L- arginine was the most potent in attenuating I/R injury as compared to all other groups (Figure 3.2 E). Thus, our studies suggest that L- arginine significantly protects I/R injury by increasing cell viability by producing NO in hepatic sinusoids. This may be due to the fact that duration of hepatic ischemia correlates with the edema and reduces dehydrogenase enzymes after liver damage during the ischemic phase due to lack of oxygen supply. L- arginine increases oxygen supply during I/R injury by increasing production of NO, which leads to vasodilatation of liver sinusoids and reduced infarct zone and hepatocellular damage.

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 decreasing reserves of cellular adenosine triphosphate. Superoxide formation by mitochondria, ultimately leads to formation of membrane permeability transition pores and breakdown of the mitochondrial membrane potential as reported by Siesjo and Bengtsson (1969). Accumulation of Ca^{+2}in mitochondria, superoxide formation is common pathophysiology in I/R mediated injury. The present investigation showed that administration of amlodipine significantly reduced infarct zone and increased cell viability as compared to I/R group rats (Figure 3.2 D and Figure 3.5 E), which indicates that protective action of calcium channel blocker-amlodipine against the I/R induced injury is mainly due to inhibitory action of Ca^2 efflux into mitochondria. This

findings can be correlated as Amlodipine, a second-generation dihydropyridine calcium channel blocker, is known to have a gradual onset and a sustained duration of action because of the selectivity of the dihydropyridines for vascular smooth muscle as reported earlier by Stepien et al. in 2002 besides the pharmacokinetic profile of amlodipine is valuable in protection of the ischemic and reperfused injury as single intravenous dose of 10 mg amlodipine resulted in an absolute bio- availability of 64% and a calculated half-life of 34 hours (Mignini et al., 2007) and this pharmacokinetic profile may contribute in prevention of I/R injury.

The present studies further showed that folic acid decreased infarct zone and increased viable hepatocytes as compared to I/R injury. This is important as folic acid prevents infarction of liver during I/R injury by attenuation of DNA damage during ischemia- reperfusion injury. This is justified because folic acid is a cofactor in 1-carbon metabolism, during which it promotes the remethylation of homocysteine and increase of homocysteine in plasma related to ischemic injury. Further, folic acid is known to promote DNA synthesis during cell proliferation (Kruman et al., 2000).

Thus, the present study proves that administration of L- arginine, amlodipine and folic acid in I/R rats reduces the infarct zone and increases cell viability. Out of these three, L- arginine proved to be most potent molecule in reducing infarct zone.

3.5. Conclusion

1. The present study showed the protective effects of L- arginine against I/R injury on rat hepatocyte, which might be partially due to its production of NO and increased vasodilatations.
2. The present investigation indicates the protective action of amlodipine against the I/R induced liver injury in rats, which may be due to its inhibitory action of Ca^{+2} influxes into mitochondria and partially due to its antioxidant property.
3. Folic acid improved the degree of infarction but it is difficult to conclude the pathophysiological mechanism by which it inhibits I/R injury. However, the fact that the folic acid altered the infarct zone provides a clue for mechanism.

3.6. References

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Chapter - 4

Folic acid, Amlodipine, L- Arginine Attenuated Hepatic Marker Enzymes and DNA & RNA in Ischemia followed by Reperfusion in Rat Liver

4. 1. Introduction

Ischemic reperfusion (I/R) injury is phenomenon whereby cellular damage occurs due to deficiency of oxygen delivery to the liver tissue. In the liver, this form of injury was recognized as a clinically important pathological disorder (Carden et al., 1993).

Aspartate aminotransferases AST (L-aspartate: 2- oxoglutarate amino transferase, SGOT) catalyses the transaminase reaction. In transamination, an amino group is transferred from an alpha amino acid to an alpha keto acid and transaminases catalyze this reaction in the presence of pyridoxal -5- phosphate as a cofactor. The activities in serum of two-transferse enzymes- aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are well known marker enzymes in I/R and related hepatic disease.

ALT, a cytosolic, enzyme is significantly increased after surgery to maximum level after 12-24h (3-6 folds over controls) and returns to normal values thereafter. AST, an enzyme located in both the cytosolic and mitochondrial sub cellular liver compartments, also showes a maximum serum activity peak at 12-24h after surgery (6 fold over controls) (Esterbauerh et al., 1991). It is postulated that I/R injury leads to changes in AST and ALT activity. Accumulated evidences indicate that oxygen derived free radicals play an important role in producing micro vascular and parenchymal cell damage in association with reperfusion following ischemia (Drugas et al., 1991). Free radicals alter the function of biological membrane such as the plasma membrane causing cell injury through chemical reactions. Earlier reports from a number of laboratories have shown that the activities of microsomal membrane bound enzymes are highly dependent on the nature of their surrounding lipid environment. Lipid peroxidation generates many potential cytotoxic products. Toxic metabolite 4-hydroxymonenal (Esterbauerh et al., 1991) has a variety of cyto - pathological effects i.e. inactivation and inhibiting of DNA and RNA synthesis, and induce heat shock protein (Esterbauerh et al., 1992).

Earlier studies suggested that microsomal drug – metabolizing enzymes are impaired during I/R injury (Metzer and Lauterburg, 1988) and this post ischemic impairment in drug metabolism might be directly related to deficits in function of cytochome P_{450}, an integral component of the hepatic microsomal oxidases. Multiple isozymes of cytochome P_{450} are associated with different spectrum of hepatic mixed function

oxidase (MFO) substrate specifity. As the regulation of cytochome P_{450} isozymes appears to be subject to independent control mechanism (Guengerich 1987; Guengerich et al., 1987), it is thought that a non-uniform effect would be present on individual cytochome P_{450} isozymes in I/R injury.

TNF-α initiates apoptosis in hepatocytes by activating different pathways, which leads to liver injury. The main apoptotic effects of TNF- α are mediated by TNFR-I. There are functional domains within TNFR-I to transducer unique intracellular signals by interacting with different intracellular adaptor proteins (Wang et al., 2000) viz. C-terminal death domain, the middle A-SMase (acidic sphingomyelinases) activating domain and the N-terminal N-SMase (neutral sphingomyelinases) activating domains. The death domain can mediate both the pro-apoptotic and anti-apoptotic pathways, while the other two sphingomyelinases pathways mainly modulate apoptotic and inflammatory responses (Muto et al., 1998). These pathways have been identified to be important for TNF- α induced apoptosis and liver injury with the mitochondria acting as the central executioner for TNF-induced hepatocyte apoptosis. TNF- α is a pleiotropic pro-inflammatory cytokine produced largely by activated macrophages and in smaller amounts by several other types of cells. It exerts a variety of effects that are mediated by TNF-receptor 1 and 2 (TNFR-I and TNFR-II). The apoptotic effects are only mediated by TNFR-I, whereas TNFR-II may serve to potentiate the effects of TNFR-I in promoting cell death or promoting inflammation (Rust and Gores, 2000). It is considered that TNF – α may be one of the regulator cytokine in I/R injury.

4.2. Materials and Methods

4.2.1. Materials

Sucrose, Dithioerytheitol (DTT), Trichloro Acetic acid (TCA) was purchased from Central Drug House, Mumbai. Absolute Ethanol, calf thymus DNA and RNA used were from Sigma (Sigma,Stlouis, Mo, USA). Folin-Ciocalteu's phenol reagent, RPMI 1640. Napthlethlenediamine dihydrochloride, Thiobarbituric acid, Sulphanilamide, Sodium Dithionate were purchased from Hymedia Ltd, Mumbai.

4.2.2. Methods

4.2. 2.1. Animal groups

Thirty Wistar rats were divided into sham-operated control group (I) (n = 6), ischemia and reperfusion group (II) (n = 6), were given 0.9 % saline (5ml kg^{-1}, p.o) for 7 days; amlodipine treated group (given amlodipine 1 mg kg^{-1} body mass daily by oral route for 7 days before induced ischemia reperfusion maneuver) group III (n=6), Folic acid treated group (given folic acid 1 mg kg^{-1} body mass daily by oral route for 7 days before inducing ischemia reperfusion maneuver) (IV) (n = 6) and L-Arginine treated group (given L-arginine 100 mg kg^{-1} body mass daily by oral route for 7 days before inducing ischemia reperfusion maneuver) (V) (n = 6).

4.2. 2. 2. Ischemia and reperfusion injury

Ischemia and reperfusion injury were same as described on page 29, chapter 3.

4. 2.2.3. Peripheral blood and tissue sampling

Peripheral blood and tissue sampling method was same as described on page30, chapter 3.

4.2.2.4. Preparation of liver microsomes (Schenman and Clinti, 1978)

100 mg liver was excised and homogenized with a loose- fitting Teflon pestle in 900 ml of 50 mM L^{-1} Tris-HC1buffer, pH 7.4, containing 0.3 M L^{-1} sucrose, 10 mM L^{-1} DTT, and 10 mM L^{-1} EDTA. The homogenate was centrifuged at 20,000 g for 15 min. The supernatant solution was centrifuged at 10,000 r.p.m for 60 min. The microsomal fraction obtained was suspended in a homogenizing medium without DTT and re- centrifuged at 10,000 r.p.m for 60 min. The resulting microsomal fraction was suspended in 0.1 ML^{-1} phosphate buffer pH 7.4, containing 1 mM L^{-1} EDTA and volume was adjusted to 10 ml with phosphate buffer.

4.2.2.5. Extraction of nucleic acids (Munro and Fleck, 1966)

200 mg of liver tissue was taken and treated with 200 ml of 10% TCA, shaken, centrifuged at 4000 r.p.m. for 15 min at 4 °C and discarded the supernatant. The residue was washed twice with 10% TCA followed by a wash with 5 ml of absolute ethanol. The residue was extracted twice with 10 ml of ether to remove phospholipids to which 2.5 ml of 10% TCA was added and centrifuged. The supernatant was preserved in a separate tube. The residue obtained was treated with 50 ml of 5% TCA and kept at 100°C for 15 to 20 min and cooled to room temperature, centrifuged at

4000 r.p.m. at 4 °C and 2.5 ml solution was taken to which 5 ml supernatant was added. it was filtered and the final residue was treated with 2.5 ml of 5% TCA, heated as above and the supernatant was preserved. 10 ml of acid extract aliquot was used for DNA and RNA estimation.

4.2.2.6. Isolation of hepatocytes (Kreamer et al., 1986)

After sacrificing rats, the left lobes were cannulated and perfused with 6 to 8 ml/min to Earle' balanced salt solution (EBSS) containing 0.25 mM of di-sodium EDTA for 4 min. The di-sodium EDTA was flushed out with EBSS for 4 min followed by perfusion with EBSS containing 1 mM calcium chloride, 0.68 mg/ml collagenase H, and 0.07 mg/ml trypsin inhibitor for 8 to 12 min until the cell matrix was ruptured. The cells were gently teased out of the liver capsule into Williams medium E (WME), pH 7.4, containing 1% (w/v) BSA, and the cell suspension was filtered through nylon mesh and centrifuged for 3 min at 100g. The supernatant was discarded and the cell pellet was resuspended in WME containing 1% BSA (w/v), and centrifuged twice. The final cell pellet was resuspended in 5 ml of WME containing 0.1% BSA, and the viability was observed using trypan blue exclusion method. All incubations were performed in microcentrifuge tubes in a Thermomixer (Eppendorf AG, Hamburg).

4.2.2.7. Estimation of alanine transaminase (ALT) and aspartate transaminase (AST)

Serum was used for the assay of ALT [EC 2.6.1.2] and AST [EC 2.6.1.1] using Merck kits (Merck India Ltd, Mumbai, India) according to the manufacturer's instructions.

4.2.2.8. Estimation of tumor necrosis factor –α (TNF – α)

Serum TNF -α was measured by enzyme immunoassay using ELISA kit (Biosource, Camarillo, CA, USA) according to the manufacturer's instructions.

4.2.2.9. Estimation of DNA by using diphenylamine reaction (Richards, 1974)

100 mg ml^{-1} DNA was prepared in distilled water and 100 µg to 1000 µg of DNA was taken in different test tube marked as standard and volume in each was adjusted to 3 ml with distilled water. For blank 3 ml distilled water was taken. In another test tube 500 µl of separated nucleic acid as described above of each group was taken and 6 ml of diphenylamine reagent was added to each tube and mixed by gently shaking. Test tubes were warmed in boiling water bath for 10 min and then cooled to room

temperature. The absorbance of the yellowish blue colored complex developed and of the blank was measured at 600 nm.

From the standard curve, the amount of DNA was calculated for the definite volume of extract used and finally amount for 10 ml extract made initially was calculated. Values were expressed for 1 g of wet tissue.

4.2.2.10. Estimation of RNA by orcinol method (Yoshida et al., 1997)

100 mg ml^{-1} RNA was prepared in distilled water and 100 µg to 1000 µg of RNA was taken in different test tubes marked as standard and volume in each was adjusted to 3 ml with RNase treated water. For blank, 3 ml RNase treated water was used. In test tube 500 µl of separated nucleic acid as describe earlier of each group was taken and volume adjusted was with 3 ml with RNase treated water and 3 ml orcinol acid reagent from a burette to all tubes including blank was added. All the test tubes were heated in a water bath for 30 min and then cooled to room temperature. The absorbance of each sample at 660 nm was measured against blank. A calibration curve was making by plotting OD against concentration of RNA. The amount of RNA in the extract was calculated from the standard curve.

4.2.2.11. Separation of protein from liver tissue

All experiments were performed at 4°C in cooling chamber-using livers stored on dry ice. 100 mg of liver tissue was added to 10 ml of ice cold ammonium sulphate saturated solution and homogenized by 10 strokes with a Potter S device (Bangalore Genni, Bangalore). Homogenates were then centrifuged for 20 min at 4°C and 14,000 r.p.m. supernatant was collected and dialyzed for overnight at 4°C at 1000 r.p.m to remove ammonium sulphate.

4.2.2.12. Determination of liver protein (Lowry et al., 1951)

100 mg ml^{-1} Bovine serum albumin (BSA) was prepared in distilled water and 100 µg to 1000 µg of BSA was taken in different test tubes marked as standard. To 0.1 ml of sample and standard 0.1 ml of 2 N sodium hydroxide was added and hydrolyzed at 100°C for 10 min in water bath, cooled to room temperature for 10 min and 1 ml of freshly prepared complex-forming reagent by mixing the 2% (v/v) sodium chloride, 1% (w/v) copper sulfate and 2% (w/v) sodium potassium tartarate in distilled water was added in 100:1:1 ratio. Solution was kept at room temperature for 10 min and Folin reagent (0.1 ml) was added and mixed using a vortex mixer. The mixture was

kept at room temperature for 1h and absorbance was measured at 750 nm. A standard curve of absorbance as a function of initial protein concentration was used to determine the unknown protein concentration.

4.2.2.13. Determination of nitric oxide production in hepatocytes (Hibbs et al., 1998)

Isolated hepatocytes of all groups were seeded (5 x10^6/ml) in RPMI 1640 phenol red free medium supplemented with 10% fetal calf serum in petri dishes and incubated at 37° C at 5% CO_2 for 4 h in Carbon dioxide incubator. After 48h, 5 ml of Griess reagent [mixture of 1:1 of napthylethlenediamine dihydrochloride (0.1% in water) and sulphanilamide (1% in phosphoric acid)] was added and incubated in the dark at 30 ^{0}C. The absorbance was measured at 546 nm and a standard curve using sodium nitrite was used to calculate the concentration of nitrite.

4.2.2.14. Determination of cytochome P_{450} (Omura and Sato, 1964).

1ml supernatant of liver microsomes was taken and 10 mg of sodium dithionate was added. Carbon monoxide was bubbled for 10 seconds and then centrifuged for 5 minutes at 3000x g. 500 µl supernatant was mixed with 5 ml Tris HCl buffer (pH 7.4) and absorbance was measured at 450 nm wavelengths using excitation coefficient of 91 mol $L^{-1}cm^{-1}$for A 450.

4.2.2.15. Determination of lipid peroxides (LPO) (Jordan and Schenkman, 1982).

To 100 µl separated microsomes in 0.1(M) phosphate buffer saline, 1ml of 28% trichloroacetic acid was added and centrifuged at x 2000 g at 4 ^{0}C for 20 minutes.1 ml of supernatant was separated and 900µl of 1% thiobarbituric acid was added and volume was adjusted to 3 ml by using phosphate buffer (pH7.0), 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 (1.56 x 10^5) of malondialdehyde.

4.3. Results

4.3.1. Levels of ALT, AST and TNF – α

ALT, AST and TNF - α levels in the sham operated rats were 84 .7 2 ± 8.2 I.U. L^{-1}, 56.02 ±11.92 I.U. L^{-1} and 37.65±3.01 $pgml^{-1}$ respectively. After 1h ischemia followed by 3h reperfusion ALT, AST and TNF - α level were increased significantly ($P< 0.01$) as compared to sham operated control rats. In folic acid treated groups ALT, AST and

TNF - α level reduced significantly (P< 0.01) as compared to I/R group rats. In, amlodipine treated group also it decreased ALT, AST and TNF – α significantly (P< 0.01), as compared to I/R group rats. ALT, AST and TNF – α levels in the L- arginine treated group were 22.24 ± 5.5 I.U. L^{-1}, 22.90 ± 7.6 I.U. L^{-1}, 44.5 7±8.2 $pgml^{-1}$ respectively which shows significantly decrease (P< 0.01) as compared to I/R treated group. L- arginine showed maximum decrease of liver function enzymes (ALT and AST) as compared to all other groups but maximum TNF - α level decreased in folic acid treated group (Table 4.1. and Figure. 4.1.) as compared to all other group.

4.3.2. Levels of DNA and RNA

Liver DNA and RNA were 2.91±1.17, 14.25±1.17 mg g^{-1} respectively in the sham operated control rats and decreased to 2.24± 0.37 and 8.23± 0.67 mg g^{-1} after 1 h ischemia followed by 3 h reperfusion. RNA levels were significantly increased in L-arginine treated rats as compared to all other groups. Ischemia and reperfusion induced toxicity reduced remarkably DNA and RNA contents as compared to sham operated control rat. L- arginine attenuated decrease of DNA and RNA content as compared to all other treated groups (Table 4.2. and Figure 4.2.).

4.3.3. Levels of NO and LPO

Activities of NO and LPO level were 8.20 ± 1.52 nML^{-1} mg^{-1} and 2.20±0.53 nmol MDA mg^{-1} respectively in I/R rat. (Table 4.3 and Figure 4.3). In the sham operated control rats, the MDA in the liver microsomes remained constant at approximately 0.5 nM MDA mg^{-1} of protein throughout the experiments. In vehicle treated I/R rats it markedly increased LPO values 4 times as compared to sham operated control rats. In L- arginine pretreated groups LPO level was significantly reduced as compared to others groups. NO production was increased significantly in L- arginine treated group as compared to other group.

4.3.4. Levels of microsomal cytochrome P_{450}

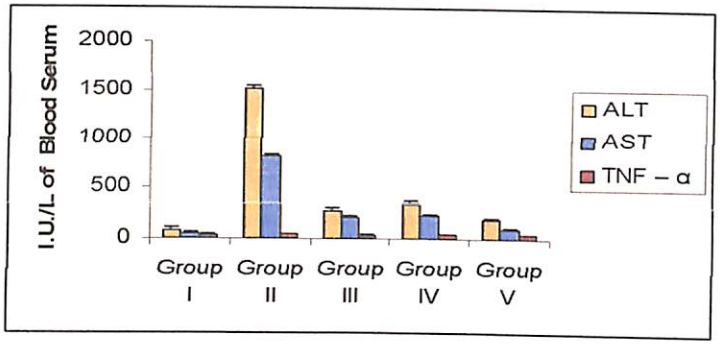
Microsomal Cytochrome P_{450} levels in the sham operated control rats were 0.74± 0.011 n ML^{-1} mg^{-1} protein which decreased to 0.40 ± 0.017 011 nML^{-1} mg^{-1} protein after1 h ischemia followed by 3 h reperfusion. The significant decrease in Cytochrome P_{450} activity that occurred after 1 h ischemia followed by 3 h reperfusion in the vehicle treated rats were significantly increased by the administration of folic acid, amlodipine and L- arginine treated rats. The decrease of Cytochrome P_{450} activity in

I/R group was maximum attenuated by L- arginine treated group as compared to all other group (Table 4.4. and Figure 4.4.).

Table: 4.1. The effect of folic acid, amlodipine and L- arginine on serum AST, ALT and TNF – α activity after I/R in rat liver.

Groups		ALT [a]	AST [a]	TNF – α [b]
Sham-operated	(Group I)	84 .72 ± 8.2	56.02 ±11.92	37.65±3.01
I/R Injury	(Group II)	1507.36 ±30.58*	817.40 ± 14.52*	48.13± 5.9 *
Folic acid treated	(Group III)	271.08 ±5.21*#	205.46± 12.14*#	35.56.±2.6
Amlodipine treated	(Group IV)	341.61 ± 7.6*#	224.85 ± 6.6* #	45.21.±4.1* #
L -Arginine treated	(Group V)	187.21± 14.52*#	105.10 ±8.92*#	44.57±8.2*#

Results are expressed as mean ± SD (n=6). * Statistically difference (P< 0.01) from sham operated rats.
Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.
[a]Expressed in I.U. L^{-1};[b]Expressed in pg ml^{-1}blood Serum



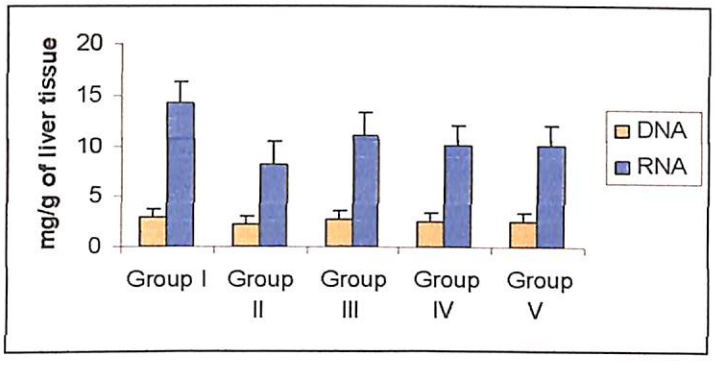
[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L-arginine treated rats].

Figure: 4.1. Graphical representation of the effect of folic acid, amlodipine and L-arginine on serum AST, ALT and TNF – α activity after I/R in rat liver.

Table: 4.2. The effect of folic acid, amlodipine and L- arginine on DNA and RNA activity after I/R in rat liver.

Groups		DNA[a]	RNA[a]
Sham-operated	(Group I)	2.91±0.97	14.25±2.17
I/R Injury	(Group II)	2.24± 0.37 *#	8.23± 0.67*#
Folic acid treated	(Group III)	2.70±0.18*#	11.20± 1.21#
Amlodipine treated	(Group IV)	2.53 ±0.72*#	10.03± 1.40*#
L -Arginine treated	(Group V)	2.55±0.28*#	10.05± 1.11*#

Results are expressed as mean ± SD (n=6). * Statistically difference (P< 0.01) from sham operated rats.
Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.
[a] Expressed as mg g^{-1} of liver tissue.



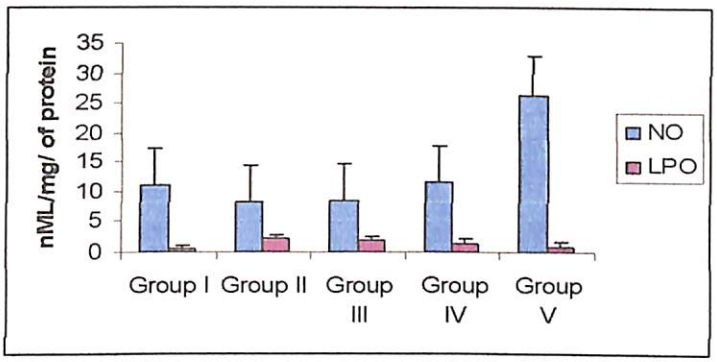
[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

Figure: 4.2. Graphical representation of the effect of folic acid, amlodipine and L-arginine on DNA and RNA activity after I/R in rat liver.

Table: 4.3. The effect of folic acid, amlodipine and L- arginine on NO and LPO activity after I/R in rat liver.

Groups		NO production[a]	LPO[b]
Sham-operated	(Group I)	11.09 ± 4.84	0.51±0.03
I/R Injury	*(Group II)*	8.20 ± 1.52*	2.20±0.53
Folic acid treated	(Group III)	8.45 ± 1.27#	1.91±0.28
Amlodipine treated	(Group IV)	11.74± 3.69##	1.49±0.47
L -Arginine treated	(Group V)	26.57± 6.32##	0.94± 0.71#

Results are expressed as mean ± SD (n=6). * Statistically difference (P< 0.01) from sham operated rats.
Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.
[a]Expressed as percentage nML^{-1} mg^{-1} of protein; [b]Expressed as nML^{-1} MDA mg^{-1} of protein



[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

Figure: 4.3. Graphical representation of the effect of folic acid, amlodipine and L-*arginine on NO and LPO activity after I/R in rat liver.*

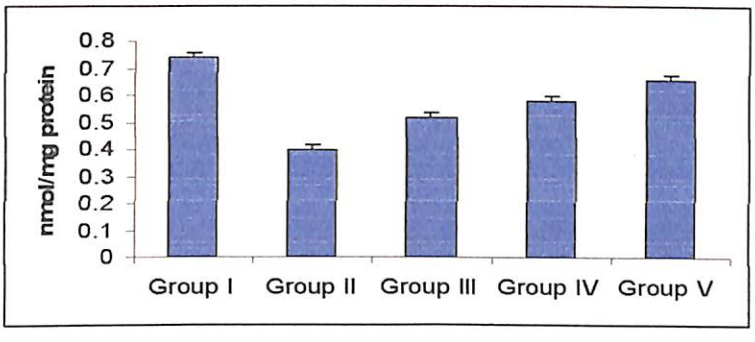
Table: 4.4. The effect of folic acid, amlodipine and L- arginine on cytochome P_{450} activity after I/R in rat liver.

Groups		Cytochome P_{450} [a]
Sham-operated	(Group I)	0.74± 0.011
I/R Injury	*(Group II)*	0.40± 0.017*
Folic acid treated	(Group III)	0.52± 0.013*#
Amlodipine treated	(Group IV)	0.58± 0.012*#
L -Arginine treated	(Group III)	0.66± 0.019*#

Results are expressed as mean ± SD (n=6). * Statistically difference ($P< 0.01$) from sham operated rats.

Statically difference ($P< 0.01$) from vehicle- treated ischemia and reperfusion.

[a]Expressed as $nML^{-1} mg^{-1}$ protein



[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

Figure: 4.4. Graphical representation of the effect of folic acid, amlodipine and L-arginine on Cytochome P_{450} activity after I/R in rat liver.

4.4. Discussion

In liver, *I/R* induced injury is characterized by reduction in the number of perfused sinusoids, heaptocellular hypoxia, leakage of enzymes (ALT, AST) from hepatocytes, decrease of microsomal cytochome P_{450} content, and decrease in total cellular DNA and RNA content (Jaeschke, 1997). The pathophysiology of I/R injury is complex and it is thought that ischemia activates kupffer cells (KC's) and subsequent reperfusion

phase forms vascular reactive oxygen (Cursio et al., 1980), which causes free radical-mediated tissue injury and by series of chain reactions produces Lipid peroxidation (LPO) (Jaeschke and Farhood, 1991). Formation of LPO leads to many pathological changes in tissue including liver necrosis (Marubayashi et al., 1986). The consequences of lipid peroxidation may be manifested as alternation in membrane integrity of membrane- associated functions in sub-cellular organelles.

The present study showed that after I/R injury ALT, AST levels were increased whereas microsomal cytochome P_{450} content, and total cellular DNA and RNA content were decreased (Table 4.1 and 4.2 & Figure 4.1 and 4.2), which is consistent with previous study (Goto et al., 1992; Colletti et al., 1996). Further, we have shown for the first time in literature that cytochome P_{450} decreased after I/R injury.

Immediately after I/R injury serum TNF-α was increased. Earlier study had shown that activation of KCs during reperfusion results in a massive release of cytokines including TNF- α (Schlayer et al., 1998) which is responsible for lung and hepatic injury and leukocyte infiltration after hepatic I/R in rats (Bevilacqu et al., 1998). Despite treatment by anti-TNF-α antibodies, pulmonary and hepatic injury after normothermic and cold hepatic ischemia reduces the levels of TNF a (Colletti et al., 1996; Cursio et al., 1998). It is also reported that TNF-α stimulates neutrophil adhesion to hepatic sinusoidal endothelial cells in rats (Colletti et al., 1996) and may activate endothelial cells to initiate coagulation. Thus, the impaired hepatic function caused by I/R injury, that might be a reason for activation of KCs, led to increase in TNF – α, as seen in the present work. It indicates that TNF- α is an important cytokine to regulate I/R injury and it is the hallmark of our study.

In our study, endothelial NO production was decreased in I/R injury and modulation of NO production leads to decrease in severity of I/R induced heaptocellular damage (Table 4.3 and Figure 4.3). Production of NO is important as it regulates vasodilation during I/R injury. It is reported that NO is a potent vasodilator, which diffuses freely across cell membranes and acts intracellularly by activation of guanylate cyclase. In response to vasoconstriction by Pringle manoeuvere technique, NO can induce vasodilatation at the level of the sinusoids as well as at presinusoidal sites (Granger et al. 1994). Thus, NO prevents oxidative damages in I/R injury is the second novel finding of the present study.

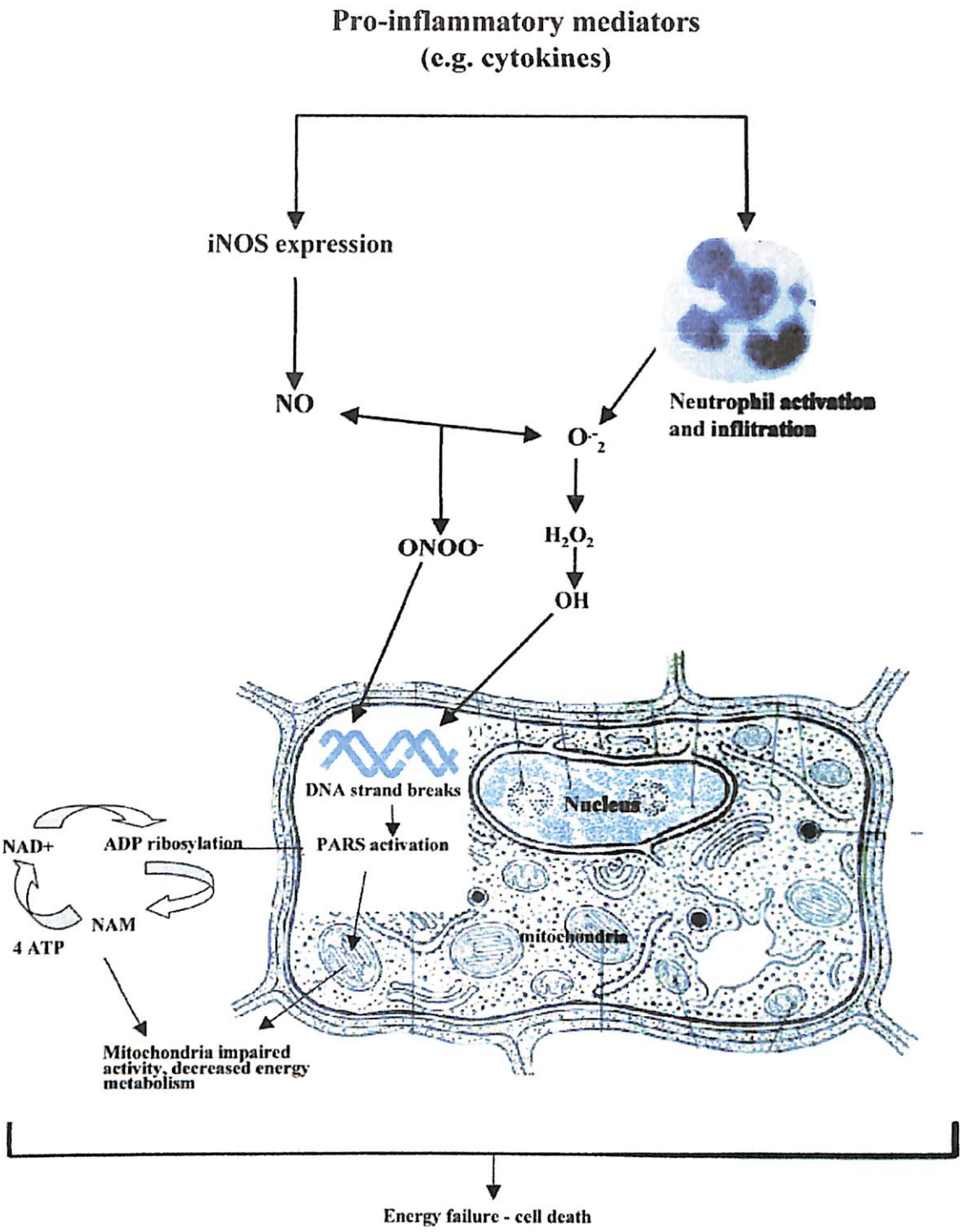


Figure: 4.5. Probable mechanism of L- arginine protection

Further, in our study, we found out that L –arginine treated group attenuated the changes in ALT, AST cytochome P_{450} content, total cellular DNA and RNA content in I/R injury rats. L –arginine also increases NO production in hepatocytes without any significant effects in modulation of serum TNF- α (Table 4.2 and 4.4). This suggests that TNF- α. mediated response is an independent phenomenon in I/R injury and there is no relationship with NO production.

Thus, our studies advocate that L- arginine significantly protects I/R injury by producing NO in hepatic sinusoids. This can be explained as L- arginine is precursor of NO and during ischemia L- arginine restored vasodilatatory effects by generation of NO in hepatic sinusoids, which attenuated the I/R injury.

Amlodipine was found to decrease AST and ALT levels, increase DNA and RNA content, increase cytochrome P_{450} levels as compared to I/R rats and reduces hepatocellular injury. Amlodipine did not have effect on NO production but decreased TNF-α levels. This can be explained by the fact that amlodipine is considered to be a long acting Ca^{+2} channel blocker and maintains Ca^{+2} homeostasis and ischemia is associated with impaired calcium homeostasis that results in increased TNF- α production leading to cell death. Ischemic tissue injury causes an increase in free intracellular calcium that leads to diminished recovery of dilatory function after ischemia (vasoconstriction), compromised membrane integrity and decrease in reserves of cellular adenosine triphosphate (Satoh et al., 2003) and enhances oxidative stress and mitochondrial calcium overload responsible for mitochondrial dysfunction (Burges and Moisey, 1994). Thus, the protective effects in I/R injury by inhibiting Ca^{+2} influx in mitochondria during I/R injury of amlodipine can be clearly explained.

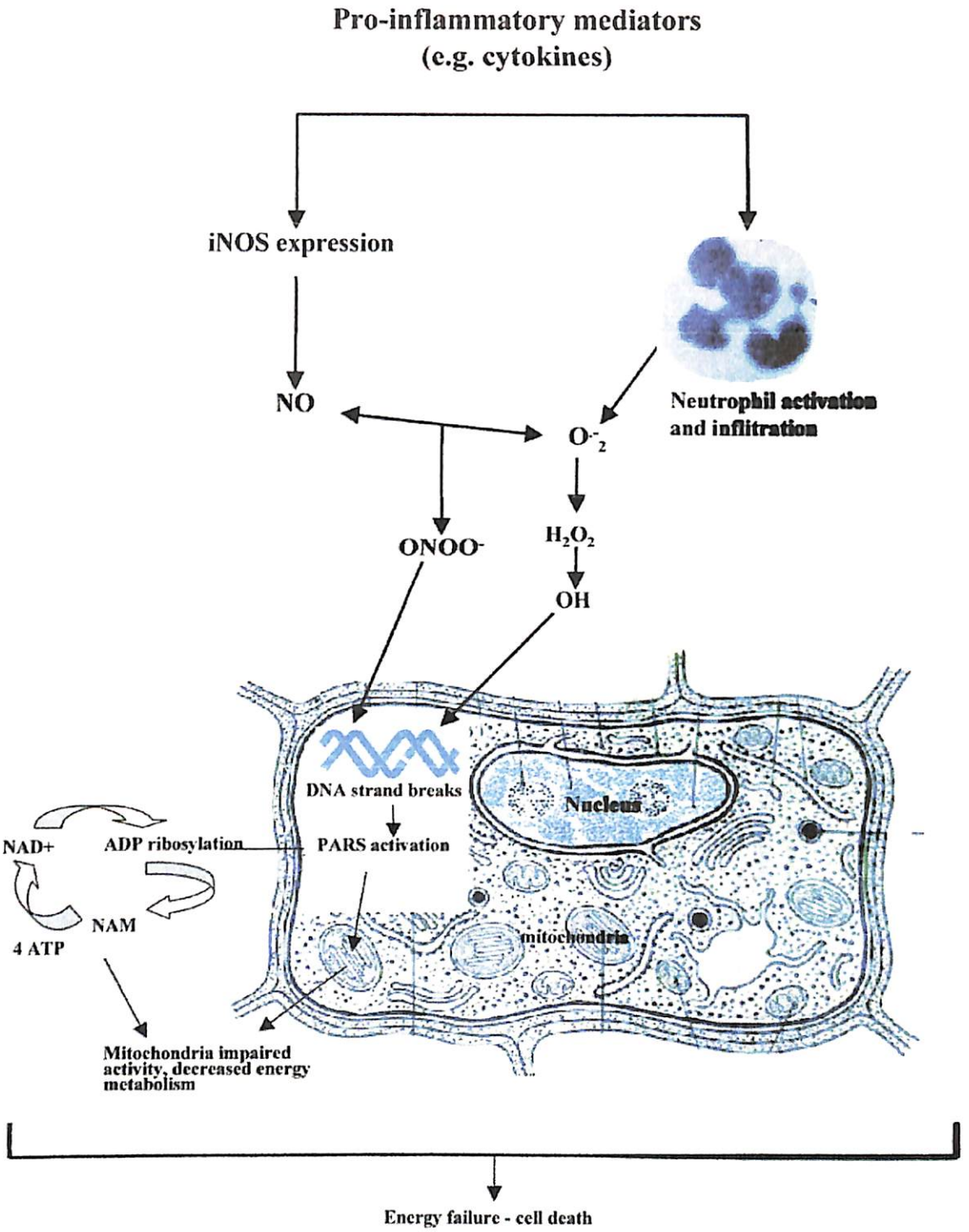


Figure: 4.5. Probable mechanism of L- arginine protection

Our study also showed that folic acid prevented I/R injury by attenuating the changes in ALT, AST cytochome P_{450}, total cellular DNA and RNA contents in I/R injury of rats. An important finding in folic acid treated group was that folic acid significantly decreased TNF-α levels but not NO level in I/R rats. This observation indicates the probable mechanism of folic acid in protection in I/R injury. TNF- α is potent inducer of hepatic chemokine synthesis and under certain circumstances this TNF- α can directly trigger apoptotic cell death. Apoptosis is mediated by the transcriptional up-regulation of adhesion molecules on endothelial cells due to the activation of the transcription factor NF-kB and increase in TNF- α level in blood serum in I/R injury. Folic acid prevents increase in TNF-α level in blood establishing that folic acid has significant role in protection from apoptosis in I/R injury. Further, folic acid deficiency inhibits cellular proliferation, disturbs cell cycling (Blount et al., 1997) causing genetic damage (Goulian et al., 1956) and eventually results in cell death. Thus, folic acid may be considered as one of the regulating factor of transcription factor NF-kB by inhibiting TNF- α level. The underlying I/R injury associated with these observed phenomena remain elusive but supports the hypothesis of inhibition of TNF-α activity by folic acid in I/R injury.

4.5. Conclusions

1. The present investigation suggests that protective action of amlodipine, a calcium channel blocker, against the I/R induced liver injury in rats is due to its inhibitory action of Ca^{2+} influx into mitochondria. This study also showed that there is a link between the reductions in I/R induced injury liver markers as achieved with calcium antagonists, and that obtained with oxygen radical scavengers.
2. The present investigation also suggests that protective effects of L- arginine against I/R injury on rat hepatocyte might be partially due to production of NO and by increased vasodilatation.
3. The protective action of folic acid, against the I/R induced injury, is mainly due to inhibitory action of TNF – α and by increase in DNA and RNA synthesis.

4.6. References

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Chapter - 5

Folic acid, Amlodipine, L- Arginine Attenuated Mitochondrial Respiratory Enzymes in Ischemia followed by Reperfusion in Rat Liver

5. 1. Introduction

Life and cellular function are dependent on oxygen consuming (aerobic) metabolism, but during limited periods with shortage of oxygen, essential processes are maintained by energy rich compounds gained through anaerobic metabolism and cause oxidative stress defined as a situation when redox- status of biological compounds is changed due to the combination of Reactive oxygen species (ROS) in relation to either a diminished or insufficient scavenging capacity and occurs with excess oxygen supply, vascular occlusion (ischemia), radiation or toxin influence. ROS and reactive nitrogen species (RNS) are well recognized for playing a dual role both as, deleterious and beneficial species. ROS and RNS are normally generated by tightly regulated enzymes, such as NO synthase (NOS) and NAD (P) H oxidase isoforms, respectively. Ischemia activates Kupffer cells (KC), which are the main sources of vascular reactive oxygen formation during the initial reperfusion period (Jaeschke and Farhood, 1991), which is only observed after no-flow ischemia (Pringle, transplantation) but not after hemorrhagic shock, i.e., low-flow ischemia (Jaeschke and Farhood, 2002). In addition to KC's-induced oxidant stress, with increasing length of the ischemic episode, intracellular generation of reactive oxygen by xanthine oxidase and in mitochondria (Gonzalez-Flecha et al., 1993) may also contribute to liver dysfunction and cell injury during reperfusion (Kumamoto et al., 1999). The presence of a phagocyte-type NADPH oxidase was recognized as a major source of superoxide formation in endothelial cells (Li and Shah 2001) and hepatocytes (Ozaki et al., 2000). Rac1, a member of the Rho family of small GTPases, regulates this enzyme. Inhibition of Rac1 attenuated the intracellular oxidant stress during the early reperfusion phase and protected against liver injury (Jaeschke et al., 1992). Pro-inflammatory cytokines, chemokines, and activated complement factors are responsible for neutrophil recruitment and the subsequent neutrophil-induced oxidant stress during the later reperfusion phase (Jaeschke et al., 2002). Stimulation of primed KC by complement factors causes the continuous activation of these macrophages (Farhood et al., 1991). The KC- and neutrophil-induced oxidant stress is an important factor in vascular and parenchymal cell injury during reperfusion (Bilzerand and Lauterburg, 1994).

The relevance of this post-ischemic vascular oxidant stress was demonstrated by the protective effect of extracellular glutathione (GSH), which can scavenge hydrogen

peroxide, hypochlorous acid, and peroxynitrite (Bilzer et al., 1999). Mainly vascular origin of the oxidant stress, reactive oxygen generated by KC (Jaeschke, 1991) or adherent neutrophils (Mathews et al., 1994) causes a substantial intracellular oxidant stress in hepatocytes. Animals deficient in glutathione peroxidase are significantly more susceptible to neutrophil-induced oxidant stress than wild-type animals (Mathews et al., 1994). Thus, that intracellular defense mechanisms appear to be critical for detoxification of reactive oxygen species generated by intracellular as well as extracellular sources and mechanism explains why antioxidants targeted to either extracellular or intracellular sites attenuated reperfusion injury in the liver (Mavier, 1988).

Substantial controversy during the last two decades was about the molecular mechanism of injury. Initially, it was assumed that any post ischemic oxidant stress leads to cell death by lipid peroxidation but lipid peroxidation is quantitatively insufficient to explain the severe cell injury during reperfusion (Kushimoto et al., 1996). Inflammatory cells also release proteases, which may be the actual cytotoxic mediators of neutrophils .The beneficial effect of protease inhibitors supported a role of proteases in the pathophysiology of reperfusion injury in experimental models and in humans (Kim et al., 2002). It was hypothesized that the role of reactive oxygen species in an inflammatory injury *in vivo* is actually not to cause cell injury but to inactivate anti-proteases of the plasma by oxidation in the vicinity of the neutrophil (Weiss, 1989), which would allow neutrophil-derived proteases to act locally without interference of anti-proteases. Proteases, which escape into the circulation, can still be inactivated to prevent systemic vascular injury (Weiss, 1989). Recent studies indicate that Kupffer cells (Jaeschke, 1991) and neutrophils (Mathews et al., 1994) can kill hepatocytes in vivo by reactive oxygen species. Generally oxidant stress-induced cell killing involves oxidation of pyridine nucleotides, accumulation of calcium in mitochondria, and superoxide formation by mitochondria, which ultimately leads to formation of membrane permeability transition pores and breakdown of the mitochondrial membrane potential.

Increases in cellular Ca^{2+} and reactive oxygen species (ROS), initiated in ischemia and then amplified upon reperfusion, are considered to be the main causes of reperfusion injury. Mitochondria are involved both in the production of ROS and as targets for the

damaging action of both ROS and calcium (Halestrap et al, 2004; Solaini and Harris, 2005).

The mitochondrial respiratory chain is one of the most important sites of ROS production under physiological conditions (Martinou, 1999; Tan et al., 1998). Mitochondria-derived ROS are vital not only because mitochondrial respiratory chain components are present in almost all eukaryotic cells but also because the ROS produced in mitochondria can readily influence mitochondrial function without having to cope with long diffusion times from the cytosol. Two sites in the respiratory chain, complex I and complex III are suggested to be the major ROS sources (Newmeyer and Ferguson-Miller, 2001). Reduced glutathione (GSH) is known to function as an antioxidant and a physiological reservoir for cysteine and is involved in DNA synthesis, protein synthesis regulation, and detoxification, etc. Cellular GSH deficiency affects the mitochondrial GSH pool and the cytosolic GSH pool. Mitochondrial GSH is important for the detoxification of ROS generated by the respiratory chain, conjugation of xenobiotics, maintenance of thiol-containing proteins, and regulation of the mitochondrial membrane potential (MMP) (Kroemer et al., 1998; Vercesi et al., 1997). Mitochondria play a central role in cell life and death by regulating the MMP as reduction of the MMP enhances the opening of mitochondrial permeability pores leading to the release of cytochrome *c* and apoptosis-inducing factor into the cytosol and thus triggering the apoptotic cascade (Esteve et al., 1999). ROS may interact with cellular biomolecules, DNA, leading to oxidative DNA damage (Cooke et al., 2003).

The depletion of ATP combined with elevated Ca^{2+} and ROS leads to a gradual decline in cellular integrity as degradative enzymes are activated and ATP-dependent repair processes are unable to operate (Halestrap et al., 1998). If the tissue remains ischemic for only short periods and the mitochondria remain sufficiently intact to generate the ATP, tissue damage is slight and can be repaired by ATP-dependent processes upon reoxygenation. Mitochondria isolated from ischemia and reperfusion exhibit reduced rates of ADP-dependent respiration (Venditti et al., 2001; Murfitt, 1978; Hardy, 1991), because mitochondria plays a critical role in the maintenance of cardiac energy and Ca2+ homeostasis but in the induction of apoptosis it is critical to define events responsible for alterations in mitochondrial activity. Coronary reperfusion is

associated with increased levels of oxygen radicals (Bolli, and Marban, 199; Zweier, 1988; Zweier et al., 1987). Studies indicate that mitochondria contribute to reperfusion-induced increase in the production of free radicals (Otani, 1988; Venditti et al., 2001). Thus it can be said that loss in respiratory function during reperfusion is partly due to oxidative modification of mitochondrial components. *In vitro* studies indicate that certain mitochondrial proteins are highly susceptible to oxidative inactivation (Humphries and Szweda, 1998; Humphries et al., 1998; Nulton-Persson and Szweda, 2001). Studies aimed at identifying alterations in protein function responsible for ischemia- and reperfusion-induced declines in mitochondrial respiration have focused primarily on the activities of electron transport chain complexes I, III, and IV, F_1F_0-ATP (synth)ase, and/or adenine nucleotide translocase (ANT) (Duan and Karmazyn, 1989; Veitch, 1992). (Figure. 4.1). The maximum rate of respiration is limited by NADH production (Humphries et al., 1989) and the degree of inactivation of these enzymes appears to be of insufficient magnitude to account for observed declines in respiration (Lucas et al., 1989). *In vitro* studies indicate that certain Krebs cycle enzymes are highly susceptible to oxidative inactivation (Gardner et al., 1994). These considerations underscore the need for evaluating ischemia/reperfusion-induced alterations in the activities of various Krebs cycle enzymes. Little is known regarding the effects of ischemia or reperfusion on enzymes critical for the supply of NADH. Previously it has been shown, that in an isolated heart model, α-ketoglutarate dehydrogenase (KGDH) activity declines during cardiac reperfusion (Lucas and Szwed, 1999) (Figure. 4.2).

Therefore, the present study was undertaken to see whether the effects of ischemia/reperfusion alter the Krebs cycle enzymes, respiratory marker enzymes or specific to those enzymes known to be susceptible to oxidative inactivation and to distinguish between functional alterations which occur during ischemia related to reperfusion on pretreatment with folic acid, L- arginine and amlodipine and to see oxidative events that contribute to decline in enzymatic and mitochondrial function.

5.2. Materials and Methods

5.2.1. Materials

Tris-HCl was purchased from Central Drug House, Mumbai. 2, 6 – Dichlorophenol Indophenol dihydrade (DCIP), Phenazine methosulphate (PMS) were purchased from

Hymedia, Mumbai. Nicotinamide adenine dinucleotide phosphate (NADH) metaphosphoric acid, Glutathione (GSH) were pyrogallol purchased from Otto Chemaei, Mumbai. 3-(4-5- dimethyl thiazol – Z- yl)- 2,5 diphenyl tetrazolium bromide (MTT), N- 2 Hydroxyethyl piperazine N- 2 – ehanesulphonic acid (HEPES) were purchased from Sisco Research Laboratories, Mumbai. ATP determination kit of Biaffin GmBH, Kassel, Germany and Cytochrome c from Sigma, St louis, Mo, USA were used. Folin-Ciocalteu's phenol reagent, Roswell Park Memorial Institute (RPMI 1640) cell culture powder, Napthlethlenediamine dihydrochloride, Thiobarbituric acid, Sulphanilamide, Sodium Dithionate were purchased from Hymedia Ltd, Mumbai

5.2.2. Methods

5.2. 2.1 Animal groups

Thirty Wistar rats were divided into sham-operated control group (I) (n = 6), ischemia and reperfusion group (II) (n = 6), were given 0.9 % saline (5ml kg^{-1}, p.o) for 7 days; amlodipine treated group (given amlodipine 1 mg kg^{-1} body mass daily by oral route for 7 days before induced ischemia reperfusion maneuver) group III (n=6). Folic acid treated group (given folic acid 1 mg kg^{-1} body mass daily by oral route for 7 days before inducing ischemia reperfusion maneuver) (IV) (n = 6) and L-Arginine treated group (given L-arginine 100 mg kg^{-1} body mass daily by oral route for 7 days before inducing ischemia reperfusion maneuver) (V) (n = 6).

5.2. 2.2. Ischemia and reperfusion injury

Ischemia and reperfusion injury were same as described on page 29, chapter 3.

5. 2.2.3. Peripheral blood and tissue sampling

Peripheral blood and tissue sampling method was same as described on page30, chapter 3.

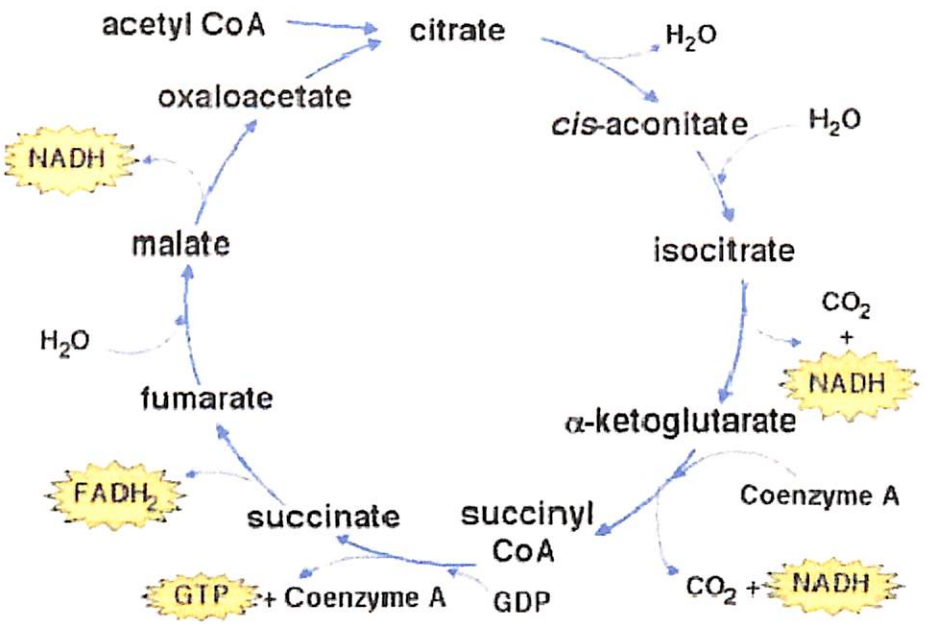

Figure: 5.1. Energy production in Citric acid Cycle.

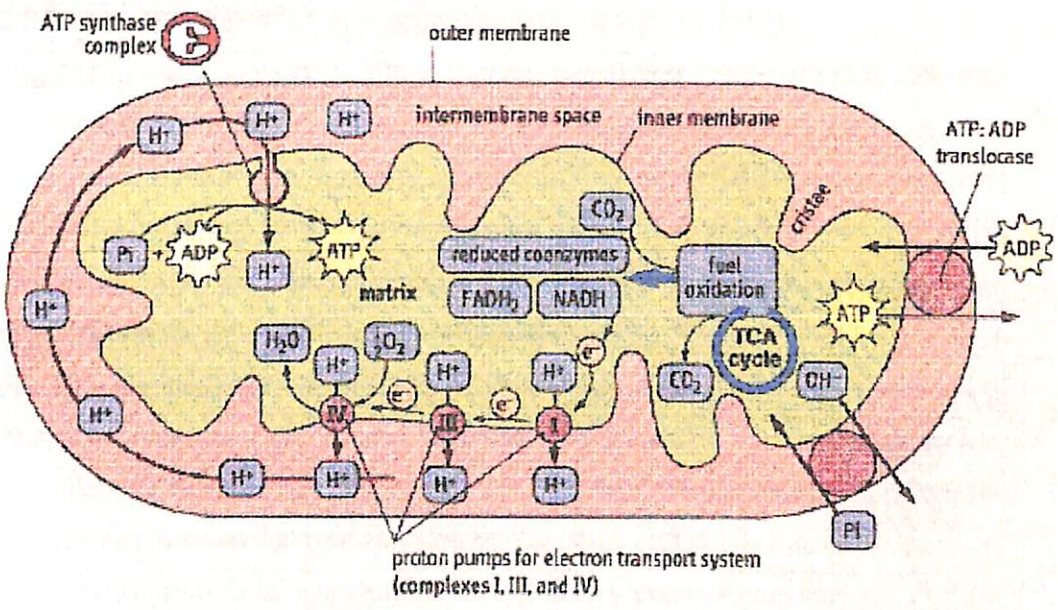

Figure: 5.2. The Mitochondrion and energy transduction.

5.2.2.4. Isolation of liver mitochondria (Starkov and Fiskum, 2003.)

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 gm L^{-1} 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 r.p.m. for 4 min. The supernatant was separated and centrifuged again at 14,000 r.p.m. 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 r.p.m for 10 min. The final pellet containing mitochondria was resuspended in isolation buffer without EGTA or BSA to a concentration of 25-30 mg of protein ml^{-1}, stored in ice, and was used within 5 h.

5.2.2.5. Assay of integrity of liver mitochondria (Mossman, 1983)

100 µg MTT was dissolved in 100 ml media containing 15mM HEPES, 5% FBS (Fetus Bovine Serum), Penicillin (100 U ml^{-1}) and Streptomycin (100 µg ml^{-1}) medium at a concentration 0.5 mg ml^{-1}. 1 ml of MTT – containing medium was added to each well containing 50 µl of mitochondrial extract. I/R group served as positive control group and sham operated control rats were served as negative control rats. After incubation for 6h at 37^0C in humidified atmosphere rich in 5% CO_2 +95% air in Carbon Dioxide incubator, the supernatant was removed and 1.25 ml of a solution of 0.4 N HCl – isoprpanol (1: 24 v/v) was added to solubilize the frormazan (colour comlex after reaction with MTT). After 30 min at room temperature, the frormazan absorbance was measured at a wavelength of 570 nm.

5.2.2. 6. Determination of mitochondrial respiratory marker enzymes

5.2.2. 6.1. Determination malate dehydrogenase (MDH) (Mehler et al., 1948)

50 µl of mitochondrial suspension was taken in 4 ml cuvette at 37 °C consisting of 0.5 ml of 0.3 M L^{-1} phosphate buffer (pH 7.4), 0.3 ml of 0.2 M L^{-1} sodium malonate, 50 µl of 0.45 M L^{-1} KCN and final volume was adjusted with water to 3 ml. After

incubation for 5 min at room temp (20°C). 60 µl DCIP (0.1% w/v solution in 0.3 M phosphate buffer) followed by 100 µl of 0.33 % w/v phenazine metosulphate (PMS) was added and the absorbance at 600 nm was recorded. Absorbance of each sample was recorded taking four different concentrations (100, 200, 300 and 400 µl) of PMS. V_{max} was estimated from a double reciprocal plot concentration (abscissa) and initial rate of the absorbance changes at 600 nm (ordinate). Activity of malate dehydrogenase was calculated from the absorbance decrease, using absorption coefficient for DCIP of 19.1 nM L^{-1}, 1 x cm^{-1} at 600 nm and expressed as micromoles of oxidized malonate x min^{-1} per mg of mitochondrial protein.

5.2.2.6.2. Determination succinate dehydrogenase (SDH) (Slater et al., 1952)

50 µl of mitochondrial suspension was taken in a 4 ml cuvette at 37 °C consisting of 0.5 ml of 0.3 M L^{-1} phosphate buffer (pH 7.4), 0.3 ml of 0.2 M sodium succinate and 50 µl of 0.45 M L^{-1} KCN and final volume was adjusted using water to 3 ml. After incubation for 5 min 60 µl DCIP (0.1% w/v solution in 0.3 M L^{-1} phosphate buffers) followed by 100 µl of 0.33 % w/v phenazine metosulphate (PMS) was added and the absorbance of each sample was recorded at 600 nm. Measurements taking four different concentrations (100 µl to 400 µl) of PMS. V_{max} was estimated from a double reciprocal plot concentration (abscissa) and initial rate of the absorbance changes at 600nM L^{-1} /min (ordinate). Activity of succinate dehydrogenase was calculated from the absorbance decrease, using absorption coefficient for DCIP of 19.1 nM L^{-1}, 1x cm^{-1} at 600 nm and expressed as micromoles of oxidized succinate x min^{-1} per mg of mitochondrial protein.

5.2.2.6.3. Determination cytochrome c oxidase activity (Ferro- cytochrome c; oxygen oxidorecductase, EC 1.9.3.1) (Pearl et al., 1963)

100 mg of cytochrome c dissolved in 10 ml cold 10 mM phosphate buffer (pH 7.4) was treated with 10 mg of potassium ascorbate. The excess of ascorbate was removed by dialysis against 1 L of 10 mM L^{-1} phosphate buffer, pH 7.4 for 24 h. Aliquots 0.5 ml of reduced cytochrome were frozen and stored at – 20 ° C for assay. 50-µl mitochondrial suspensions was taken in 4 ml cuvette at 37 °C in a medium consisting of 1ml 45 mM phosphate buffer, pH 7.4 and 50 µl reduced cytochrome c and volume was adjusted to 3 ml by distilled water. Absorbance at 550 nm at interval of 30 seconds was taken to studying the rate of reaction. After 3 min the reaction was

terminated by addition of 10 µl of saturated potassium ferrocyanide solution to oxidize the remaining reduced cytochrome c. The difference between the initial and final absorbance was used to determine the initial substrate concentration. The activity of the enzyme was calculated as V_i, by multiplication of the initial substrate concentration by the estimated first order velocity constant. Results are expressed as micromoles of oxidized cytochrome c x min $^{-1}$ per mg of mitochondrial protein.

5.2.2.6. 4. Determination NADH dehydrogenase (Minakami et al., 1962)

50 µl mitochondrial fraction was taken in a 4 ml cuvette at 37 °C containing medium consisting of 100 µl of 250 µM L^{-1} NADH, 50 µl 10 mM L^{-1} KCN, 100 µl 40µM L^{-1}DCIP and final volume was adjusted to 2.9 ml by Tris- HCl (pH 7.5) . After incubation for 5 min, the mixture was added to 60-µl di chloro indo phenol (DCIP) (0.1% w/v solution in 0.3 M L^{-1} phosphate buffer) and the absorbance was recorded at 600 nm. V_{max} was estimated from a double reciprocal plot concentration (abscissa) and initial rate of the absorbance changes at 600 nm min^{-1} (ordinate). Activity of NADH dehydrogenase was calculated from the absorbance decrease, using absorption coefficient for DCIP of 19.1 nM L^{-1}, 1 x cm $^{-1}$ at 600 nm and expressed as micromoles of oxidized dehydrogenase x min $^{-1}$ per mg of mitochondrial protein.

5.2.2.6.5. Respiratory complex I assay (Humphries and Szweda, 1998)

500 µl liver mitochondria were solubilized in hypotonic solution (5 ml of 10 mM MOPS, pH 7.4) to produce 100 µl ml^{-1} concentration and freeze-thawed using liquid nitrogen. Frozen and thawed mitochondria were then diluted to 5 µl ml^{-1} in a solution containing 10 mM L^{-1} MOPS, 10 nM L^{-1} antimycin A and 20 µM L^{-1} KCN and then 40 µM L^{-1} ubiquinone-1.NADH (5-40 µM) was immediately added to initiate complex I activity. Complex I activity was measured as the rate of NADH oxidation (340 nm, $\varepsilon = 6200\ M^{-1}.cm^{-1}$) using UV spectrophotometer.

5.2.2.6.6. Determination of ketoglutarate (Reed and Mukherjee, 1969)

In a eppendorf tube 0.1 M-Tris-HCl, pH 8.0, l0 mM-$MgCl_2$, mM-EDTA, 0.1 mM-thiamin pyrophosphate, 2.5mM L^{-1}-cysteine hydrochloride, 0.1 mM L^{-1}-CoA, 0.5 mM L^{-1}-NAD^+ and 1 mM L^{-1}- ketoglutarate were taken to produce 900 µl by adding with distilled water. The mixture were incubated for 10 min at 25°C and NADH (250 µML^{-1}) was added to give a total volume of 1.0 ml. Lineweaver-Burk plots were drawn by absorbance at Y axis and substrate concentration at X axis. The values of activity (v)

and substrate concentration (A) were fitted to the equation v = (Vmax.A)/ (Km+A) and concentration of α ketoglutarate was calculated at 600nm using UV spectrophotometer.

5.2.2.6.7. Determination intracellular ATP

50µl of mitochondrial suspension was taken in cell culture tube and 3 ml ice cold 6% (w/v) perchloric acid was added. It was then sonicated and left at 0°C for 20 min after filtration neutralized with ice cold 6 M L^{-1} KOH followed by 100 µl of 0.5 M morpholine sulphonic acid was added. ATP content was determined in neutralized cellular extracts by the luciferase- luciferin assay by using ATP determination kit (Biaffin GmBH, Kassel, Germany) as per manufacturer's guidelines.

5.2.2.6.8. Determination mitochondrial calcium content

50µl of mitochondrial suspension was taken in cell culture tube. Mitochondrial calcium was estimated using diagnostic kit (Quilagens diagnostics, India) as per manufacturer's guidelines.

5.3. Results

5.3.1. Mitochondrial integrity

The mitochondrial integrity in the sham operated control rats were 0.631 O.D. Mitochondrial integrity decreased to 0.210 O.D. in I/R rats. The decrease of mitochondrial integrity activities in I/R group were attenuated in folic acid, L-arginine and amlodipine treated groups as compared to I/R group. L- Arginine and amlodipine treated groups showed the nearly similar results in MTT assay as compared to all other treated groups. (Table.5.1 and Figure. 5.3).

5.3.2. Ca^{+2} content and mitochondrial levels of NADH, cytochrome –c (respiratory marker enzymes)

The mitochondrial Ca^{+2} levels in the sham operated control rats were 7.11±0.39 nM L^{-1} mg^{-1} protein. After 1 h ischemia followed by 3 h reperfusion Ca^{+2} content was increased to 12.36 ± 1.54 nM L^{-1} mg^{-1} protein in I/R group. Accumulations of Ca+2 were 4 times higher in I/R perfusion rats as compared to sham-operated rats. The increase of Ca^{+2} activities in I/R group was maximum attenuated in amlodipine treated group as compared to all other experimental groups (Table 5.2 and Figure 5.4, 5.5).

Activities of cytochrome c and NADH levels in the sham operated control rats were 0.32±0.07 and 121.47 ±9.72 nM L^{-1} mg^{-1} protein respectively whereas after 1 h ischemia followed by 3 h reperfusion injury in the vehicle – treated cytochrome c and NADH levels were 0.25±0.05 and 60.44 ±7.54 nM L^{-1} mg^{-1} protein respectively. Decrease of cytochrome c and NADH levels were significantly ($P< 0.05$) suppressed by folic acid, amlodipine, L- arginine. L- arginine attenuated maximum the decrease of mitochondrial respiratory level (NADH, Cytochrome –c) as compared to all other experimental groups (Table 5.2 and Figure 5.4, 5.5).

5.3.3. Levels of mitochondrial MDH, SDH, α- KGDH (TCA marker enzymes)

Activities of MDH, SDH, α- KGDH were 198.11 ± 24.2 and 107.02 ± 15.11 nM L^{-1} mg^{-1} protein respectively in I/R group which were significantly lower as compared to sham operated control rats. Amlodipine and folic acid treated groups also attenuated decreased levels of TCA marker enzymes. MDH, SDH, α- KGDH which were 202.14± 11.54, 174.52± 12.05, and 39.87±7.05 nM L^{-1} mg^{-1} protein respectively in folic acid treated group and 209.7± 27.18, 182.32± 24.50, 41.57±4.87 nM L^{-1} mg^{-1} protein respectively in amlodipine treated group. L- arginine treated group showed maximum protection in attenuation of TCA marker enzymes as compared to all other experimental groups. MDH, SDH, α- KGDH in L- arginine treated group were 244.50 ± 15.70, 193.4 1 ± 26.14, 62.10±6.17 nM L^{-1} mg^{-1} protein respectively (Table.5.3 and Figure. 5.6).

5.3.3. Levels of mitochondrial respiratory complex I

The mitochondrial respiratory complex I levels in the sham operated control rats was 158.31± 9.87 nM L^{-1} mg^{-1} protein. After 1 h ischemia followed by 3 h reperfusion injury respiratory complex I content was decreased to 104.56 ± 8.75 nM L^{-1} mg^{-1} protein in I/R group. Administration of folic acid and L- arginine significantly increase respiratory complex I activity as compared to I/R group. The decrease of respiratory complex I activities in I/R group was maximum attenuated in amlodipine treated group as compared to all other experimental groups (Table. 5.4 and Figure. 5.7).

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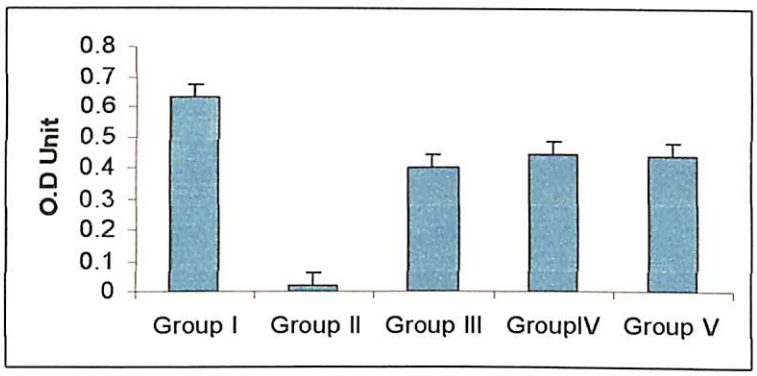
Table: 5.1. Effect of folic acid, amlodipine and L- arginine on mitochondrial integrity after I/R in rat liver.

Groups		[a] MTT assay (Δ^{570})
Sham-operated	*(Group I)*	*0.631 ± 0.020*
I/R Injury	(Group II)	0.210 ± 0.041*
Folic acid treated	(Group III)	0.403± 0.012*[#]
Amlodipine treated	(Group IV)	*0.441± 0.039*[#]*
L -Arginine treated	(GroupV)	0.437 ± 0.028*[#]

Results are expressed as mean ± SD (n=6). * Statistically difference (P< 0.01) from sham operated rats.

[#] Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.

[a] Expressed as respective Optical density of fromazan formation at Δ^{570}



[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L-arginine treated rats].

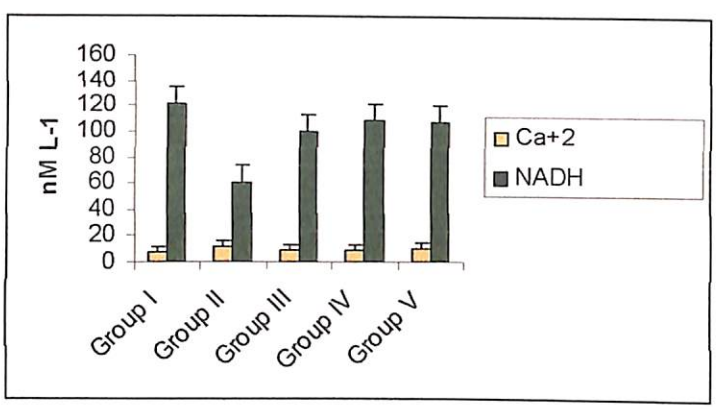
Figure: 5.3. Graphical representation of the effect of folic acid, amlodipine and L-arginine on mitochondrial integrity after I/R in rat liver.

Table: 5.2. The effect of folic acid, amlodipine and L- arginine on Ca^{+2} content and mitochondrial respiratory levels (NADH) after I/R in rat liver.

Groups		Ca^{+2} [a]	Cytochrome c [b]	NADH[c]
Sham-operated	(Group I)	7.11±0.39	0.32±0.07	121.47 ±9.72
I/R Injury	(Group II)	12.36±1.54*	0.25±0.05*	60.44 ±7.54 *
Folic acid treated	(Group III)	9.12±0.8 6*#	0.26±0.07*#	100.25±13.55*#
Amlodipine treated	(Group IV)	8.70±0.9 2*#	0.27 ± 0.04*#	108.44 ± 17.47#
L -Arginine treated	(GroupV)	10.21 ± 4.58*#	0.30 ± 0.02* #	106.84 ±15.21*#

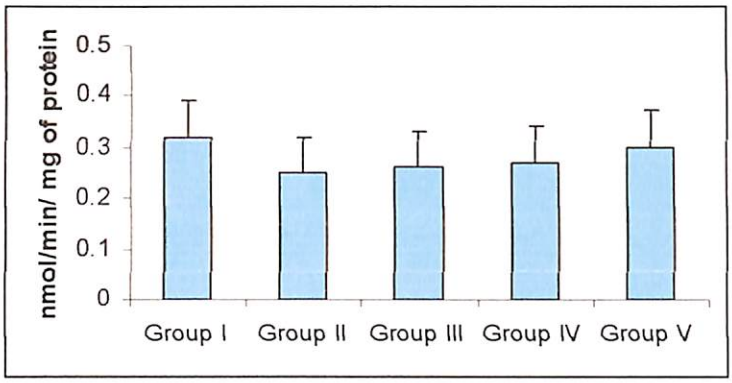
Results are expressed as mean ± SD (n=6). * Statistically difference (P< 0.01) from sham operated rats. # Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.

[a]Expressed as nM L^{-1} mg^{-1} of protein; [b]Expressed as nM L^{-1} $min^{-1}mg^{-1}$ of protein; [c]Expressed as nM L^{-1} of NADH oxidized per min per mg protein for NADH dehydrogenase.



[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L-arginine treated rats].

Figure: 5.4. Graphical representation of the effect of folic acid, amlodipine and L-arginine on Ca^{+2} content and mitochondrial respiratory levels (NADH) after I/R in rat liver.



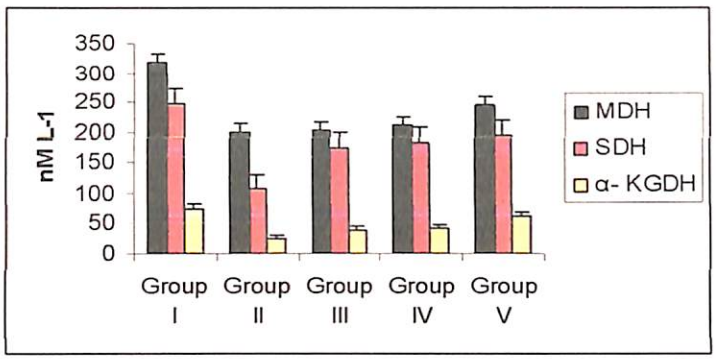
[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L-arginine treated rats].

Figure: 5.5. Graphical representation of the effect of folic acid, amlodipine and L-arginine on cytochrome – c levels after I/R on rat's liver.

Table: 5.3. The effect of folic acid, amlodipine and L- arginine on TCA cycles marker enzymes (MDH, SDH, α- KGDH) after I/R in rat liver.

Groups	MDH[a]	SDH[b]	α- KGDH[c]
Sham-operated (Group I)	319.11 ± 14.0	247.44 ± 24.8	74.20± 5.81
I/R Injury (Group II)	198.11 ± 24.2*	107.02 ± 15.11*	24.51± 9.04*
Folic acid treated (Group III)	202.14± 11.54	174.52± 12.05*#	39.87±7.05*#
Amlodipine treated (Group IV)	209.7±27.18*	182.32± 24.50*#	41.57±4.87*#
L -Arginine treated (GroupV)	244.50 ± 15.70*#	193.41 ±26.14*#	62.10±6.17*#

Results are expressed as mean ± SD (n=6). * Statistically difference (P< 0.01) from sham operated rats. # Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion. [a]Expressed as nM L^{-1} of NADH oxidized min^{-1} mg^{-1} protein for MDH; [b]Expressed as nM L^{-1} of succinate oxidized per min per mg protein for SDH; [c] expressed as nM L^{-1} of ferrocyanide formed h^{-1} mg^{-1}protein for α KGDH.



[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L-arginine treated rats].

Figure: 5.6. Graphical representation the effect of folic acid, amlodipine and L-arginine on TCA cycles marker enzymes (MDH, SDH, α- KGDH) after I/R in rat liver.

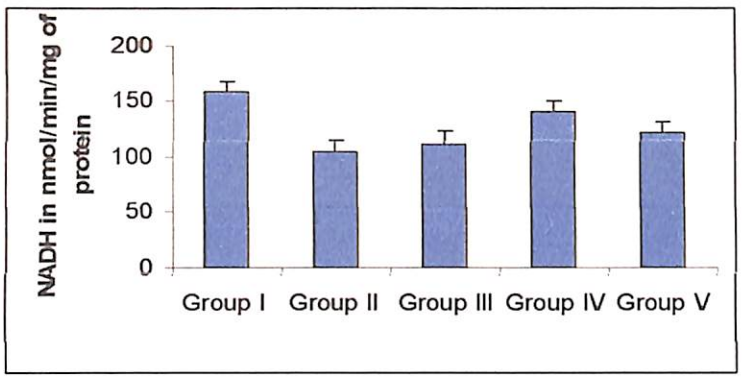
Table: 5.4. The effect of folic acid, amlodipine and L- arginine on respiratory complex I activity after I/R in rat liver.

Groups		Complex I [a]
Sham-operated	(Group I)	158.31± 9.87
I/R Injury	*(Group II)*	*104.56 ± 8.75**
Folic acid treated	(Group III)	111.34 ± 7.32* [#]
Amlodipine treated	(Group IV)	139.20 ± 7.81* [#]
L -Arginine treated	*(Group V)*	*120.65 ± 8.92** [#]

Results are expressed as mean ± SD (n=6). * Statically difference (P< 0.01) from sham operated rats.

[#] Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.

[a] Expressed as nM L^{-1}mol of NADH oxidized min^{-1} mg $^{-1}$protein



[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L-arginine treated rats].

Figure: 5.7. Graphical representation the effect of folic acid, amlodipine and L-arginine on respiratory complex activity I after I/R in rat liver.

Table: 5.5. The effect of folic acid, amlodipine and L- arginine on ATP production after I/R in rat liver.

Groups		ATP[a]
Sham-operated	(Group I)	2.53±0.49
I/R Injury	(Group II)	0.821 ±0.04
Folic acid treated	(Group III)	1.16± 0.45 *#
Amlodipine treated	(Group IV)	1.70± 0.75*#
L -Arginine treated	(Group V)	1.62± 0.88 *#

Results are expressed as mean ± SD (n=6). * Statically difference (P< 0.01) from sham operated rats.

Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.

[a]Expressed as μM g^{-1} tissue

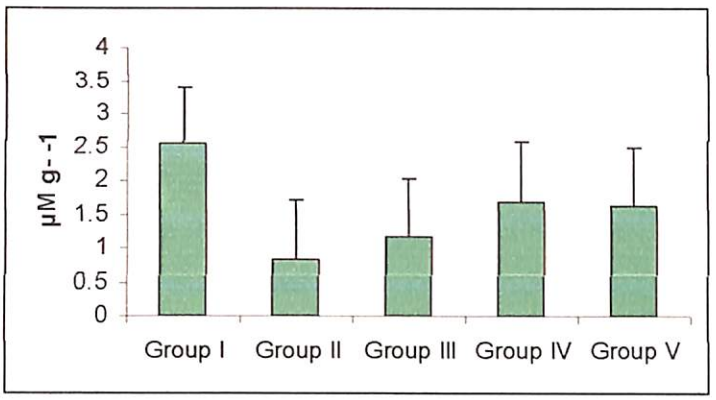


Figure: 5.8. Graphical representations the effect of folic acid, amlodipine and L-arginine on ATP production after I/R in rat liver.

5.4. Discussion

Mitochondrion is most susceptible organelle for oxidative damage during I/R. Ex- vivo models of I/R have demonstrated that decline in activities of mitochondrial electron transport chain components as well as Krebs cycle marker enzymes leads to mitochondrial damage (Lucas and Szweda, 1998; Lucas and Szweda, 1999). The magnitude of decline in activity of electron transport chain components is not directly proportional to decline in ADP linked respiration in I/R injury (Lucas and Szweda, 1999). Moreover, defects in Krebs cycle enzymes are detrimental factors and are rate limiting steps in mitochondrial respiration in I/R injury (Weiss, 1993). KGDH and complex I are two main components, which regulate the mitochondrial Krebs cycle, and electron transfer chain respectively. KGDH is highly sensitive to oxidative stress predominant in I/R injury (Humphries and Szweda, 1998; Humphries et al., 1998; Lucas and Szweda, 1998; Lucas and Szweda, 1999; Nulton-Persson and Szweda, 2001; Bulteau et al., 2003)). Also loss of activity of KGDH inactivates Krebs cycle during I/R injury.

Oxidative stress is particularly important phenomenon for loss of complex I activity (Kaur and Bhardwaj, 1998; Mizuno, 1995; Parker et al., 1998). Complex I inhibition by oxidative stress decreases mitochondrial activity. During I/R injury release of arachidonic acid from cell is universally accepted phenomena during inflammation. Earlier study showed that respiratory complex I activity is inhibited by arachidonic acid during ischemia followed by reperfusion (Van der Vusse et al., 1997) besides treatment

of isolated mitochondria with arachidonic acid results in the inhibition of complex I and increased generation of oxygen radicals (Cocco et al. 1999). Therefore it is another indirect evidence that complex I activity is inhibited during I/R injury.

During ischemic conditions 30-50% ATP is produced by TCA using glucose of liver and there is continuous electron flows in respiratory chain (Niu et al., 1996; Malarkodi et al., 2003). The inhibition of mitochondrial respiration by low oxygen supply results in stimulation of glycolytic flux is known as Pasteur effect and indicates that in hepatocytes, Krebs cycle and mitochondrial respiration are functionally closely connected (Nishida et al., 1994; Venditt et al., 2001; Malarkodi et al., 2003). Therefore, investigation of Krebs cycle marker enzymes and mitochondrial respiration were most important parameter in our study .

In the present study, respiratory and Krebs cycle marker enzymes, ATP production was decreased in I/R group rats, which is in agreement with earlier reported studies. Further, we have observed:

1. I/R does not result in generalized reduction in activities of Krebs cycle marker, respiratory marker and red- ox marker enzymes at the same levels. Further, mitochondrial redox enzymes were found to be more susceptible to inactivation in I/R injury, which indicates a potential mechanism of inactivation of mitochondrial enzymes, by free radicals. It provides a clue for rational drug design for preconditioning of liver graft to prevent I/R injury (Table 5.3 and Figure 5.6).
2. Respiratory complex I exhibits loss in activity upon I/R which suggests that generation of free radicals in I/R deactivate respiratory complex I. This is an agreement with previous studies where it was demonstrated that inhibition of respiratory complex I can result in increases in free radical generation (Boveris, 1985; Kwong and Sohal, 1998) and it is known that free radical production also affects the ATP levels during I/R injury.
3. Depletion of ATP was observed during I/R injury, which provides a evidence that the rate of mitochondrial free radical production is inversely proportional to the rate of electron transport and this observation supports previous work. (Boveris, 1984; Cadenas and Davies, 2000).

4. The decline in mitochondrial ADP-linked respiration was observed which could result from deficit in activities of Krebs cycle enzymes, electron transport chain enzymes, and/or ATP synthetic machinery. Thus decline of ATP production in I/R provided a clue for further investigation to study the role of ATP in pathogenesis of I/ R hepatotoxicity (Table 5.5 and Figure 5.8).
5. I/R mitochondria was found to have a significant increase in total mitochondrial calcium concentration which was in agreement with the previous reports of reperfusion induced mitochondrial calcium overload (Reimer, and Jennings, 1992; Ferrari, 1996). It explains that calcium overload could potentially play a role in the observed decline in mitochondrial function.
6. Activities of respiratory marker enzymes, NADH dehydrogenase and cytochrome c oxidase were lowered in I/R induced rats which is in agreement with earlier work. It is reported that activity of mitochondrial and respiratory marker enzymes in ischemic condition decreases (Guppy et al., 1990).

Moreover the effect of three molecules L- arginine, amlodipine and folic acid was: -

- L –arginine treated group attenuated the changes in mitochondrial antioxidant, respiratory and Krebs cycle marker enzymes and depletion of ATP in I/R injury rats indicating that L- arginine significantly protects I/R injury by producing NO in hepatic sinusoids which can be explained as L- arginine is precursor of NO and during ischemia L-arginine restored vasodilatatory effects by generation of NO in hepatic sinusoids, which may leads to deactivation of ET_B receptor which attenuated the I/R injury (Table 5.2 and Figure 5.4) .
- Pre- treatment with amlodipine prevented the increase in mitochondrial Ca^{2+} concentration thereby restoring the mitochondrial function that can be explained as restoration of complex I activity prevents endogenous production of super oxide anion. Earlier study showed that inhibition of Ca^{2+} influx in mitochondria promotes complex I activation by inhibiting production of sulfhydryl reactive compounds (Kotlyar et al., 1992). Thus our study provides an important findings that amlodipine also potentates respiratory complex –I activity in I/R injury.

- Administration of folic acid in I/R rats showed decrease production of mitochondrial; antioxidant enzymes and increase in activity of respiratory marker enzymes and ATP production that indicates that folic acid attenuates mitochondrial dysfunction in I/R injury (Table 5.3 and 5.6). 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 mitochondria, which prevents mitochondria from I/R injury. However role of folic acid in protection of mitochondrial antioxidant enzymes is not reported. Recognition of the ancillary actions of folic acid is important for understanding the mechanisms of action.

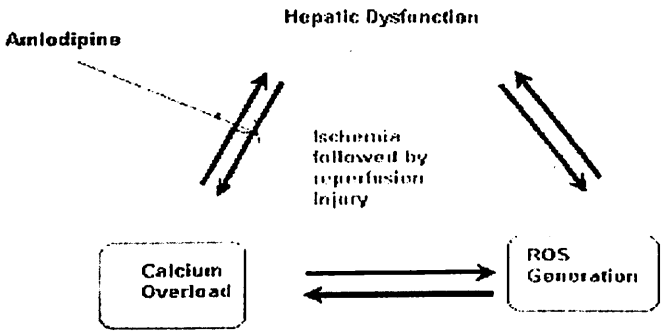


Figure: 5.9. Schematic diagram of mechanism of Ca^{+2} association in I/R injury

5.5. Conclusion

1) The present studies show a correlation of Ca^{2+} homeostasis with I/R injury. I/R injury decreases Krebs cycle enzymes and citric acid cycle enzymes in mitochondria. Amlodipine prevents decrease of Krebs cycle and citric acid cycle marker enzymes in I/R rats.

- Ca^{2+}homeostasis are regulated by amlodipine by inhibition of Ca^{+2} entries in mitochondria during I/R. Amlodipine not only antagonizes mitochondrial Ca^{2+} entry, but also prevents ATP depletation during I/R.
- Ca^{2+} signaling pathway utilized by a key endogenous regulator in attenuation of mitochondrial damage during I/R injury provide new insight and potential molecular approaches to modulate ischemia followed by reperfusion pathophysiological events.

2) The enhanced production of NO through administration of L-arginine appear to reduce oxygen free radicals, increase the mitochondrial antioxidants, increase mitochondrial enzymes and subsequently decrease the amount of Ca^{2+} entry by removing substrate for free radical generation by vasodilatation mechanisms. L – arginine protects hepatobiliary function by attenuation of mitochondrial injury in hepatic ischemic and reperfused liver induced injury.
3) Treatment with folic acid improved the mitochondrial dysfunction. Although it is difficult to analyze and conclude the pathophysiological mechanism by which folic acid inhibit I/R injury but our finding that the folic acid altered the mitochondrial Krebs cycle enzymes and citric acid cycle enzymes provide a clue that folic acid prevents DNA damage from I/R injury.

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Chapter - 6

Folic acid, Amlodipine, L- Arginine Attenuated Anti-oxidant Enzymes in Ischemia followed by Reperfusion on Rat Liver

6.1. Introduction

Oxidative stress has been recognized to play a pivotal role in influencing pathophysiology of I/R leading to organ failure. The role of Reactive Oxygen Species (ROS) and Reactive Nitrogen-Oxygen Species (RNOS) in modulating cell signaling, proliferation, apoptosis, and cell protection have now been clearly identified. Apart from this, ROS and RNOS are capable of attacking proteins, polysaccharides, nucleic acids, and polyunsaturated fatty acids; the ultimate outcome being the cellular damage and tissue dysfunction (Lovat and Preiser, 2003). In septic shock and in critical illness ROS are generated from mitochondrial dysfunction (Brealey et al., 2002) and mitochondrial respiratory marker enzymes, NADPH oxidase, stimulate neutrophils, macrophages and xanthine oxidase resulting in conversion of xanthine dehydrogenase during ischemia/reperfusion injury (Lovat and Preiser, 2003). ROS/RNOS may also trigger release of cytokines from immune cells, activate inflammatory cascades increase the expression of adhesion molecules and mediated through inducing the expression of nuclear factor κB (Grimble. 1994). Inflammation and tissue injury result in the accumulation of granulocytes in organs that leads to increase generation of ROS, which further perpetuates or amplifies the inflammatory response and subsequent tissue injury (Hammerman and. Kaplan, 2000). These pathways and cycles are principle cause of pathophysiology of I/R injury.

Reperfusion is associated with ROS production (Kevin et al., 2003). Some may be produced by xanthine oxidase and NADPH oxidase but it is likely that most are formed by complex I and complex III of the respiratory chain (Becker, 2004; Solaini and Harris, 2005; Pacher et al., 2006).

Mitochondrial proteins are especially susceptible to ROS induced damage and this is reflected in the impaired respiratory chain activity in isolated mitochondria (Chen et al., 2006). ROS have direct effects on several respiratory chain components, especially on complex I and complex III, and other iron sulphur proteins such as aconitase. Inhibition of complex I associated with increased production of ROS in I/R injury cause heaptocellular damage. Also ROS cause oxidation of glutathione to form mixed disulphides with proteins. Such protein modifications likely have inhibitory effects on ion pumps; exacerbate the effects of ATP deprivation on ionic homeostasis, which could be another reason for heaptocellular injury (Eaton et al. 2002, Hool et al., 2006).

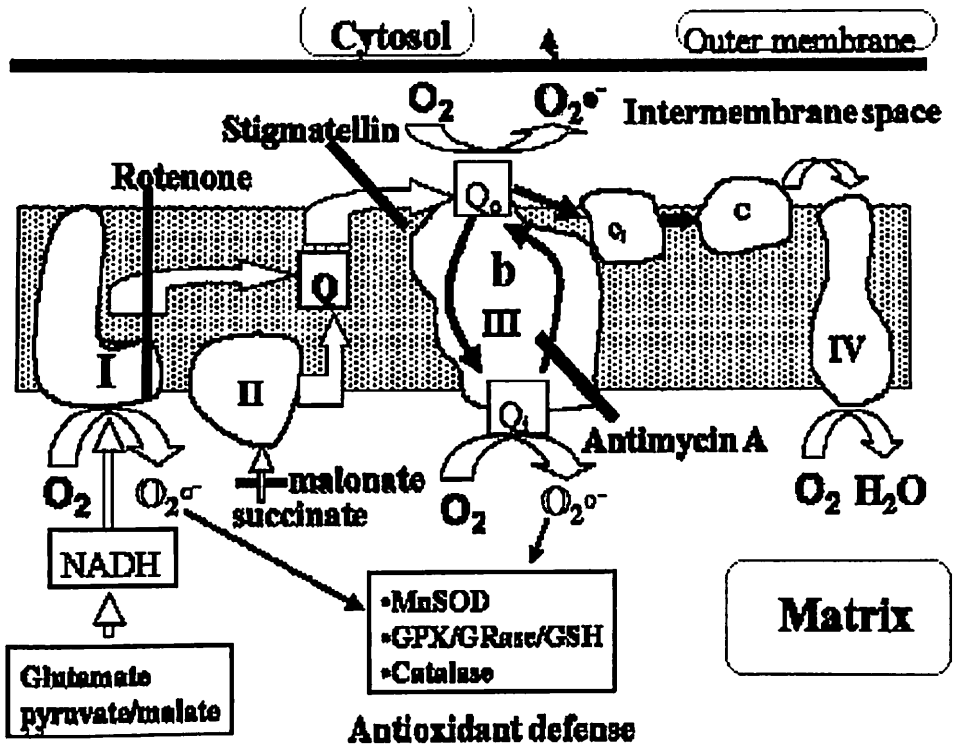


Figure: 6.1. Antioxidant defense mechanism of Mitochondria during Ischemia followed by reperfusion injury.

The combined effect of ROS and elevated mitochondrial [Ca^{2+}] which increases in I/R injury plays a critical role in the transition from reversible to irreversible reperfusion injury as mitochondria are the major target of these agents which lead to the opening of the mitochondrial permeability transition pores, which play a critical role in reperfusion injury (Halestrap et al., 1998; Halestrap et al., 2004; DiLisa, 2006).

Mechanisms responsible for the increase in mitochondrial free radical production during ischemia/reperfusion suggested future therapeutic strategies for intervention. There are number of events that occur during ischemia reperfusion that would be expected to result in increases in oxygen radical production viz. decreased levels of the terminal electron acceptor O_2 during ischemia resulting in increased reduction of various respiratory chain components (Baker and Kalyanaraman, 1989; Grill, 1992), decrease in cytochrome c levels in the inner mitochondrial membrane resulting in decrease of maximum rate of electron transport (Chen, 2001; Czerski et al., 2003), decline in the activities of electron transport complexes I, III, and IV, ATP synthase, and adenine nucleotide translocase

ANT) during ischemia and/or reperfusion (Chen et al., 2001). Each of these events are considered to increase ratio of reduced and oxidized components of the electron transport chain promoting mitochondrial generation of oxygen radicals during reoxygenation (DiLisa, 2006).

Free radicals are highly reactive, short-lived molecules that exert their effect in close proximity to the site of their production (Berlett and Stadtman, 1997). Mitochondria, in addition to being a major source of production of free radicals, are also a primary target for free radical mediated effects which is supported by the evidence of involvement of the electron transport chain components in the production of ROS in addition to the susceptibility of multiple redox sensitive mitochondrial enzymes to oxidant damage (Malis and Bonventre, 1986; Borutaite et al., 2000).

It was, therefore, decided to study: 1) whether the effects of ischemia/reperfusion are due to Krebs cycle enzymes, respiratory marker enzymes or those specific enzymes known to be susceptible to oxidative inactivation and, 2) Functional alterations which occur during ischemia relative to reperfusion with pretreatment with folic acid, L- arginine and amlodipine and, 3) Oxidative events that contribute to decline in enzymatic and mitochondrial function.

6.2. Materials and Methods

6.2.1. Materials

5,5, dithio bis 2 nitro benzoic acid (DNTB), Ethylene diamine tetra acetic acid (EDTA), Tris (Hydroxymethyl) amino methane were purchased from Sisco Research Laboratories, Mumbai., Hydrogen peroxide (H_2O_2), pyrogallol, sodium azide. Potassium dihydrogen phosphate ($KH_2\ PO_4$), Tri carboxyl acid (TCA) Ferric chloride ($FeCl_3$), hematoxylin were purchased from Otto Chemei, Mumbai.

6.2.2. Methods

6.2. 2.1 Animal groups

Thirty Wistar rats were divided into sham-operated control group (I) (n = 6), ischemia and reperfusion group (II) (n = 6), were given 0.9 % saline (5ml kg^{-1}, p.o) for 7 days; amlodipine treated group (given amlodipine 1 mg kg^{-1} body mass daily by oral route for 7 days before induced ischemia reperfusion maneuver) group III (n=6), Folic acid treated group (given folic acid 1 mg kg^{-1} body mass daily by oral route for 7 days before inducing ischemia reperfusion maneuver) (IV) (n = 6) and L-Arginine treated group (given L-

arginine 100 mg kg^{-1} body mass daily by oral route for 7 days before inducing ischemia reperfusion maneuver) (V) ($n = 6$).

6.2. 2.2 Ischemia and reperfusion injury

Ischemia and reperfusion injury were same as described on page29, chapter 3.

6.2. 2.3. Peripheral blood and tissue sampling

Peripheral blood and tissue sampling method was same as described on page30, chapter 3.

6.2. 2.4. Isolation of mitochondria

Isolation procedure of mitochondria method was same as described on page 72, chapter 5.

6.2.2.5. Estimation of reduced glutathione (GSH) (Habig et al., 1974).

Samples for estimation were prepared by adding 0.2 ml mitochondrial enzyme to 2 ml distilled water followed by 3.0 ml of precipitating mixture (1.67g 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 r.p.m.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.

6.2.2.6. Estimation of superoxide dismutase (SOD) (Hodgson and Fridovich, 1975).

50 µl of the mitochondrial enzyme was mixed with 75mM L^{-1} Tris-HCl buffer (pH 8.2), 30 mM L^{-1} EDTA and 2 mM L^{-1} 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.

6.2.2.7. Estimation of catalase (CAT) (Takahara et al., 1960)

20 µl of the lysate was added to a cuvette containing 2 ml of phosphate buffer (pH 7.0) and 1 ml of 30 mM L^{-1} H_2O_2 and catalase activity was measured at 240 nm after 1 min using spectrophotometer. The molar extinction coefficient of H_2O_2, 43.6 M L^{-1} $c.m^{-1}$ was used to determine the catalase activity. One unit of activity is equivalent to one mM L^{-1} of H_2O_2 degraded per minute and is expressed as units per mg of protein

6.2.2.8. Estimation of glutathione peroxide (GPx) (Rotruck and Ganther, 1973)

20 µl of the lysates was added to a cuvette containing 0.2 ml of 100mM L^{-} Tris buffer (pH 7.0), 0.2 ml of 30 mM L^{-1} EDTA, 1.0 ml of sodium azide and then to this, 0.2 ml of glutathione followed by 0.1 ml of hydrogen peroxide was added. The contents were mixed

well and incubated at 37 °C for 10 min alongwith a control tubes containing all the reagents expect tissue homogenate. After 10 min, the reaction was arrested by addition of 0.5 ml of 10 % TCA. Solution was centrifuged at 5000 r.p.m. for 5 min. 1 ml supernatant was added to 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. The glutathione peroxides activity was expressed as mcg glutathione utilized min^{-1} mg^{-1} protein.

6.2.2.9. Estimation of hydroxyl radicals (OH^-) (Hallwell and Aruoma, 1987)

20 µl of the lysates were added to a cuvette containing 1 ml of reaction mixture consisting of 2.8 mM L^{-1} NaH_2PO_4 (pH 7.0), 20 mM L^{-1} KH_2PO_4 (pH 7.4), $FeCl_3$ (100 mM), 104 µM L^{-1} EDTA, 1mM L^{-1} H_2O_2 and 100 µM L^{-1} ascorbate which was then incubated at 37 °C for 1 h and absorbance was measured at 532 nm.

6.2.2.10. Estimation of superoxide (O_2^-) (Martin and Sugrman, 1987)

20 µl of the lysates were added to a cuvette consisting 1 ml of reaction mixture containing 0.1 ML^{-1} EDTA, (pH 7.4), EDTA (0.1 mM), and 50 μM^{-1} hematoxylin and incubated at 25 °C for 1 h and absorbance was measured at 560 nm.

6.3. Results

6.3.1. Levels of mitochondrial antioxidant GSH, SOD and CAT

Activities of GSH, SOD and CAT were 58.21±4.3 mM L^{-1}, 14.32±2 U and 2.25 ± 0.24 mM L^{-1} respectively in sham operated rat which decreased to 38.51±2.1 mM L^{-1}, 5.20± 4.3 U and 0.08 ± 0.002 mM L^{-1} after 1 h ischemia followed by reperfusion. Levels of antioxidant enzymes in folic acid and amlodipine treated were significantly increased as compared to I/R rat. Treatment with L -Arginine protected decrease in antioxidant enzymes level as compared to all other groups (Figure 6.2 and Table .6.1).

6.3.2. Levels of mitochondrial GPx, OH, O_2

Activities of GPx, OH, O_2 were 0.581±0.022 mcg, 0.212± 0.036 mg and 0.309± 0.073 mM L^{-1} respectively in sham operated rats which decreased to 0.194 ± 0.051 mcg, 0.687± 0.029 mg and 0.680± 0.095 mM L^{-1} after 1 h ischemia followed by reperfusion. Treatment with L -Arginine protected decrease in antioxidant enzymes level as compared to all other groups. (Figure 6.3 and Table 6.2)

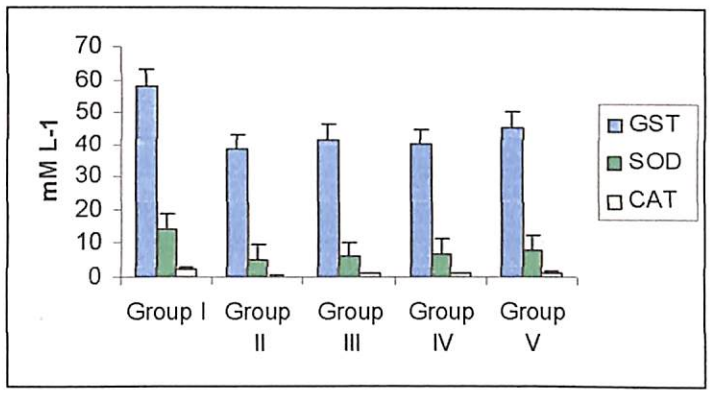
Table: 6.1. The effect of folic acid, amlodipine and L- arginine on mitochondrial GST, SOD and CAT levels after I/R in rat liver.

Groups		[a]GST	[b]SOD	[c]CAT
Sham-operated	(Group I)	58.21±4.3	14.32±2.6	2.25 ± 0.24
I/R Injury	(Group II)	38.51±2.1*	5.20± 4.3*	0.08 ± 0.002*
Folic acid treated	(Group III)	41.54±2.98*#	6.18±0.49*#	0.87±0.06*#
Amlodipine treated	(Group IV)	40.29±4.87*#	6.98± 0.91*	0.94±0.08*#
L -Arginine treated	(Group V)	45.65±3.0*#	8.11± 2.9 *#	1.29± 0.35*#

Results are expressed as mean ± SD (n=6). * Statistically difference ($P< 0.01$) from sham operated rats.

Statically difference ($P< 0.01$) from vehicle- treated ischemia and reperfusion.

[a] Expressed as mM L^{-1} of 1-chloro 2,4-dinitrobenzene (CDNB) conjugated min^{-1} 100 mg^{-1} protein for GST; [b] Expressed as Units per mg per 100 mg protein for SOD; [c] Expressed as mM L^{-1} of H_2O_2 decomposed per min per mg protein for CAT.



[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

Figure: 6.2. Graphical representation of the effect of folic acid, amlodipine and L-arginine on mitochondrial SOD and CAT levels after I/R in rat liver.

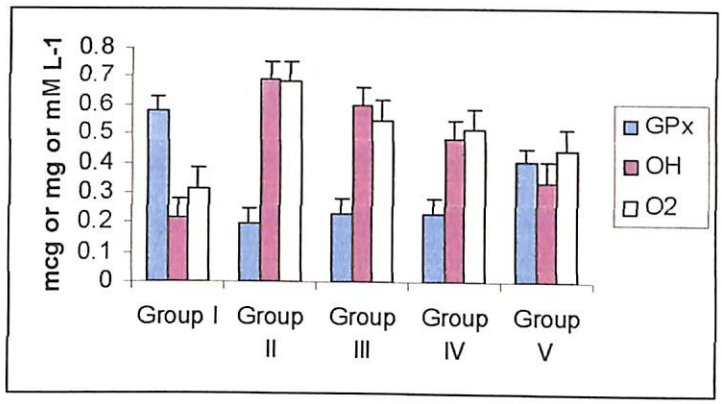
Table: 6.2. The effect of folic acid, amlodipine and L- arginine on mitochondrial GPx, OH and O_2 levels after I/R in rat liver.

Groups		[a]GPx	[b] OH^-	[c] O_2^-
Sham-operated	(Group I)	0.581±0.022	0.212± 0.036	0.309± 0.073
I/R Injury	(Group II)	0.194 ± 0.051*	0.687± 0.029	0.680± 0.095
Folic acid treated	(Group III)	0.228± 0.036*#	0.597± 0.033*#	0.544± 0.044#
Amlodipine treated	*(Group IV)*	*0.226± 0.027*	*0.481± 0.025*#*	*0.512± 0.027*#*
L -Arginine treated	(Group III)	0.401± 0.019*	0.334± 0.068*#	0.441± 0.061*#

Results are expressed as mean ± SD (n=6). * Statistically difference (P< 0.01) from sham operated rats.

Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.

[a] Expressed as mcg glutathione utilized min^{-1} mg^{-1} protein; [b] Expressed as mg hydroxyl ion production min^{-1} mg^{-1} protein; [c] Expressed as mM L-1 of hematoxylin decomposed min^{-1} mg^{-1} protein for O_2.



[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

Figure: 6.3. Graphical representation of the effect of folic acid, amlodipine and L-arginine on mitochondrial GPx, OH and O_2 levels after I/R in rat liver.

6.5. Discussion

The mitochondrial respiratory chain is an important source of non-enzymatic generation of reactive species. During oxidative phosphorylation process, electrons are transferred by electron carriers NADH and $FADH_2$, through four complexes in the inner mitochondrial membrane to oxygen during generation of ATP (Essani et al., 1997). Under normal conditions, $^{\bullet}O_2^-$ is immediately eliminated by natural defense mechanisms. The liver injury is mediated by free radicals or by the depletion of endogenous pool of antioxidants from mitochondria. Reactive species can be eliminated by a number of enzymatic and non-enzymatic antioxidant mechanisms. Generations of reactive species of I/R injury are NOS, NADPH oxidase and xanthine oxidase (De Groot et al., 1989). Oxidant stress-induced cell damage associated with oxidation of pyridine nucleotides in nucleus, accumulation of calcium in mitochondria as well as super oxide formation in mitochondria leads to formation of membrane permeability transition pores and breakdown of the mitochondrial membrane potential (Carden et al., 1993). The intracellular signaling mechanism and the mitochondrial membrane permeability transition was observed during hepatic ischemia-reperfusion (Nieminen et al., 1997). Lipid peroxidation is one of the major outcomes of free radical- mediated tissue injury and is an indicator for oxidative damage by series of chain reactions (Fortunato et al., 2006). Increased level of MDA, the end product of LPO, was observed in I/R injured rats. Major antioxidants like SOD, CAT and GST are important for cellular protection due to their ability to detoxify free radicals such as ROS. A number of studies have reported diverse results for the changes of these antioxidant enzyme activities in I/R induced hepatic injury (Younes and Strubelt, 1998; Lee and Clemens, 1999). In view of above, the aim of present study was to study weather after induction of I/R injury hepatic mitochondria undergoes oxidative damage and to see the effect of L-arginine, amlodipine and folic acid in protection from oxidative damage.

It is known that NO is a potent vasodilator synthesized from L-arginine and diffuses freely across cell membranes and acts intra cellularly by activation of guanylate cyclase and is also NO is inducer of vasodilatation at the level of the sinusoid as well as at presinusoidal sites (Curran et al., 1989; Curran et al., 1991). NO is an endothelium-derived relaxing factor, is formed from the terminal guanidino nitrogen atom of L-arginine by NO synthase (Moncada 1991) and binds to the haem moiety of guanylate cyclase and increases its activity by 400-fold, catalyzing the conversion of guanosine triphosphate to cyclic

guanosine monophosphate (cGMP). Elevation of cGMP relaxes the smooth muscles in blood vessels inhibits platelet aggregation and adhesion, and blocks the adhesion of white cells to the blood vessel wall (Ozturk *et al.*, 2001; Russwurm and Koesling 2002; Vallance 2003). In addition to its vasodilatory effect, NO suppress the super oxide formation (Granger et al., 1994).

Conforming with earlier reported work, the present work showed that the enhanced production of nitric oxide through administration of L-arginine reduces oxygen free radicals, increases the mitochondrial antioxidants, increases respiratory marker mitochondrial enzymes and subsequently decreases the amount of lipid peroxidation by removing substrate for free radical generation by physiological mechanisms.

Further, mitochondria are involved in the generation of NO· via the nitric oxide synthase (NOS) reaction. O_2· and NO· react to form another oxidant, peroxynitrite (ONOO), which represents a potential source for the more powerful and aggressive hydroxyl radical (·OH) (Ozturk et al., 2001). The present studies showed that activation of iNOS by L- arginine leads to release of NO, dilates sinusoids of liver and reduces ·OH production in sinusoids, which is another important observation indicating how L- arginine prevents I/R injury.

Earlier work showed that ischemia is associated with impaired calcium homeostasis and 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 ATP (Satoh et al., 2003). Calcium homeostasis undergoes fluctuations in balance during reperfusion, largely owing to the release of calcium from intracellular stores, particularly from the sarcoplasmic reticulum (Krause et al., 1989; Temash et al., 1999). Experimental observations of calcium signal transmission between endoplasmic reticulum and mitochondria suggest the existence of stable mitochondria–reticulum interaction. Mitochondria are the principal targets in the development of ischemia – reperfusion (I/R) induced hepatic injury (Detmers et al., 1999; Elimadiet al., 2001).

Increase in the production of superoxide radicals at the level of the mitochondrial respiratory chain stimulated by Ca^{+2} has been alleged as a major participant in I/R injury (Sohal and Dubey, 1994). Oxidant stress induced cell killing involves oxidation of pyridine nucleotides, accumulation of calcium ions in mitochondria, and superoxide formation by mitochondria, ultimately leading to formation of membrane permeability transition pores

and breakdown of the mitochondrial membrane potential (Nieminen et al., 1995; Nieminen et al., 1997). Alternation of mitochondrial enzymes activity, intracellular Ca^{2+} accumulation and Ca^{2+} overloading, contributed to irreversible cell damage (Doroshow, 1983). Rise in the activities of mitochondrial antioxidant enzymes in amlodipine pre-treated group highlights the protective role of amlodipine in combating the oxidative insult (Wang et al., 1993). However, there is no report to support the assertion that amlodipine directly affects mitochondrial antioxidant enzymes.

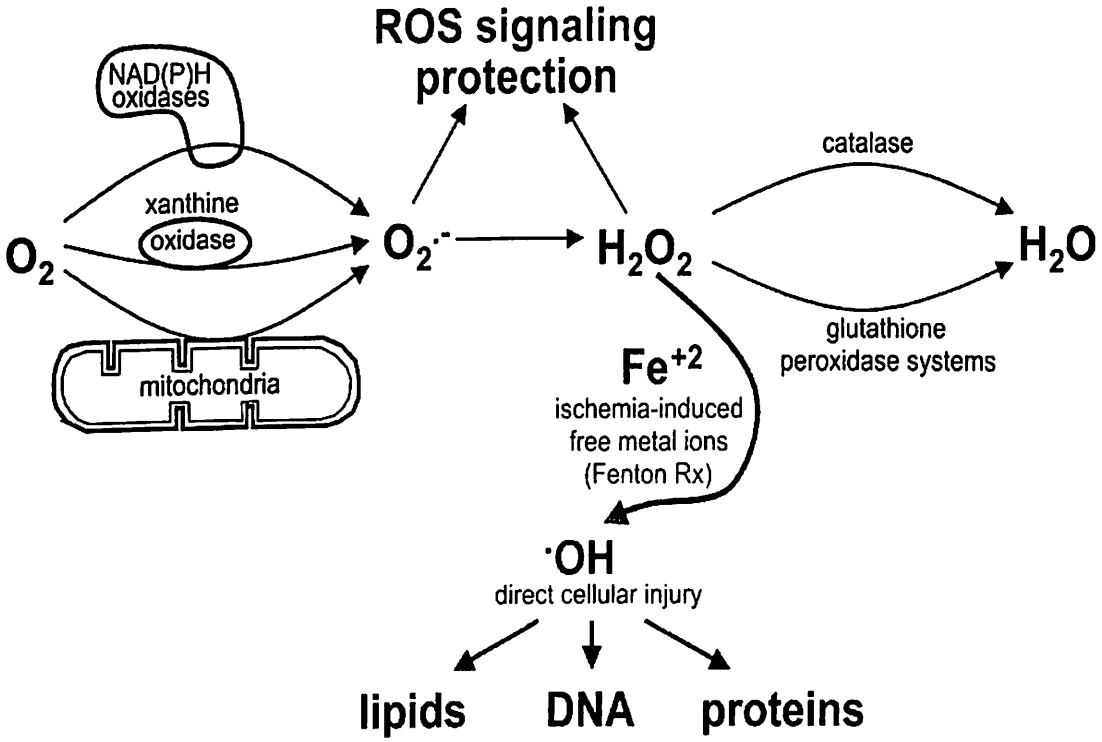


Figure: 6.4. Reactive oxygen metabolism in Ischemia followed by reperfusion injury.

In the present study it was observed that, ischemic tissue injury causes an increase in free intracellular calcium that lead to diminish recovery of dilation function after ischemia (vasoconstriction), compromised membrane integrity and decreasing reserves of cellular adenosine triphosphate. Also, the present studies showed that amlodipine protects mitochondrial antioxidant system by Ca^{2+} inhibition in mitochondria. Amlodipine specifically blocks L-type Ca^{2+} channels, which are exclusively localized to the plasma membrane. The results of present work support earlier work that amlodipine regulates membrane fluidity and cholesterol depositions, acts as antioxidant and regulates matrix deposition by inhibition Ca^{+2} ions.

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 mitochondria 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 BH_4 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 1^{st} pass metabolism. Thus, our study suggests that folic acid is biologically active to protect mitochondria from oxidative injuries.

However effective dose of folic acid to counteract mitochondria oxidative injuries in the present study was found at micro molar levels, which are much greater than those encountered in the human physiological system (nanomolar levels) and one will raise the question whether effects of folic acid found in the present study have physiological relevance, therefore, further studies are required to prove the folic acid in nanomolar level in attenuation of mitochondrial injury in I/R rats.

6.6. Conclusion

1. Antioxidant property of amlodipine, calcium channel blocker against the I/R induced liver injury in rats is due to its inhibitory action of Ca^{2+} influx into mitochondria.
2. The enhanced production of NO on administration of L-arginine appeared to reduces oxygen free radicals, increases the mitochondrial antioxidants, increases respiratory marker mitochondrial enzymes and subsequently decreases the amount of lipid peroxidation by removing substrate for free radical generation by physiological mechanisms.
3. Antioxidant action of folic acid protects against mitochondria oxidative injuries. Supplemental folic acid attenuates the oxidative defects in mitochondria of pro-oxidant-treated hepatocytes. Implication of increased folic acid levels as a possible determinant of mitochondria function may unveil a new potential target to reduce intracellular super oxide overproduction.

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Chapter - 7

Folic acid, Amlodipine, L- Arginine Attenuated Necrosis and Apoptosis in Ischemia followed by Reperfusion Injury in Rat Liver

7.1. Introduction

Apoptosis is permanent cell death where nucleus undergoes fragmentation whereas necrosis is reversible cellular changes. Under favorable condition necrotic cells become a normal cell and revert to their original size, shape and function. During I/R injury, cells die 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 (Gujral et al., 2001). These morphological features are characteristic of oncotic necrosis. It was postulated that most liver cells actually die by apoptosis (Jaeschke, 1991; Cursioet al., 1999; Detmerset al., 1999;Gujral et al., 2001) which is morphologically characterized, by cell shrinkage, formation of apoptotic bodies with intact cell organelles (Figure 6.1 and 6.2) and the absence of inflammation Manjo and Joris, 1995). On the basis of the terminal deoxynucleotidyl transferase-(mediated dUTP nick-end labeling (TUNEL) assay, it was reported that 50-80% of liver endothelial cells and hepatocytes die through apoptosis during the first 3-6 h of reperfusion (Kohli et al., 1999). Immediate cell contents release and inflammation are not consistent with apoptosis as the only mode of cell death (Gujral et al., 2001) and interventions such as overexpression of Bcl-2 can prevent both apoptotic and necrotic cell death (Jaeschke, 1991; Jaeschke, 2000; Redaelli et al., 2002). More than 90% of cells die by oncotic necrosis during ischemia-reperfusion and that apoptotic cell death can trigger neutrophil transmigration with massive aggravation of the apoptotic cell injury (Jaeschke et al., 1998, Kobayashi et al., 1998).

The Bcl-2 gene was first identified at the chromosomal breakpoint of t (14:18)-bearing B cell lymphomas (Draetta and Beach, 1988) and was found to act as a new class of oncogenes that functions to prevent apoptosis instead of directly promoting cellular proliferation (Droin et al., 1998; Du et al., 2000). Regulation of mitochondrial membrane permeabilization is the major mechanism by which Bcl-2-like proteins exert their regulatory effect on apoptosis. As a site of convergence for multiple death-inducing stimuli, the mitochondria a pivotal decision center controls life and death by releasing apoptogenic factors in the cytosol (Figure7.3).

These death-inducing molecules are located within the mitochondrial intermembrane

space including cytochrome c (Jost et al., 1997) which in addition to their central role in controlling cell death also indirectly controls cell cycle progression by controlling Bcl-2 family gene. Bcl-2 itself was shown to slow entry from the quiescent G_0 into the G_1 phase of the cell cycle prior to DNA replication in multiple cell lineages and transgenic mice (Domina et al., 2000; Fan et al., 2000) and Bcl-2 homologue known to function as an anti-apoptotic protein (Kozopas et al., 1993), inhibits cell-cycle progression through the S phase of the cell cycle (Schmitt et al., 2007).

Inhibition of Bcl-2 increases G_2 / Mit cell cycle ratio and I/R injury initiates G_2 / Mit Cell cycle (Figure 6.2). Bcl-2 is apoptosis regulators promote cell survival or facilitate cell death (Fujise et al., 2000). Bcl-2 is thus founder of a large family apoptosis regulator that either promotes cell survival or facilated cell death.

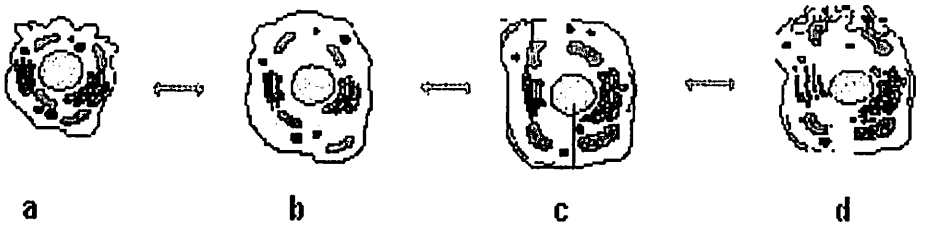
In view of the above, present study was undertaken to: 1) determine whether the effects of ischemia/reperfusion are due to Bcl-2 gene and apoptosis; 2) distinguish between functional alterations which occur during I/R and after pretreatment with folic acid, L- arginine and amlodipine and 3) provide insight into necrosis and apoptosis that contribute to decline in hepatic function.

7.2. Materials and Methods

7.2.1. Materials

Dithiothreitol was purchased from Sisco Laboratories, Mumbai, India. CGT-CAT-AAC-TAA-AGA-CAC-CCC and TTC-ATC-TCC-AGT-ATC-CGA-CTC sequence primer were customized synthesized from Integrated DNA technologies, Inc.milpitas, CA 95035, USA. Ampli Taq Polymerase and 1 kbp DNA ladder were purchased from Bangolore Genni, India. AMV reverse transcriptase and RNase were procured from Boehringer, Mannheim, Germany. Protease inhibitor procured from Amersham Pharmacia Biotech, Uppsala, Sweden, Mouse Bcl-2 antibody and glyceraldehyde–3 – phosphate dehydrogenase (G3PDH) from Bio source international, USA and Propidium iodides, Annexin V-FITC, 4, 6-diamidino-2-phenylindole Hoechst 33258 were procured from Sigma Chemical Co., St. Louis, MO, USA were used.

A. Process of necrosis



B. Process of apoptosis

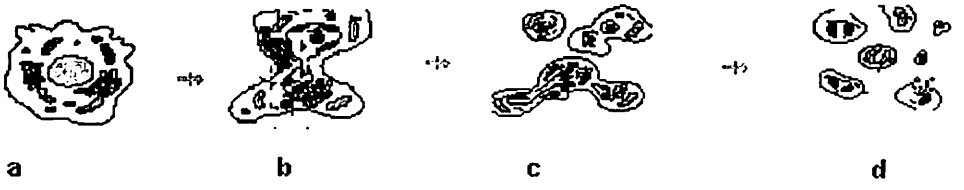


Figure: 7.1. Process of necrosis and apoptosis. **(A)** Process of necrosis. **(a-c)** Cell morphology changes and cell swells. **(d)** Loss of cell integrity but no nuclear changes. **(B)** Process of apoptosis. **(a)** Irreversible changes in cell morphology were observed. **(b)** Nuclear condensation. **(c-d)** Nuclear fragmentation of cells.

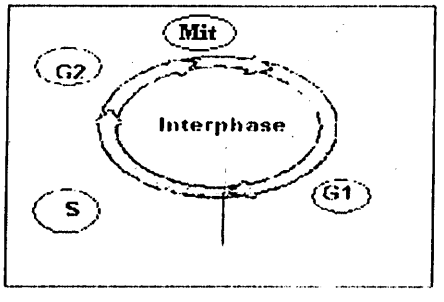


Figure: 7.2. The eukaryotic cell cycle. Mitotic phase (Mit), during which first nuclear division (mitosis) and then cell division (cytokinesis) take place. G_1, S and G_2 are the interphases of cell division.

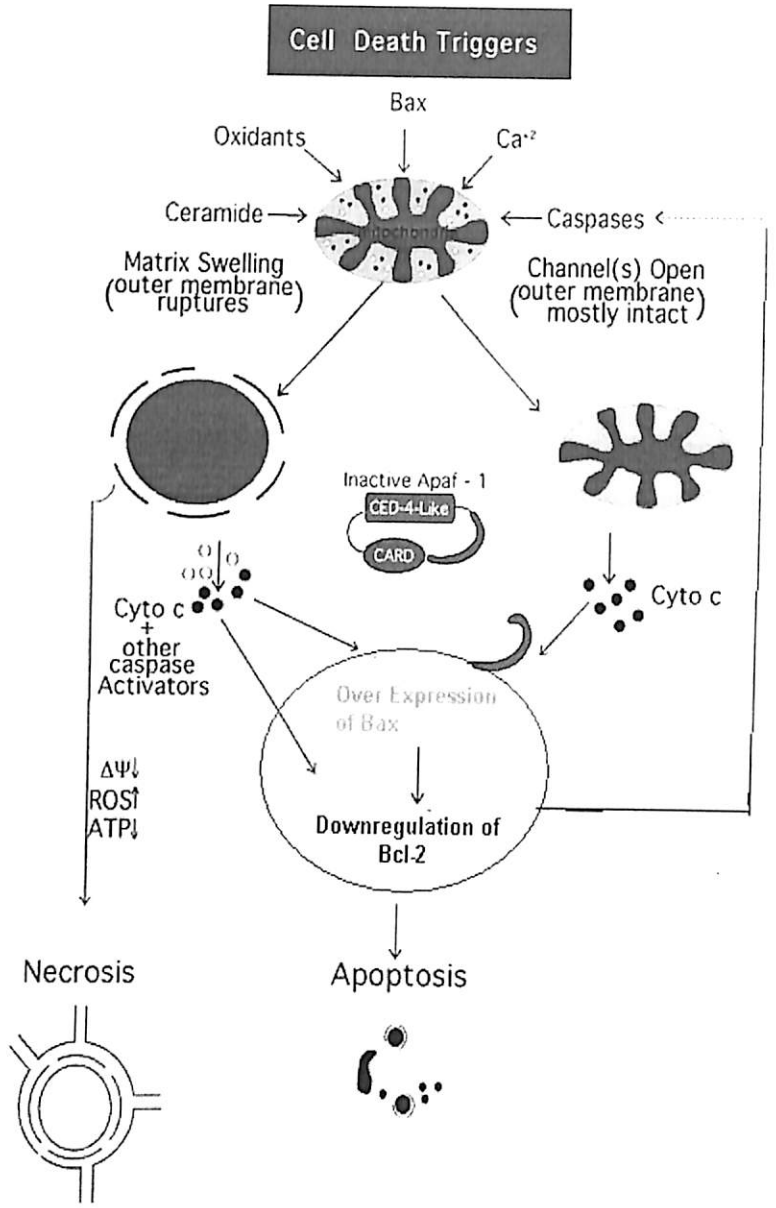


Figure: 7.3. Molecular events in apoptosis during I/R. Activation of Cytochrome c and decrease of ATP levels leads to Bax activation and suppression Bcl-2 gene leads to apoptosis.

7.2.2 Methods

7.2. 2.1. Animal groups

Thirty Wistar rats were divided into sham-operated control group (I) (n = 6), ischemia and reperfusion group (II) (n = 6), were given 0.9 % saline (5ml kg^{-1}, p.o) for 7 days; amlodipine treated group (given amlodipine 1 mg kg^{-1} body mass daily by oral route

for 7 days before induced ischemia reperfusion maneuver) group III (n=6), Folic acid treated group (given folic acid 1 mg kg^{-1} body mass daily by oral route for 7 days before inducing ischemia reperfusion maneuver) (IV) (n = 6) and L-Arginine treated group (given L-arginine 100 mg kg^{-1} body mass daily by oral route for 7 days before inducing ischemia reperfusion maneuver) (V) (n = 6).

7.2. 2. 2. Ischemia and reperfusion injury

Ischemia and reperfusion injury were same as described on page 29, chapter 3.

7. 2.2.3. Peripheral tissue sampling

Peripheral tissue sampling method was same as described on page30, chapter 3.

7.2.2.4. Amplification o Bcl-2 Gene

7.2.2.4.1. Isolation of RNA

RNA was isolated using RNA isolation kits (Bangalore Genni, Bangalore) as per manufacturer's instruction.

7.2.2. 4. 2. Reverse transcriptase polymerase chain reaction

Identity and purity of isolated RNA was confirmed by spectrophotometery 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 Reverse transcripts (equivalent to125 ng of total RNA) were used directly for each amplification reaction using a light cycle rapid thermal cycle (Eppendorf, Germany). Amplification of using Bcl-2 gene was a 21 sence primer –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 gave the product length was 234 kbp and the PCR profile was then set for denaturation for 1 min at 94°C, annealing 90 s at 56°C and then further for 2 min at 72°C, and semi- quantization was optimized to 35 cycles. G3PDH transcript abundance was used as an endogenous control. The amplified cDNA was further amplification by PCR. Amplified product was then 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 was run on every gel to confirm expected molecular weight of the amplification product. Bands were quantitatively measured by densitometry analysis system. Image Analysis System Version 1.5 and the data are expressed in relative optical density (OD) units.

7.2.2. 5. Western blot analysis

Protein expressions of Bcl-2 protein in rat livers were confirmed using 4°C cooling chamber by Western blot analysis.

7.2.2. 5.1. Separations of liver protein

Isolated frozen 100 mg of liver tissue was added to 1 ml of ice-cold lysis buffer and homogenized by 10 strokes with a Potter S device. Homogenates were then centrifuged for 20 min at 4°C at 14,000 r.p.m. Clear supernatant was separated and used for the protein determination and protein electrophoresis.

7.2.2. 5. 2. Protein sample preparations for electrophoresis

1ml (200 μg ml^{-1}) of isolated liver protein were mixed with 500 μl of 4% SDS and 500 μl of 4% β-mercaptoethanol and the mixture was kept in a boiling water bath for 3 min. After cooling to room temperature (26°C), sucrose at the rate of 100 mg ml^{-1} and one drop of 0.005% bromophenol blue was added.

Denatured isolated liver protein loaded in wells of vertical electrophoresis system was added to stacking gel and separating gel (1:3) after complete polymerization. 3 μl molecular weight standards diluted with 32 μl were placed in another well. Electrophoresis was carried out at 100 V for 21 min followed by at 200 V for 36 min. After Western blotting, SDS-page gels were stained for 15 min with Coomassie staining solution and then, were destianed with destaining solution for 15 min 3 times and finally stored in H_2O.

7.2.2. 5.3. Protein transfer by semidry blotting

Separated proteins by SDS- PAGE analysis was transferred to Nitrocellulose membrane (NCB)(Schleicher and Schuell, BA 85, 0.45) and which was cut to the size of the separating gel and placed for 5 min each in methanol, H_2O and electrode buffer respectively.

A sandwitch of stack was made by placing perpex board (drilled with holes to permit free passage of buffer) over Porous pad followed by three sheets of Whatman 3MM filter paper (moistened with electrode buffer). Finally Gel was placed on stack of membranes and subsequently covered with three sheets of Whatman 3 MM filter paper, porous pad followed by perpex board moistened with electrode buffer. Placed a number of elastic bands over "sandwich" set so as to hold the layers tightly. Loaded the "sandwich" into the electrophoresis transfer chamber with NCB sheet lying

between the gel and anode. Electrode buffer was placed in the chamber and run Blotting was performed at a current of 0.8 mA per cm^2 of blotting surface at 115 volt for 12 h in air-cooled room. Removed nitrocellulose sheet soaked in buffer B for 1 h at room temperature followed by buffer A with slow shaking and blot excess liquid with filter paper. The membrane was then dried for 30 min at 80°C and was blocked overnight at 4°C in a 5% (w/v) solution of low fat milk powder in Tris buffer saline (TBS) pH 8.0.

7.2.2. 5.4. Western blot using antibodies

For Bcl-2 Western blotting, a monoclonal mouse Bcl-2 antibody and G3PDH served as first antibody, and a polyclonal anti-mouse served as secondary antibody conjugated with horseradish peroxides (HRP)

Bcl-2 and G3PDH antibodies were diluted (1:1000) with a solution of 1% in low fat milk powder in TBS pH 8.0. After incubating prepared blotted NCB membrane for 60 min with the primary antibody on a shaking platform, washed for 10 min with buffer A followed by 2 times with 100 ml of buffer C and finally washed with buffer A. The second antibody was incubated for 60 min and again washing steps were performed similar as primary antibodies. The two detection solutions (Western Blot Chemiluminescence's Reagent Plus, NEN Life Science Products, Cologne) were mixed in 1:1 ratio according to the manufacturer's instruction and the membrane was incubated for 1 min. Bands were quantitatively measured by densitometry analysis system. Image Analysis System Version 1.5 and the data were expressed in relative optical density (OD) units.

7.2.2. 6. Apoptosis assessment techniques

7.2.2. 6.1. Flow cytometry analysis (Suzuki and Toledo-Pereyra, 1994)

200 µl of Hepatocytes (1×10^9 L^{-1}) washed with PBS, exposed to 100 µl of propidium iodide (PI) 50 mg L^{-1} and 100 µl of Annexin V-FITC (AV) 50 mg L^{-1}, 50 µl of 0.1% Triton X-100, 50 µl of 0.01 mM L^{-1}, EDTA $(Na)_2$ and Rnase 50 mg L^{-1} at normal temperature in darkness for 12-24 h. Specimen were then presented to the FACS-420 Flow Cytometry Analyzer 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 AV. Cell cycle distribution and cell

proliferation were determined by flow cytogram by using Cell quest software and Modift 3.0 DNA software.

The proliferation index was calculated according to formula:

$$\text{Proliferation index} = \frac{(S + G_2/G)}{G_1 + S + G_2/Mit} \times 100$$

Where S= DNA synthesis phase; G_1= Gap 1 phase; G_2= Gap 2 phase; Mit = Mitosis phase.

7.2.2.6.2. Determination of hepatocytes viability by 4,6-diamidino-2- phenylindole (DAPI) staining

The liver tissues were soaked in 30% sucrose in PBS overnight at 4°C and then embedded in acrolein. Series of 8 um-thick sections were cut at -20°C in refrigerated microtome and placed on poly-L-lysine-coated microscopic slide and stained with,6-diamidino-2-phenylindole (DAPI). Positive cells were observed in frozen sections. Fluorescence imaging was performed with an Olympus BX51 microscopy equipped with Fluorescence and CCD camera. From each section, 6 light microscopic slides were used to identify DAPI-positive cells.

7.2.2. 6.3. Determination of apoptosis by terminal deoxynucleotidyl transferase (TdT)-mediated dUDP-biotin nick end labeling (TUNEL) assay (Wei et al., 2005). Reported method was slightly modified and was used for the assay. Liver specimens were fixed in paraffin and 2 μm slices were prepared by using rotary microtome. After liver specimens were de- paraffinized and immersed in PBS containing 0.3% hydrogen peroxide for 10 min at room temperature in a petri dish, they were incubated with 20 mg mL^{-1} proteinase K for 15 min. 75 micro liters of equilibration buffer was added to the specimens and kept for 10 min at room temperature, followed by 55 mL of TdT enzyme and incubated at 37 °C for 1 h followed by fluorescein-labeled dUTP at 37°C in a moist chamber for 60 minutes. The reaction was terminated by transferring the slides to pre-warmed wash buffer for 30 min. After that the sections were incubated with a peroxidase-labeled anti-fluorescein antibody at 37°C for 30 minutes. 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. TUNEL-positive cells showed clear nuclear labeling corresponding to apoptotic cells.

7.2.2. 6.4. Determination of apoptotic hepatocytes by fluorescence microscopy

Hepatocytes were washed with PBS and fixed with PBS-buffered (pH 7) containing 4% formaldehyde, 1.5% methanol solution (CDH, Mumbai) at 4°C for 15 min, then washed three times with PBS. 25µl cells were taken in a PCR Tubes and 5µl ethidium bromide (EB) (100µl ml^{-1}) and acridine orange (100µl ml^{-1}) was added. Stained hepatocytes were mounted on slides coated with buffered mounting medium consisting of 90% glycerol, 10% PBS with 0.1% NaN_3 and 3% triethylenediamine. Inverted Olympus microscope was used for analysis at maximum excitation was performed by a 543-nm line of the internal He-Neon lamp, and fluorescence emission was observed above 570 nm with long pass barrier filter LP-570. Images were converted to TIFF format, and the contrast level and brightness of the images were adjusted by using the microscope program.

7.2.2.6.5. Determination of chromatin condensation by Hoechst 33258 stating

Liver specimens were fixed in paraffin and prepared 2 µm slices by using rotary microtone. Liver specimens were then de- paraffinized and immersed in phosphate-buffered saline. The specimens were counterstained by with 8 µg ml^{-1}. Hoechst 33258 in H_2O_2 and mounted in glycerol, and observed by fluorescence microscopy. Stained nuclei were scored according to the condensation and staining characteristics of the chromatin. Cells with condensed or fragmented chromatin were considered apoptotic.

7.2.2. 6.6. Determination of DNA aberration by electrophoresis

The isolated hepatocytes of each group were lysed in mixture of Tris-HCl 20 mM L^{-1} (pH 8.0), EDTA 10 mM/L, and 0.5% lauryl sarcosyl containing 200 mg L^{-1} of proteinase K at 55°C overnight for cellular degdration. DNA was isolated by successive extraction with phenol/ Chloroform/ Isoamylalcohol (25:24:1) followed by precipitation with ethanol. Precipitated DNA was dried and re-suspended in TE buffer. The DNA was then loaded to 1%-2% agarose gel containing 1.0µg ml^{-1} EB and the gel were run in Genni mini- submarine electrophoresis unit at 50 V for 3 h with TAE running buffer and photographed under UV transillumination.

7.4. Results

7.4.1. The effect of folic acid, amlodipine and L- arginine on Bcl-2 expression after I/R in rat's liver.

7.4.1. 1. Bcl-2 gene expression by RT-PCR analysis

Anti-apoptotic Bcl-2 and house keeping G3PDH gene amplified by RT-PCR were separated by electrophoresis by staining with ethidum bromide. PCR products of Bcl-2, glyceraldehydes-3 phosphate dehydrogenase (G3PDH) were expressed at 234 bp and 510 bp respectively (Figure 7.4).

Expression of Bcl-2 gene in I/R group rats was negligible in comparison to sham-operated group after 1 h ischemia followed by 3h reperfusion. Pre treatment with folic acid, amlodipine and L- arginine significantly increased Bcl-2 gene expression as compared to I/R group rats. Bcl-2 expression of the L- arginine treated group wasmarkedly increased compared to folic acid and amlodipine treated rats. In L-arginine treated group, the amounts of Bcl-2 mRNA transcript were comparable with sham operated control rat Figure 7.5).

7.4.1.2. Protein expression by SDS PAGE analysis

Major protein band were observed at 45.0, 35.0 and 14.4 KDa respectively in all groups. No different or extra protein was observed in sham operated control rat and all other groups (Figure. 7.6).

7.4.1.3. Bcl-2 gene expression by Western Blot technique

Anti-apoptotic Bcl-2 proteins and house keeping G3PDH proteins identified by western blot technique. Bcl-2 and G3PDH were expressed at 25 KDa and 37 KDa. Bcl-2 protein level was increased 4 fold in sham operated control rats as compare to I/R rats. The significant decrease expression of Bcl-2 protein that occurred after 1 h ischemia followed by 3 h reperfusion in the vehicle treated rats were significantly increased by the administration of folic acid, amlodipine and L- arginine. Expression of Bcl-2 protein of L- arginine treated rats was nearly similar to those of the sham groups (Figure. 6.7 & 6.8).

7.4.2. The effect of folic acid, amlodipine and L- arginine on necrosis and apoptosis after I/R in rat's liver.

7.4.2.1. Determination of necrosis and apoptosis by flow cytometry analysis

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. The decreases were restored to the level observed in sham operated control rats by 100 mg kg^{-1} of L- arginine. In folic acid and amlodipine treated rats necrosis and apoptosis were also significantly decreased as compared to I/R rats (Table 7.1; Figure 7.9 & 7.10.)

Hepatocytes isolated from different experimental groups techniques showed changes in cell cycle distribution characterized by an increase and decrease in the number of cells in Go/G_1as well as S and G_2 /M phases respectively. I/R rats showed significantly decrease in DNA synthesis (S) phase. L- arginine treated rats showed significantly increased S phase and HPI as compared to amlodipine and folic acid treated groups (Table 7.2 & Figure 7.11).

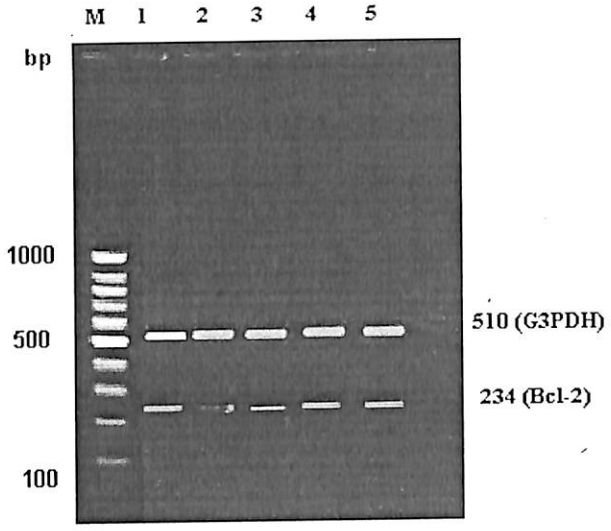
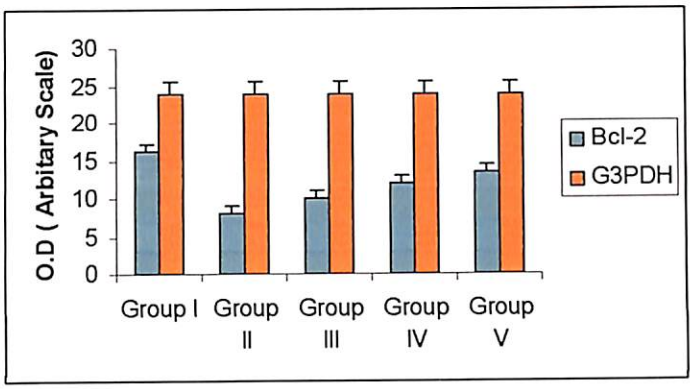


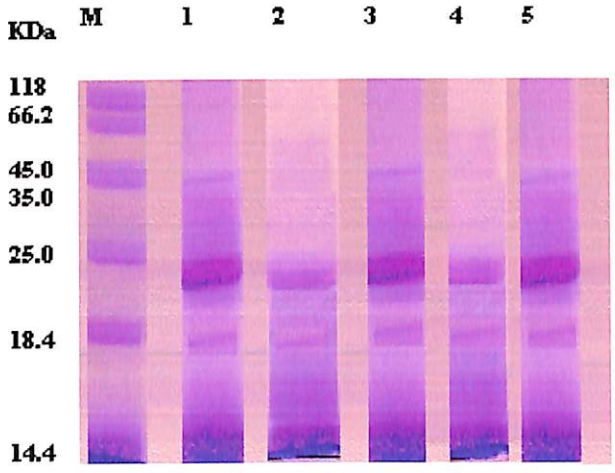
Figure: 7. 4. Bcl-2 gene expression after treatment with folic acid, amlodipine and L- arginine after I/R in rat liver.

Lane M: Marker; **Lane 1:** Sham-operated group; **Lane 2:** I/R control group, **Lane 3:** Folic acid treated group; **Lane 4:** Amlodipine treated group; **Lane 5:** L-arginine treated group.



[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

Figure: 7.5. Bcl-2 transcriptions of folic acid, amlodipine and L- arginine treated rat's abundance are normalized to house keeping G3PDH gene amplified by RTPCR after 1h of hepatic ischemia followed by 3 h reperfusion.

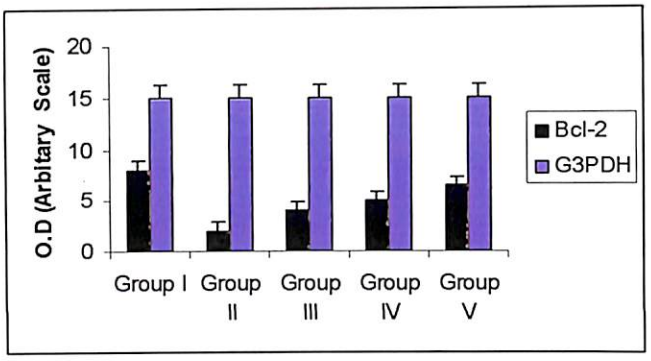


[M: Marker; **Lane 1:** Sham operated group; **Lane 2:** I/R group; **Lane 3:** Folic acid treated group; **Lane 4:** Amlodipine treated group. **Lane 5:** L- arginine treated group]

Figure: 7.6. Photomicrograph of SDS PAGE after treatment with folic acid, amlodipine and L- arginine after I/R in rat liver.

[**M:** Marker; **Lane 1:** I/R group; **Lane 2:** Sham operated group **Lane 3:** Folic acid treated group; **Lane 4:** Amlodipine treated group. **Lane 5:** L- arginine treated group]

Figure: 7.7. Western Blot of Bcl- 2 protein after treatment with folic acid, amlodipine and L- arginine in I/R rats.



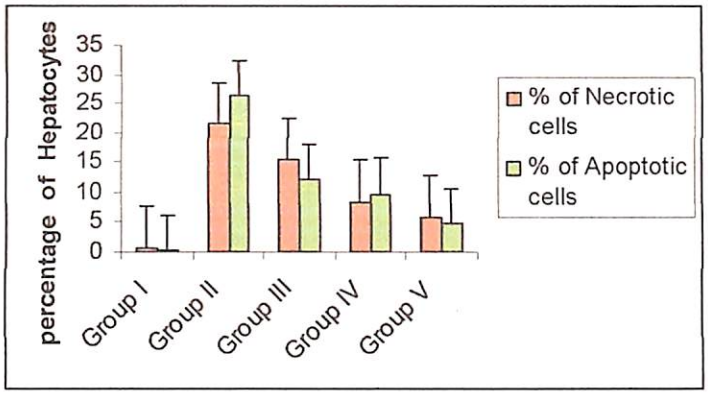
[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

Figure: 7.8. Graphical representation of Bcl-2 protein transcriptions of different treated rat's abundance are normalized to house keeping G3PDH gene amplified by western blot after 1h of hepatic ischemia followed by 3 h reperfusion.

Table: 7.1. The effect of folic acid, amlodipine and L- arginine on necrosis and apoptosis after I/R in rat liver (Flow cytometry analysis).

Groups		% of Necrotic cells	% of Apoptotic cells
Sham-operated	(Group I)	0.56 ± 0.21	0.20± 0.08
I/R Injury	(Group II)	21.54 ±7.14*	26.44±6.04*
Folic acid treated	(Group III)	15.32± 2.87* #	12.10± 3.2*#
Amlodipine treated	(Group IV)	8.21±0.22*#	9.65±0.96*#
L -Arginine treated	(Group V)	5.68 ±0.85*#	4.70±0.49*#

Results are expressed as mean ± SD (n=6). * Statistically difference (P< 0.01) from sham operated rats. # Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.



[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

Figure: 7.9. Graphical representation of the effects of folic acid, amlodipine and L-arginine on necrosis and apoptosis after I/R in rats. (Flow Cytometry analysis).

Table: 7. 2. The effect of folic acid, amlodipine and L- arginine on cell –cycle after I/R in rat liver.

		Cell Cycle %		
Groups		G_o/G_1	S	G_2/Mit
Sham-operated	(Group I)	60.11± 2.45	24.11±1.64	5.91±2.06
I/R Injury	(Group II)	92.13 ± 1.43*	1.27± 0.76*	0.34±.002*
Folic acid treated	(Group III)	37.91± 1.67*#	16.89±1.23*#	2.55±0.66*#
Amlodipine treated	(Group IV)	44.10± 2.81*#	17.50±2.47*#	2.76±0.81*#
L -Arginine treated	(Group V)	52.49±4.66*#	20.90±3.11*#	4.20±0.87*#

Results are expressed as mean ± SD (n=6). * Statistically difference (P< 0.01) from sham operated rats. # Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.

[S: DNA synthesis phase; G_1: Gap 1 phase; G_2: Gap 2 phase; Mit: Mitosis Phase]

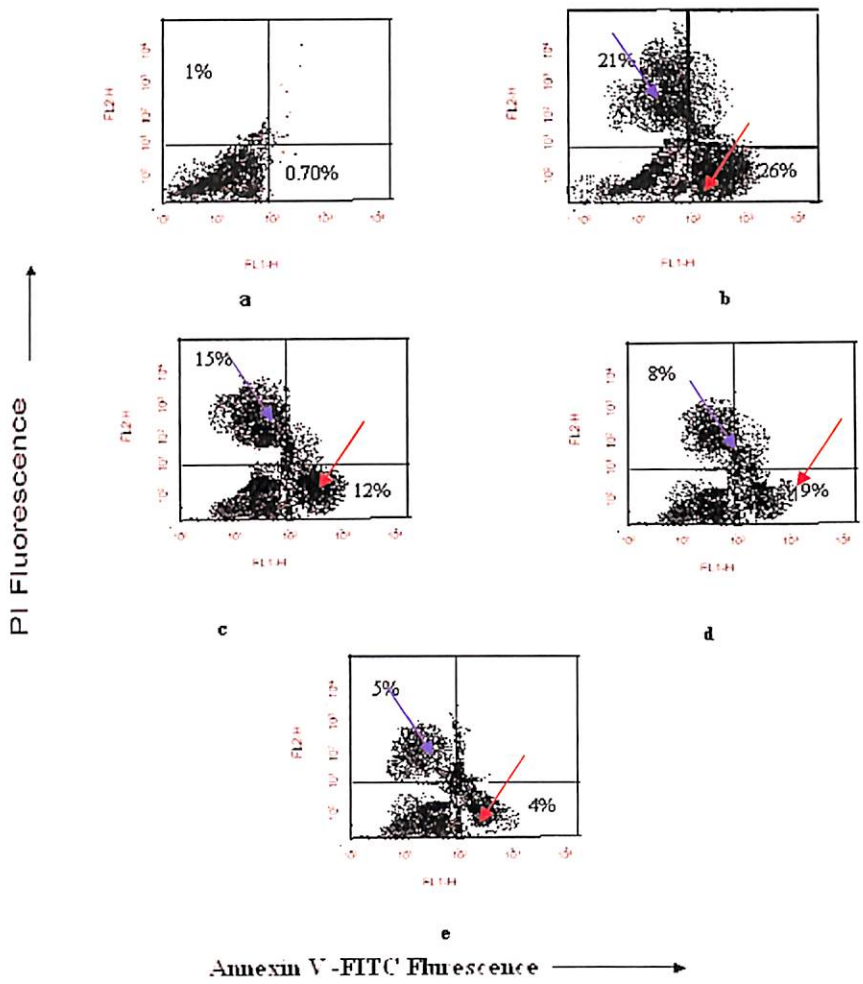
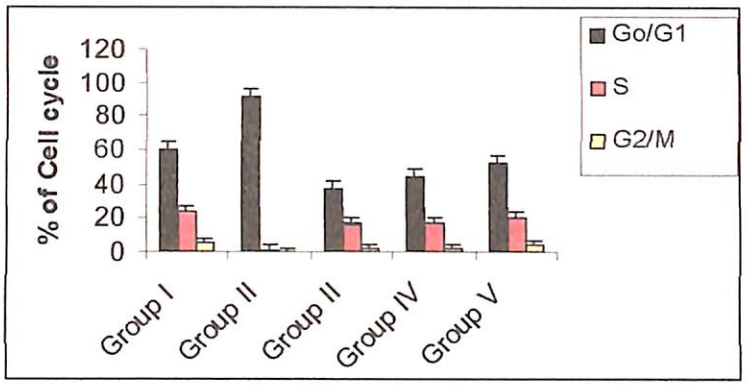


Figure: 7.10. (a-e). Photomicrograph of flow cytometric analysis for the effect of folic acid, amlodipine and L- arginine on necrosis and apoptosis after I/R in rat liver. **a:** Flow-cytogram of sham operated control rats; **b:** Flow - cytogram of ischemic and reperfused rats ; **c:** Flow- cytogram of folic acid treated rats; **d:** Flow- cytogram of amlodipine treated rats .**e:** Flow- cytogram of L – arginine treated rats ; Red arrow (→) denotes apoptotic hepatocytes; Blue (→) arrow denotes necrotic cells .



[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

Figure: 7. 11. Graphical representation of the effect of folic acid, amlodipine and L-arginine on cell –cycle after I/R in rat liver.

7.4.2.2. Determination of viable hepatocytes by DAPI staining technique

Viable hepatocytes were evaluated by DAPI positive staining. Maximum viable hepatocytes were observed in peri- infract zone. L-arginine treated rats showed maximum viable hepatocytes as compared all experimental animals. I/R rats showed the decrease in hepatocytes as compared to I/R rats (Table 7.3; Figure 7.12 & 7.13).

7.4.2.3. Determination of apoptotic hepatocytes by TUNEL assay

Apoptotic cells were not detected in the sham operated control group. The apoptotic – labeling index (AI) in 100 hepatic cells ranged between 0% to a maximum 24.11 %in I/R groups. L- Arginine significantly decreased A.I as compared to other groups. (Table 7.4; Figure 7 .14 &7.15). The ascending orders of inhibition of apoptosis were as follows:

Group I (Sham operated rats) >Group V (L- arginine treated) >Group IV (Amlodipine treated) >Group III (Folic acid treated > Group II (I/R Rats).

7.4.2.4. Determination of apoptotic and viable hepatocytes by fluorescence microscopy

Saline-treated group showed viable cells marked with green fluorescence where as I/R group showed apoptotic cells marked with orange fluorescence. Viable cells marked

with green fluorescence were increased in folic acid treated group as compared to I/R groups.

L- Arginine treated group showed maximum improved results in prevention of apoptosis as compared to folic acid treated group and amlodipine treated group in I/R rats. Apoptotic bodies were significantly decreased and viable hepatocytes marked with green fluorescence were increased as compared to folic acid treated and amlodipine treated groups. (Table 7.5; Figure .7.16 & 7.17).

7.4.2.5. Determination of chromatin condensation and fragmentation by Hoechst 33258 staining

Sham operated control rats showed that nuclear chromatin was faintly stained and homogeneously dispersed throughout the whole nucleus whereas I/R rat's apoptotic nuclei were clearly stained with typical condensed nuclei, condensed chromatin marginally located at the nuclear membrane or nuclear fragments consisting of condensed masses of chromatin were observed. L- Arginine treated rats showed reduced condensation of nuclear chromatin as compared to all other experimental groups (Table.7.6; Figure 7.18 & 7.19).

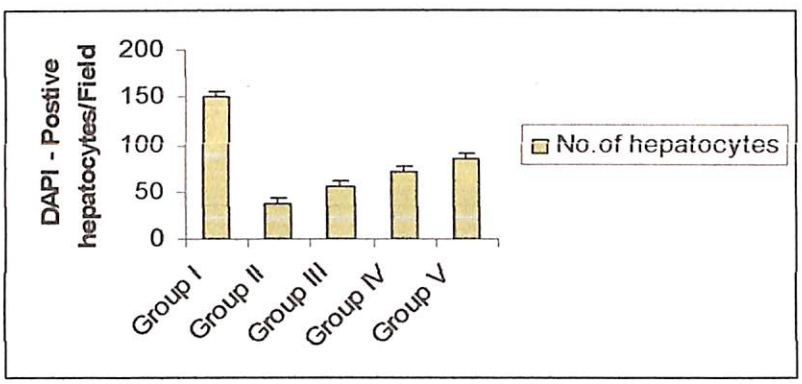
7.4.2. 6. Determination of DNA fragmentation by electrophoresis

Apoptosis was evaluated based on some distinct morphological features such as cell shrinkage, chromatin condensation, oligonucleosomal DNA fragmentation and finally break down of the cell into smaller units (apoptotic bodies) and DNA ladder formation (smear). Reperfusion of the ischemic liver caused severe hepatocellular apoptosis after 1 h ischemia followed by 3 h reperfusion in I/R groups and formed DNA ladder. In, Lane Sham operated rats there was no sign of apoptosis and intact DNA band was observed. Groups treated with folic acid and amlodipine showed less characteristics DNA ladder formation. In groups treated with L- arginine DNA characteristics were similar to sham operated control rats without any smear formation (Figure 7.20).

Table: 7.3. The effect of folic acid, amlodipine and L- arginine on DAPI positive hepatocytes per field after I/R in rat liver.

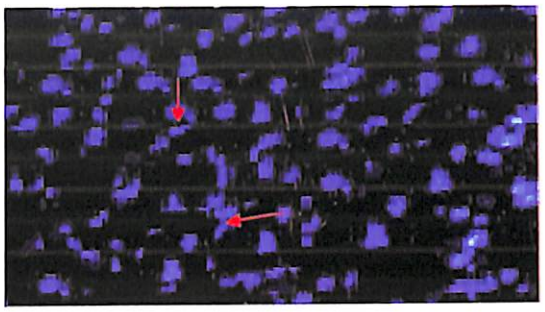
Groups		Number of Positive DAPI hepatocytes Field^{-1}
Sham-operated	(Group I)	150 ± 5.76
I/R Injury	(Group II)	38 ± 2.89*
Folic acid treated	(Group III)	55 ± 3.03*$^{\#}$
Amlodipine treated	(Group IV)	72 ± 2.67*$^{\#}$
L -Arginine treated	(Group V)	85 ± 4.98*$^{\#}$

Results are expressed as mean ± SD (n=6). *Statically difference (P< 0.01) from sham operated rats.
$^{\#}$ statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.

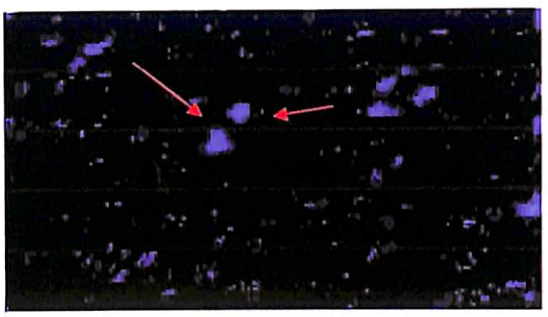


[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

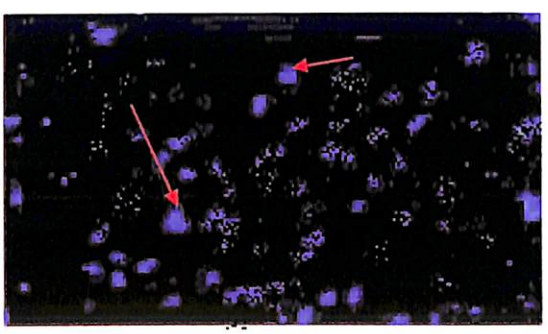
Figure: 7.12. Graphical representation of the effect of folic acid, amlodipine and L-arginine on DAPI positive hepatocytes per field after I/R in rat liver.



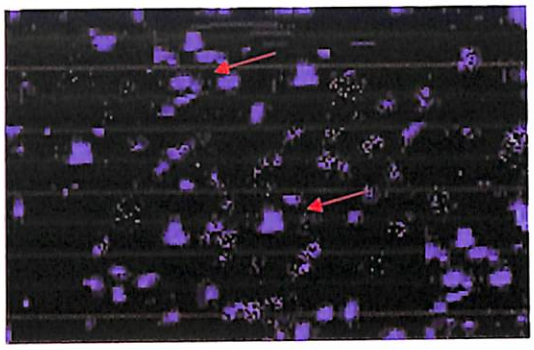
A: Number of viable hepatocytes in Sham operated control rats.



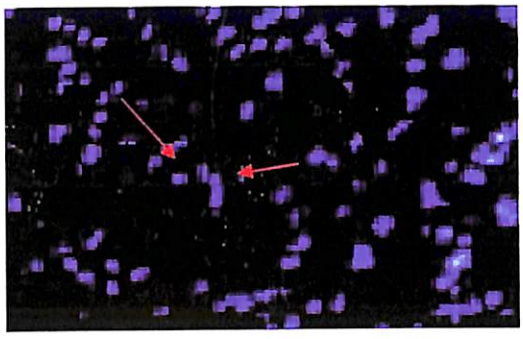
B: Viable hepatocytes decreased in I/R rats



C: Viable hepatocytes with red mark arrow were evident in Folic acid treated rats after I/R injury.



D: Administration of Amlodipine increased viable hepatocytes



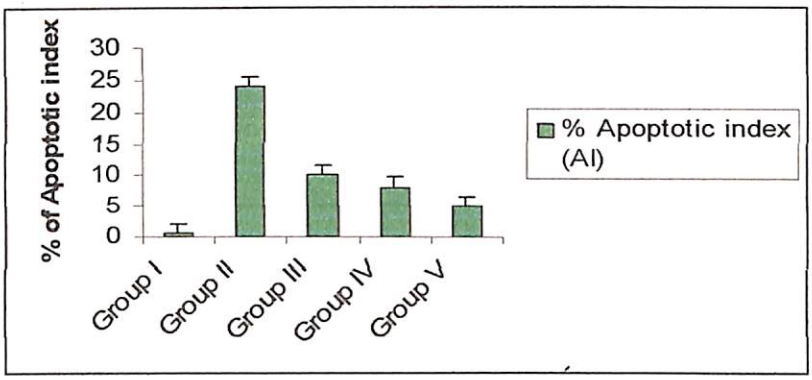
E: L - arginine treated I/R rat's showing number of viable hepatocytes.

Figure: 7.13. Immunofluorescence of DAPI positive viable hepatocytes in folic acid, amlodipine and L- arginine treated rats after 1h ischemia followed by 3h reperfusion.

Table: 7.4. The effect of folic acid, amlodipine and L- arginine on apoptosis after I/R in rat liver (TUNEL assay).

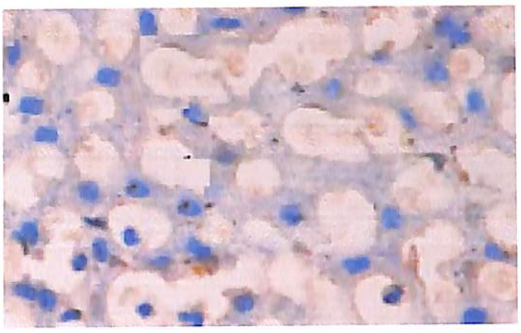
Groups		% Apoptotic index (AI)
Sham operated	(Group I)	0.45 ±0.021
I/R rats	*(Group II)*	24.11± 1.2 6*
Folic acid treated	(Group III)	10.19± 1.54 *#
Amlodipine treated	(Group IV)	8.14± .0. 65*#
L- arginine treated	(Group V)	5.10 ± .0. 75

Results are expressed as mean ± SD (n=6). *Statically difference (P< 0.01) from sham operated rats.
Statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion.

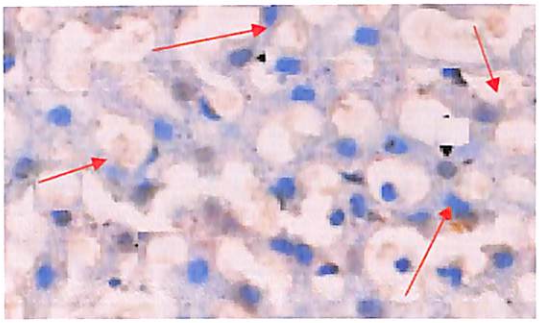


[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

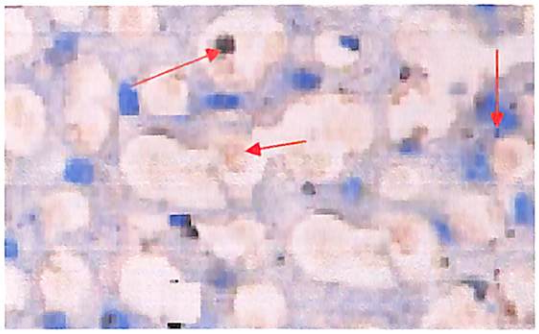
Figure: 7. 14. Graphical representation of the effect of folic acid, amlodipine and L-arginine on apoptosis after I/R in rat liver (TUNEL assay).



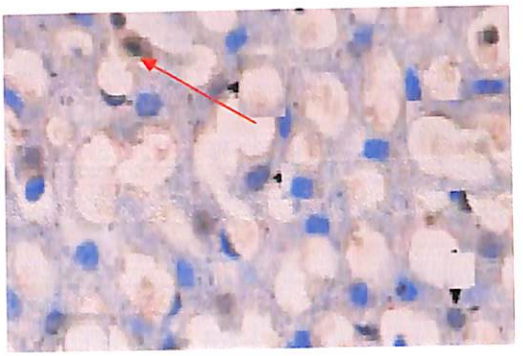
A: Sham operated control rat showing normal hepatocytes



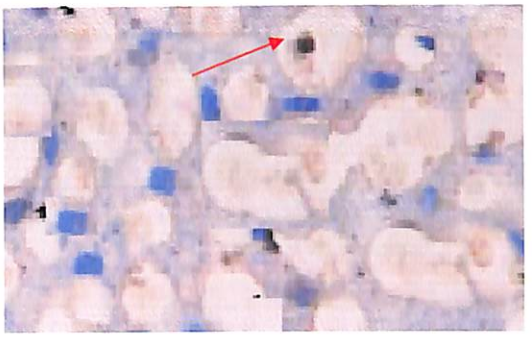
B: I/R rat's showing number of apoptotic (TUNEL Positive) hepatocytes marked with arrow.



C: Few apoptotic hepatocytes in Folic acid treated I/R rats.

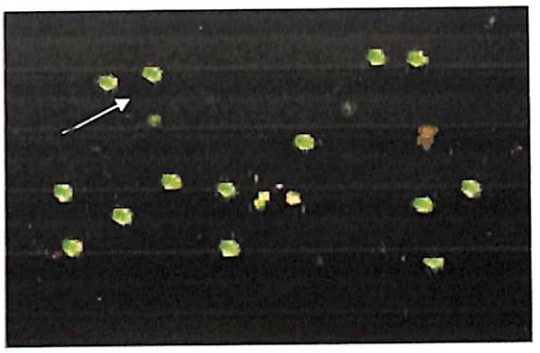


D: Amlodipine treated rats showing decreased apoptotic hepatocytes in I/R rats.

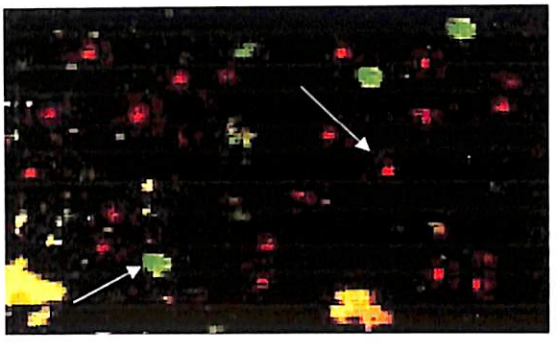


E: L- arginine treated rats showing decreased apoptotic hepatocytes in I/R rats.

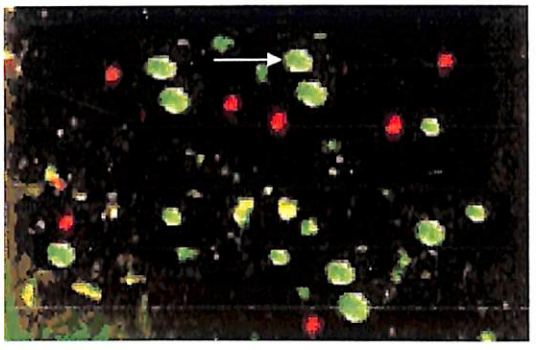
Figure: 7.15 (A-E). Photomicrographs of TUNEL assay showing the effects of folic acid, amlodipine and L- arginine on apoptosis after I/R in rat liver.



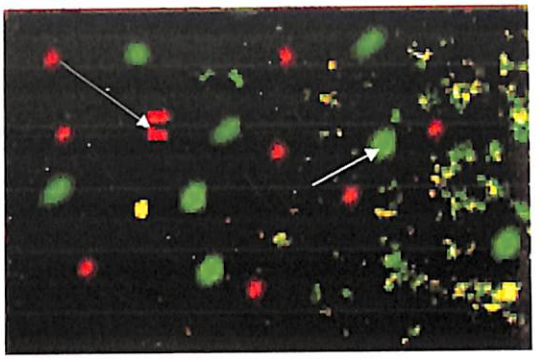
A: Viable hepatocytes showing green fluorescence of in sham operated control rats.



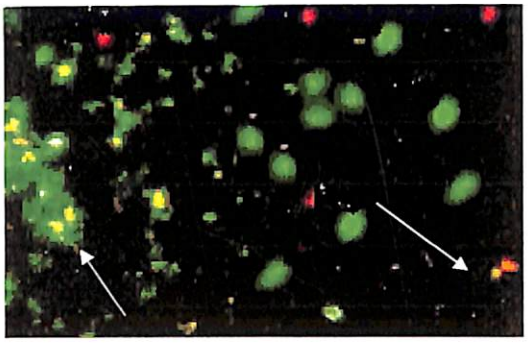
B: Viable hepatocytes showing reduced numbers of green fluorescence and apoptotic hepatocytes increased numbers of red fluorescence in I/R rats.



C: Folic acid treated I/R rats showing increased viable hepatocytes with green fluorescence.



D: Amlodipine treated rats I/R showing increased viable hepatocytes with showing as green fluorescence and reduced apoptotic hepatocytes showing red fluorescence.



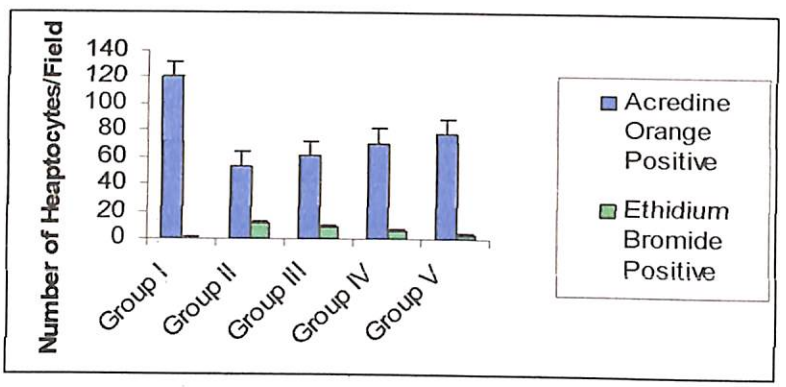
E: L –arginine treated I/R rats showing increased viable hepatocytes with green fluorescence and very few numbers of apoptosis hepatocytes showing red fluorescence.

Figure: 7.16 (A-E). Photomicrographs of fluorescence microscopy studies showing the effect of folic acid, amiodipine and L- arginine on apoptosis after I/R in rat's liver (x 400). Green fluorescence correspondences to viable hepatocytes marked with single arrow. Red fluorescence correspondences to hepatocytes marked with single arrow.

Table: 7.5. The effect of folic acid, amlodipine and L- arginine Acridine orange positive viable hepatocytes shown green fluorescence and ethidium bromide positive apoptotic hepatocytes showing red fluorescence per field after I/R in rat liver.

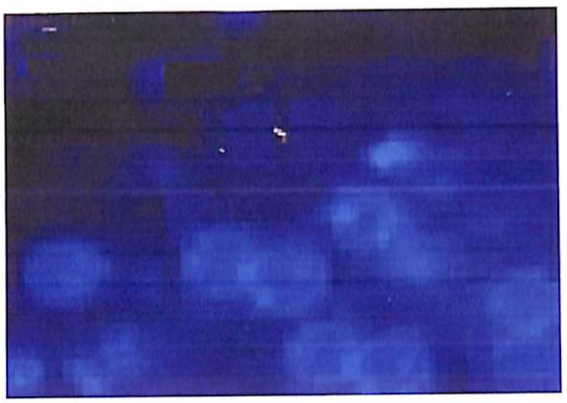
Groups		[a]Acredine orange Positive	[a]Ethidium Bromide Positive
Sham-operated	(Group I)	120 ± 11.57	0.54± 0.037
I/R Injury	(Group II)	52± 6.37*	11 ± 1.01*
Folic acid treated	*(Group III)*	*61± 4.98*#*	*8.8 ± 0.70*#*
Amlodipine treated	(Group IV)	70 ± 2.21*#	6.5 ± 0.59*
L -Arginine treated	(Group V)	77 ± 5.66*#	3.2± 0.52*#

Results are expressed as mean ± SD (n=6). *Statically difference (P< 0.01) from sham operated rats. # statically difference (P< 0.01) from vehicle- treated ischemia and reperfusion. [a] Measured number of hepatocytes Field^{-1}

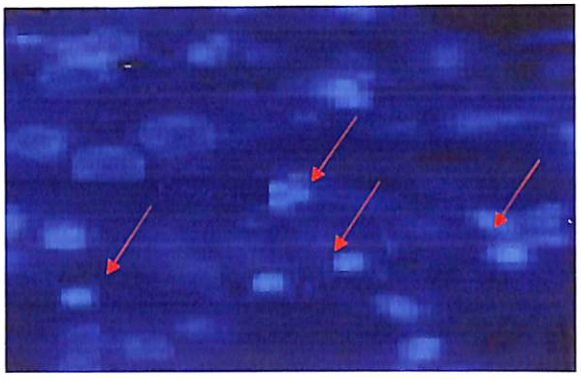


[Group I: Sham operated control rats; Group II: I/R rats; Group III: Folic acid treated rats; Group IV: Amlodipine treated rats; Group V: L- arginine treated rats].

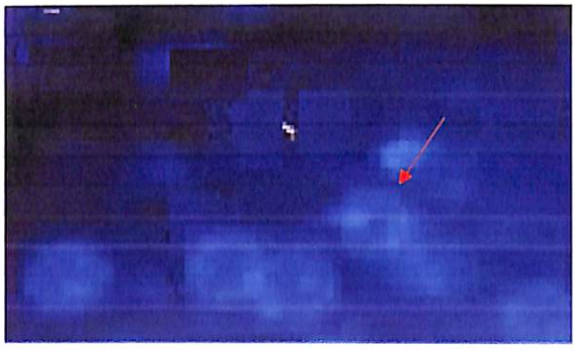
Figure: 7.17. Number of viable hepatocytes (Acridine orange positive) and apoptotic *cells (Ethidium bromide positive) in different groups after 1h ischemia* followed by 3 h reperfusion.



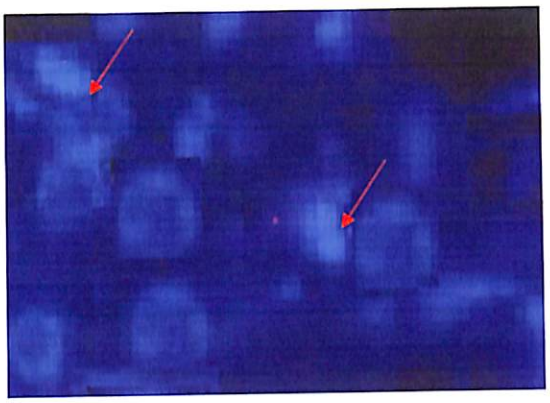
A: Sham operated control rats showing no chromatin aberration in liver.



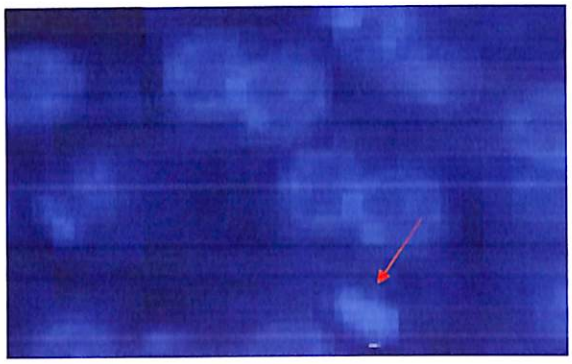
B: I/R rats showing number of abortive chromatin in liver marked as red arrow.



C: Folic acid treated I/R rats showing reduced number of abortive chromatin in liver marked as red arrow.

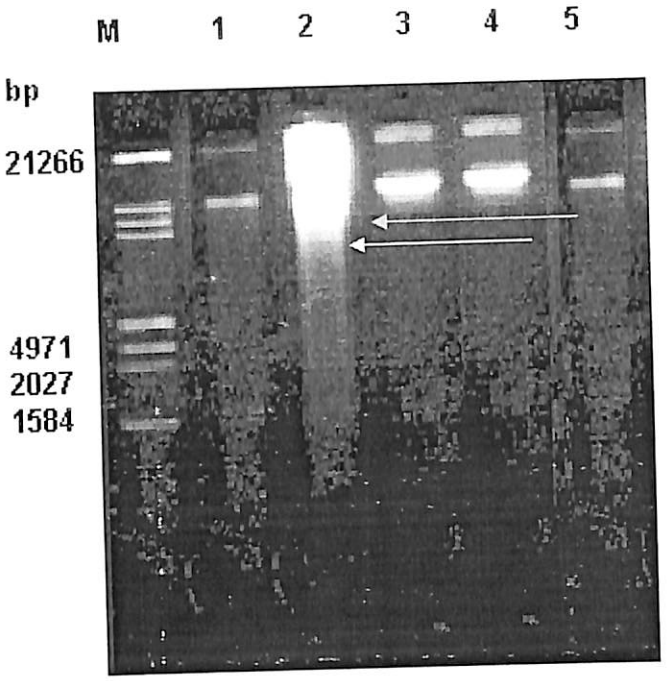


D: Amlodipine treated rats liver showing reduced number of abortive chromatin marked as a red arrow.



E: No sign of abortive chromatin in L- arginine pretreated group of I/R rat's.

Figure: 7.19 (A-E). Hoechst 33258 positive abortive chromatin after I/R and in treated rats.



[Lane M: Marker; Lane 1: Sham-operated; Lane 2: I/R control; Lane 3: Folic acid treated; Lane 4: Amlodipine treated; Lane 5: L- arginine treated].

Figure: 7.20. DNA fragmentation after 1 h ischemia followed by 3 h reperfusion and in treated groups.

7.5. Discussion

Bcl-2 gene family plays a critical role for the common pathway of apoptosis. Over expression of Bcl-*2* family prevented apoptosis in chronic infective hepatocytes, marrow stem cells, and nerve cells (El-Deiry et al., 1993; Xiong et al., 1993; Agarwal et al. 1995). Earlier study showed that Bcl-2 is an anti-apoptotic protein and plays an important role in cellular apoptosis Gorczyca et al., 1993). Earlier studies suggest that Bcl-2 may have a more general role in regulating mitochondrial metabolism and function (Oltvai et al., 1993; Bartling et al., 1998; Celli et al., 1998; Sun K et al., 2004) apart from 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 (Shimizu et al., 1999) and Bcl-2 promotes VDAC closure (Jurgensmeier et al., 1998; Shimizu et al., 2000).

Ca^{2+} is also one of the major ions in regulating VDAC opening and closer. 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 (Paradies et al., 1999; Crompton, 1999; Bernardi et al., 2001; Satoh et al., 2003). However, no work on the effect of Ca^{2+} ion, NO stimulator and DNA stabilizer agent in inhibition of Bcl-2 gene and VDAC regulation is reported so far.

Therefore, the aim of this study was to look for the mechanism by which specific over- expression of Bcl-2 suppresses cell death as well as potential mechanisms by which Bcl-2 might modulate cellular metabolism and to evaluate role of calcium channel blocker- amlodipine, NO oxide precursors- L- arginine and DNA stabilizer- folic acid in expression of Bcl-2 as well as in apoptosis.

The present study demonstrated that after 1 h ischemia followed by 3 h reperfusion Bcl-2 expression was inhibited and increased numbers of necrotic cells and apoptotic cells was found (Figure 7.6. & 7.7 and Table 7.1). Moreover, abortive DNA, cytochrome c level and viable hepatocytes was increased (Figure 7.20 lane 2 and Figure 7.13 B), which indicated that I/R injury caused necrosis and apoptosis in the rat liver.

Further, most important finding of our study is that, after I/R injury, Bcl-2 expression decreased as compared to sham operated control rats (Figure 7.4, Lane 2 and Figure 7.4). This result can be explained on the basis of findings of Chen et al., 2001, 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.

Interphase is known as regulator of cell cycle. "S" sub phase of interphase is involved

in resynthesis of nucleic acids for cell cycle and apoptosis is triggered by inhibition of "S" phase (G2 Gap). This is often observed in response to the suppression of DNA replication or DNA repair in I/R injury (El-Deiry et al in 1993, Xiong et al., 1993, Agarwal et al., 1995 and Yonish-Rouach 1996). Present study shows that after I/R injury, "S" phase gap decreased 23 fold in cell cycle as compared to sham operated control rats (Table 7.2 and Figure 7.1). Thus, I/R injury caused necrosis and apoptosis through suppression of Bcl-2 and reducing "S" phase gap in cell cycle.

L- arginine was potent cytoprotective in vivo and modulated necrosis and apoptosis. In addition, it showed increased "S" phase time gap in cell cycle as well as an increase in cell viability as compared to all other treated groups (Figure 7.13 D and E). Previous report (Curran et al., 1991) indicated that L -arginine-NO pathways have beneficial effects in liver transplantation models and the L -arginine-NOS pathway plays important role during infection, inflammation, organ injury, and transplant rejection. This can be further explained on the basis of work of Wu et al 1995 as NO exerts protective effects after I/R by improving blood flow, antagonizing neutrophil activation and adhesion, neutralizing free radical injury, and eliciting antiapoptotic effects. Thus, the present study provides clue that production of NO in hepatic sinusoids by L – arginine inhibits apoptosis and necrosis in I/R rats by vasodilatation.

Studies in the present work showed that pretreatment with amlodipine significantly up regulated Bcl-2 expression, which resulted in diminished hepatic necrosis and apoptosis caused by liver I/R in rats (Figure 7.4 and 7.5). This observation can be explained based on the fact that 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 (Farrow et al., 1995). Therefore present studies support that treatment with amlodipine restores Bcl-2 function after I/R injury by maintaining low Ca^{+2}. Gibson et al (1996) reported that amlodipine restored Bcl-2 expression indirectly 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), therefore, it reduced apoptosis in I/R rat. However, this present study provides new insight into the mechanism by blocking calcium cannels, by which it mediates protection from apoptosis by indirectly causing Bcl-2 up regulation.

Pre - treatment with folic acid showed considerable prevention of necrosis and

apoptosis in I/R rats by over expressing Bcl-2 and increased gap of "S" phase in cell cycle. These results are in agreements with those reported in patients with pre-malignant gastric lesions in which folic acid therapy decreases Bcl- 2 expression in gastric mucosa and decreases "S" phase (Cao et al., 2005, Kim et al., 2002; Kim, 2004; Wang et al., 2004). However the present studies support that folic acid increased " S" phase by increased nucleic acid synthesis to counter the insults of I/R injury (Table 7.1). This can be rationalised as folic acid is a co- factor in 1- carbon metabolism (Bilbao et al., 1969) and prevents mitochondrial DNA damage. Besides folic acid deficiency causes uracil mis-incorporation into DNA and chromosomal breakage (Manjo and Jaris, 1995). Thus, present study showed that amlodipine and folic acid are effective in I/R injury to prevent necrosis and apoptosis but L- arginine appeared as the most potent molecule to attenuation I/R injury.

7.6. Conclusion

The present study showed: -

1. Administration of L-arginine reduced necrosis and apoptosis through NO production in liver sinusoids and also L–arginine acid protects hepatobiliary function and unregulated Bcl- 2 gene in I/R rats.
2. Protective action of calcium channel blocker-amlodipine in apoptosis and necrosis in I/R induced injury is mainly due to inhibitory action of Ca^2 efflux into mitochondria.
3. Treatment with folic acid improved the hepatocellular structure and reduced apoptotic cells besides altereing the expression levels of Bcl-2 gene. This provides a strong evidence for pathophysiological mechanism by which folic acid protects from I/R injury.

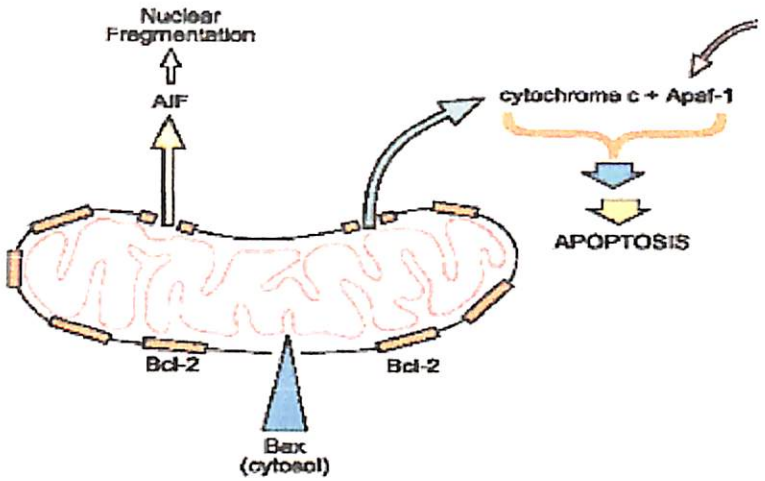


Figure: 7.21. Regulation of cell cycle in ischemia followed by reperfusion injury.

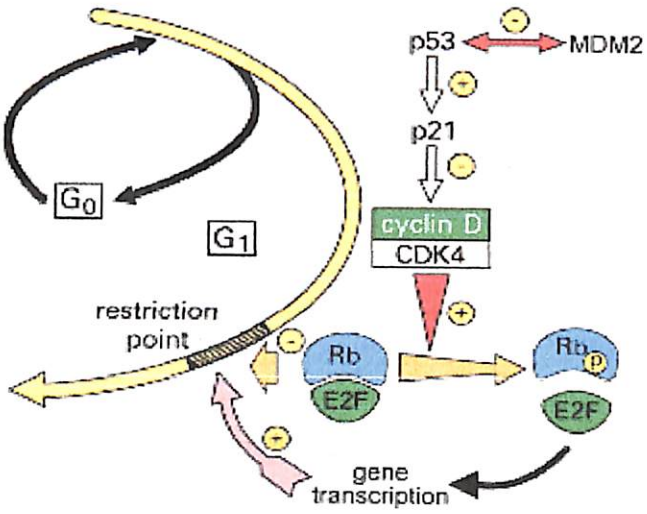


Figure: 7.22. Mitochondrial regulation of Bax and Bcl-2 gene during apoptosis process.

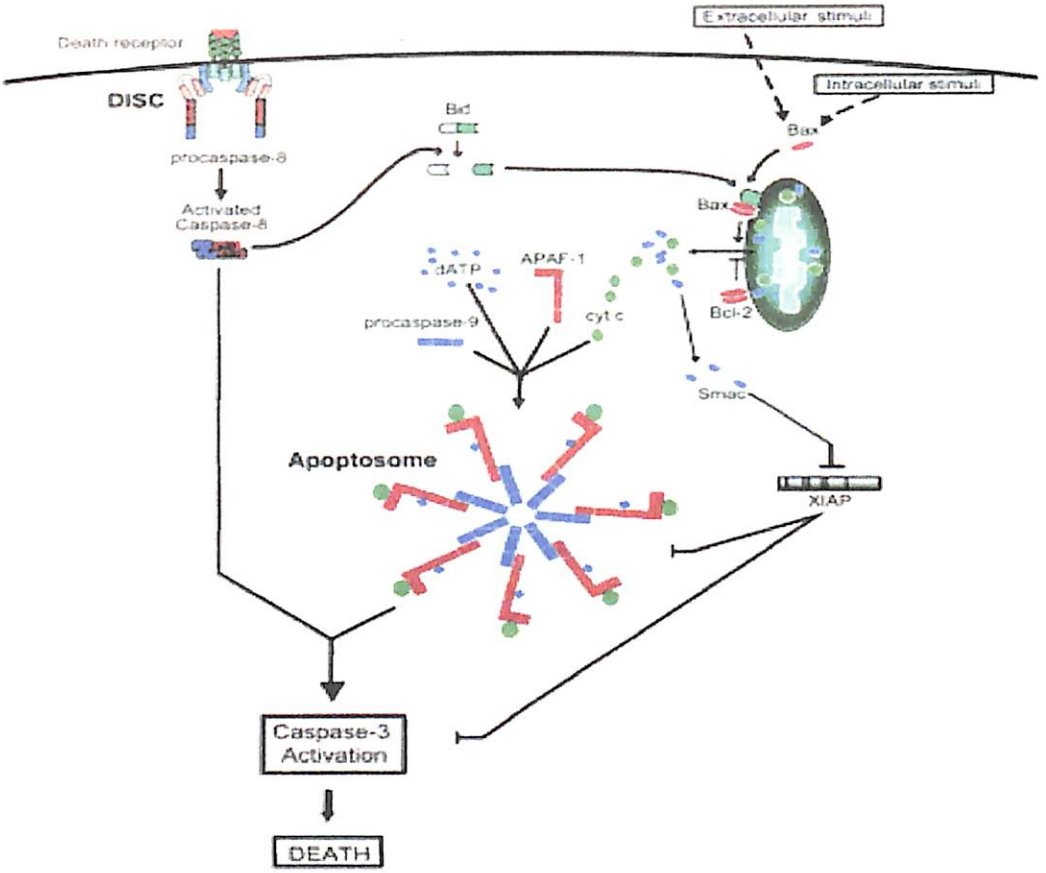


Figure: 7.23. Events of ischemia – reperfusion that generates apoptosis stimulus during apoptosis.

7.7. References

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Chapter - 8

Folic acid, Amlodipine, L- Arginine Attenuated Microscopical Changes in Ischemia followed by Reperfusion Injury in Rat Liver

8.1. Introduction

I/R injury is a pathophysiological process whereby hypoxic organ damage is caused due to deprivation of oxygen delivery to the compromised tissue. Hepatic ischemia occurs during solid organ transplantation, trauma, hypovolemic shock, and elective liver resection, when inflow occlusion or total vascular exclusion is used to minimize blood loss. The pathophysiology of liver I/R injury includes both direct cellular damage as a result of the ischemic insult as well as delayed dysfunction and damage resulting from activation of inflammatory pathways (Clavien et al., 2001; Fondevila et al., 2003; Debonera et al., 2004).

Like other parenchyma, the normal liver contains an epithelial component (hepatocytes), an endothelial lining, tissue macrophages (Kupffer cells), and perivascular mesenchymal cells called the stellate cells (Figure 8.1). In I/R injury, liver parenchyma (hepatocytes) and endothelial lining (kupffer cells) are destroyed and stellate cells plays virtually key role in I/R injury (Murawaki et al., 1997; Iredale et al., 1998).

The cellular elements of the liver are organized within sinusoids with the sub- endothelial space of Disse separating the hepatocytes from the sinusoidal endothelium. The space of Disse contains a basement membrane-like matrixallowing maximized passage of molecules from the fenestrated sinusoidal endothelium to hepatocytes providing structural integrity to the liver parenchyma (Friedman, 2000; Benyon, 1998). The main components of the endothelium collagen matrix (ECM) in normal liver are collagens type I, III, IV, V, and VI in addition to other types of collagen present in small proportions. There are also many non-collagenous matrix components viz. fibronectin, laminin, elastin and proteoglycans.(Schuppan, 1990; Woessner 1991; Murphy and Docherty, 1992; Okazaki et al.,2000). Interruption and subsequent restoration of the blood flow is unavoidable in transplantation of organs. Organ injury caused by this transient ischemia followed by reperfusion is one of the main causes of initial poor function after OLT. (Maring et al., 1997). The spectrum of clinical manifestations of ischemia and reperfusion (I/R) injury can range from asymptomatic elevation of liver enzymes to primary non-function of the liver. Hepatic I/R injury is a complex, multifactorial pathophysiologic process that affects all types of hepatic cells, such as Kupffer cells, endothelial cells, hepatocytes and neutrophils. (Fan et al., 1999; Serracino-Inglott et al.,

2001). The histopathological characteristics of ischemic preservation include hepatic vacuolisation and swelling of endothelial cells. Within minutes after reperfusion with oxygenated blood, endothelial cells round up and eventually detach from the connective tissue matrix, consisting of cords of collagen linked to the cells through intermediate molecules such as fibronectin. This process of detachment appears to be mediated by proteases (Clavien, 1998). Hepatocytes retain their viability and initially appear to be minimum affected and thereafter, Kupffer cells become activated, as indicated by degranulation, increased phagocytosis, release of oxygen free radicals and inflammatory mediators, such as TNF-α, IL-1, and platelet activating factor. Kupffer cell activation and endothelial cell injury leads to profound microcirculatory disturbances and sinusoidal accumulation of leukocytes and platelets (Farmer et al., 2000).

In the late phase of injury, neutrophils infiltrate the liver in response to chemo attractants released by activated Kupffer cells and expression of intercellular adhesion molecules on endothelial cells (Lichtman and Lemasters, 2001). Accumulation of activated neutrophils within the hepatic parenchyma causes further hepatocyte damage several hours after reperfusion through the release of oxidants and proteases (Lentsch et al., 2000). Microcirculatory changes appear to reach maximum level within 48 hours after reperfusion (Farmer, 2000).

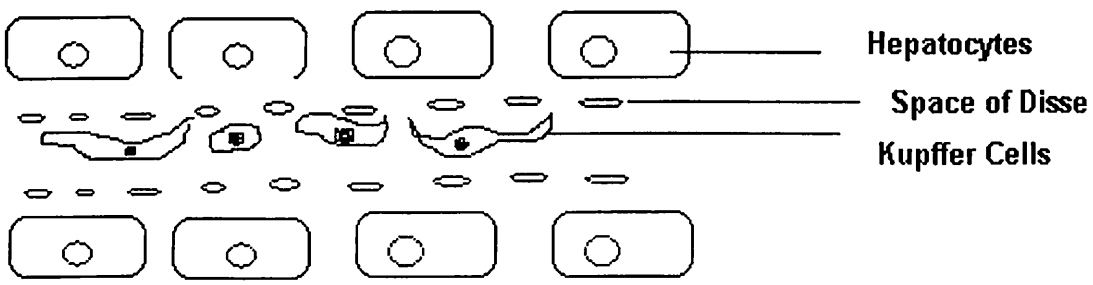


Figure: 8.1. Structure of sinusoids.

NO is known to have an important role in regulating liver physiology and blood flow. NO and citrulline are produced by the family of nitric oxide synthases (NOS) from the substrate L-arginine (Wu and Morris, 1998).

Microcirculatory disturbance during hepatic I/R are attenuated by NO through vasodilatation and decrease releases of mediators. NO is inducers of vasodilatation at the level of the sinusoid as well as at presinusoidal sites (Curran et al., 1989; Curran et al., 1991). NO has been shown to exert protective effects in the liver by improving blood flow, antagonizing neutrophil activation and adhesion, neutralizing free radical injury, and eliciting antiapoptotic effects (Pryor and Squadrito, 1995). The beneficial effects of the L-arginine-NO pathway have also been reported in liver transplantation models. In addition to its vasodilatory effect, NO reacts with superoxide to form the potent oxidant peroxynitrite (Granger et al., 1994).

The exaggerated Ca^{+2} influx into cells is an important signal, which may lead to cell death. Number of studies showed that heapto-toxic drugs are associated with an increase in intracellular Ca^{+2} (Thomas and Reed 1989; Nicotera et al., 1992).

Folate is a cofactor in 1-carbon metabolism, during which it promotes the remethylation of homocysteine. In addition to augmenting the risk for vascular events, low folate and high homocysteine may directly increase the susceptibility of neurons to brain injury (Mattson et al., 2003). Folate deficiency causes uracil misincorporation into DNA and chromosomal breakage, which has implications in neuronal damage.

Further, in I/R injury DNA damage is caused by oxidative stress. Oxidative stress-induced cell killing involves oxidation of pyridine nucleotides, accumulation of calcium in mitochondria, and superoxide formation by mitochondria, which ultimately leads to formation of membrane permeability transition pores and breakdown of the mitochondrial membrane potential (Carden et al., 1993) which was supported by observation of the mitochondrial membrane permeability during hepatic ischemia-reperfusion (Nieminen et al., 1997).

The effect of Folic acid, Amlodipine and L- arginine therapy on microscopical changes in hepatic ischemia has not yet been conclusively elucidated. Therefore, it was decided to see 1) the effects of ischemia/reperfusion in ultra structural and microscopical alternation; 2) difference between histological alterations which occur during ischemia following reperfusion on pretreatment with folic acid, L- arginine and amlodipine; 3) oxidative events that contribute to declines in histological changes; 4) pathological changes in the different experimental treated groups.

8.2. Materials and Methods

8.2.1. Materials

Glutarldehyde, Hematoxylin and Formaldehyde were purchased from S.D Fine Chemicals, Mumbai, India. Ethanol was purchased from Bengal Chemicals, Calcutta. Osmium tetroxide from Fluka, USA and Araldite resin from Ducupan, Fluka Biochemika, Ronkokoma, KX were used.

8.2.2 Methods

8.2.2.1. Animals treatment

Thirty Wistar rats were divided into sham-operated control group (I) (n = 6), ischemia and reperfusion group (II) (n = 6), were given 0.9 % saline (5ml kg^{-1}, p.o) for 7 days; amlodipine treated group (given amlodipine 1 mg kg^{-1} body mass daily by oral route for 7 days before induced ischemia reperfusion maneuver) group III (n=6), Folic acid treated group (given folic acid 1 mg kg^{-1} body mass daily by oral route for 7 days before inducing ischemia reperfusion maneuver) (IV) (n = 6) and L-Arginine treated group (given L-arginine 100 mg kg^{-1} body mass daily by oral route for 7 days before inducing ischemia reperfusion maneuver) (V) (n = 6).

8.2.2.2. Animals Model

Ischemia and reperfusion injury were same as described on page 29, chapter 3.

8.2.2.3. Peripheral tissue sampling

Peripheral tissue sampling method was same as described on page30, chapter 3.

8.2.2.4. Histopathology

6 μm stains of formalin –fixed and paraffin-embedded were taken and stained with hematoxylin –eosin (HE). The liver pathology was scored as follows:

Steatosis (the percentage of liver cells containing):

1+= 05 to 25% of cells containing fat

2+ = 26% to 50% of cells containing fat.

3+= 51% to 75% of cells containing fat.

4+= > 75% of cells containing fat.

Inflammation and necrosis

1 + = one focus/ lobule

2+ = two or more foci/lobules

3+= apoptosis

Two types of eosinophilic hepatocellular changes are presumed to be apoptotic in origin viz round and detached from surrounding hepatocytes (classical Councilman bodies) and shrunken compared to adjacent hepatocytes, but still firmly attached were counted in 5 to 20 fields from each liver to count at least 100 stellate –Abs. The following changes were calculated /100 stellate cells: -

1. If nucleus fragmented present or absent
2. If nucleus Pyknotic (or those in intact nuclei)
3. If nucleus Pyknotic, fragmented or absent
4. If adjacent inflammation present
5. If located in an acidophilic domain
6. If nodular hyperplasia
7. If atrophy present
8. If apoptosis present
9. If necrosis present

8.2.2.5. Transmission electron microscopy (TEM)

Liver tissues were fixed in Karnovsky's solution (2.5% glutarldehyde and 2% formaldehyde in 0.2 M L^{-1} phosphate buffers(pH 7.4)for 4 h at 4°C. After fixation and an overnight wash with sodium cacodylate buffer at 4°C, the specimens were postfixed with 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.4), dehydrated with ethanol and then embedded in Araldite resin and sections (1 μm) thick were cut.

Ultra thin sections (40-60nm thick) were placed on copper mesh grids (200 mesh) and stained with uranyl acetate and lead citrate. Sections were examined using a transmission electron microscope and photomicrographs were taken.

8.2.2.6. Scanning electron microscopy (SEM)

Liver tissues were fixed in Karnovsky's solution pH 7.4 for 4 h at 4°C. After fixation and an overnight wash in sodium cacodylate buffer at 4°C, the specimens were post fixed with 1% osmium tetroxide in 0.1M phosphate buffer (pH 7.4), dehydrated with ethanol

and coated with gold. The tissue surface was examined using a scanning electron microscopy (Leo, 435 VP, Cambridge, U.K.) and photomicrographs were taken.

8.2. Results

8.2.1. Histopathology

Liver histopathology was evaluated based on sinusoidal congestion, cytoplasmic vacuolization, hepatocellular necrosis, and neutrophil infiltration (Table.8.1, 8.2 and Figure 8.2). 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 & dilation. There was chronic inflammatory cells infiltrate in the portal tracks. There was also extensive hepatocellular necrosis, sinusoidal congestion, and neutrophil infiltration. Shrunken acidophilic hepatocytes were both round and detached from surrounding hepatocytes (round – Abs) and stellate – shaped firmly attached to adjacent hepatocytes (stellate – Abs). In 45% of round – Abs, the nucleus was fragmented or absent and among those with an intact nucleus, 80% had nuclear pyknosis Stellate - Abs fragmented or absence of nuclei in 4% and, among those with an intact nucleus, 24 % of nuclei were pyknotic. Stellate – Abs often clustered in acidophilic domain usually without significant lymphoid infiltrate or necrosis. Round- Abs were usually not clustered and seen more often with an adjust lymphocytes infiltrate. Apoptotic bodies, in the form of round or stellate – Abs, were seen.

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 (acidophilic domain) were often present in livers, especially in regions with stellate – ABs and in regions with atrophy. 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.

In amlodipine pretreated rat there were patchy spots of mild necrosis in various areas of the liver tissue observed at centrilobular region. Acidophilic domains were seen to be situated between portal tracts and the adjacent hepatic veins, though the cells were part of a single circulatory unit, e.g an acinus. Portal and / or hepatic veins were often obliterated and the outer perimeters of the domains were often concave, giving the impression that acidophilic domains in these livers were atrophic acini that had lost volume. This impression was confirmed by a semiquantative analysis as livers with acidophilic domains usually had atrophy within those domains. In the center of cirrhotic, nodules and some eoisinophilic nucleus with mild atrophy, sinusoidal dilation and rarely with additional findings of coagulative necrosis and apoptosis or sinusoidal fibrosis were observed.

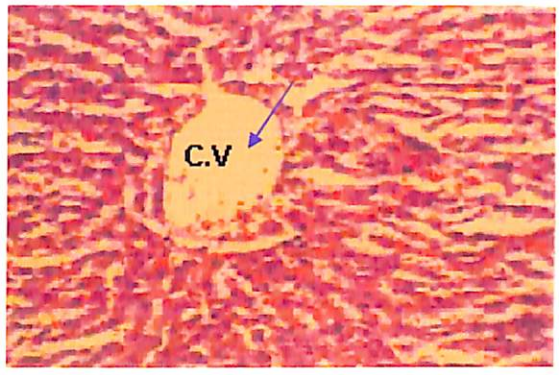
L- arginine pretreated rats showed less severe damage than those of the amlodipine and folic acid treated rats. Prominent nucleoli, kupffer cells, absence of apoptosis and mild necrosis were observed which were comparable to sham operated rats. L- arginine treated showed maximum protection from ischemic followed by reperfusion injury as compared to folic acid treated and amlodipine treaded rats. At the margins of infracts of centrilobular regions there were irregular tongues of atrophic hepatocytes admixed with normal parenchyma giving quite distinguishable appearances. Hepatocytes between nodules were small and usually slightly more eoisinophilic than the cells of the nodules. Portal tracts were usually found in the centers of nodules.

Table: 8.1. Shows histopathological changes in folic acid amlodipine and L-arginine treated I/R rats.

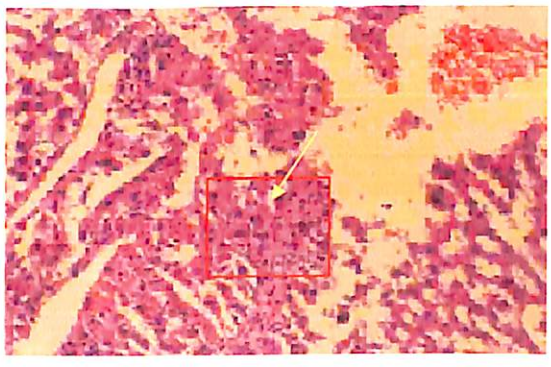
Histological Features	Groups									
	I (Sham Operated)		II (I/R rats)		III (Folic acid treated)		IV (Amlodipine Treated)		V (L-Arginine Treated)	
	Round AB	Stellate AB	Round-AB	Stellate AB	Round AB	Stellate AB	Round AB	Stellate AB	Round AB	Stellate AB
Nucleus fragmented or absent	7/105 (6%)	11/410 (2%)	63/121 (52%)	40/650 (6%)	40/120 (33%)	29/500 (5.8%)	47/168 (27%)	20/475 (4.2%)	24/170 (14%)	11/385 (2.8%)
Nucleus Pyknotic (intact Nucleus)	3/ 100 (3%)	10/200 (5%)	47/60 (78%)	170/600 (28%)	35/50 (70%)	145/620 (23%)	40/70 (57%)	150/750 (20%)	45/60 (75%)	107/400 (27%)
Nucleus Pyknotic, fragmented or absent	Not significant	2/100 (2%)	104/120 (87%)	210/525 (40%)	100/135 (74%)	190/500 (38%)	85/130 (65%)	175/550 (31%)	24/95 (25%)	150/400 (37%)
Adjacent inflammation present	5/ 104 (5%)	3/92 (9%)	107/143 (74%)	254/502 (50%)	56/109 (51%)	181/450 (40%)	80/170 (47%)	45/134 (33%)	61/187 (32%)	46/210 (21%)
Located in an acidophilic domain	3/105 (2.%)	5/140 (3%)	113/190 (59%)	105/180 (58%)	103/205 (50%)	74/160 (46%)	74/254 (29%)	40/140 (28%)	45/170 (26%)	47/200 (23%)
Nodular hyperplasia	4/100 (4%)	11/120 (9%)	125/140 (89%)	107/130 (82%)	100/180 (55%)	102/189 (53%)	45/135 (33%)	22/85 (25%)	30/ 151 (19%)	24/111 (21%)
Atrophy	2/87 (2%)	4/107 (3%)	117/153 (76%)	101/189 (53%)	74/102 (72%)	25/70 (35%)	54/91 (59%)	20/89 (22%)	12/70 (17%)	22/107 (20%)

Table: 8.2. Shows steatosis, inflammation and necrosis hepatocytes in folic acid amlodipine and L-arginine pretreated I/R rats.

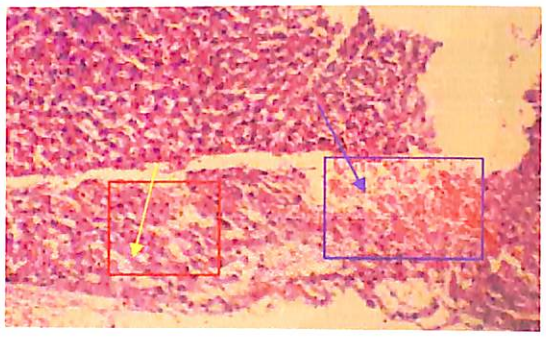
Histological Features		**Groups**				
		I (Sham Operated)	**II (I/R rats)**	**III (Amlodipine Treated)**	**IV (Folic acid Treated**	**V L-Arginine Treated**
Steatosis	**Scale** 1+	√				
	2+					√
	3+			√	√	
	4+		√			
Inflammation and necrosis	**Scale** 1+	√				
	2+			√	√	
	3+		√			



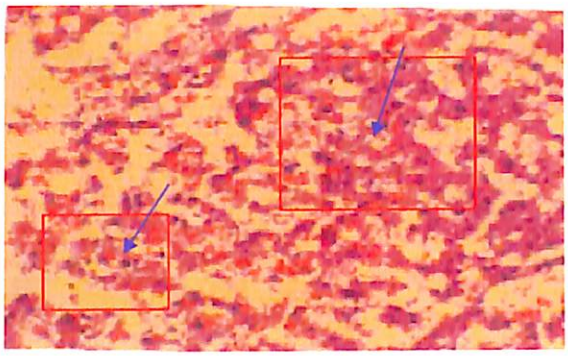
A: Sham operated control rats showing central vein (C.V) (H& E., x 400).



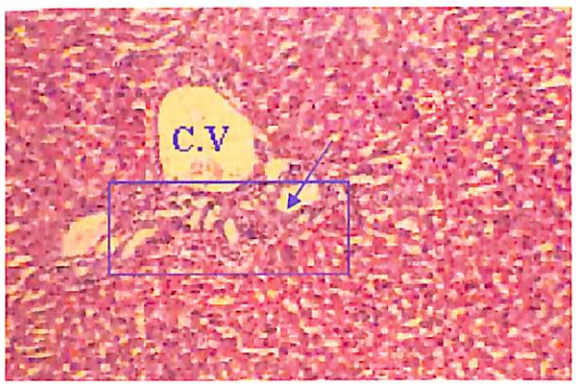
B: I/R rats showing hepatocytes with steatosis, inflammation and accumulation of erythrocytes in sinusoids (H & E., x 400).



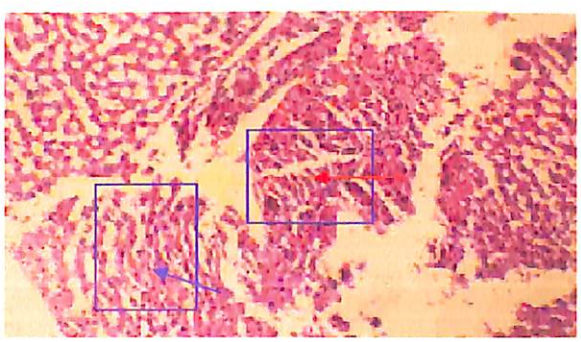
C: I/R rats showing pyknotic nucleus (yellow arrow), acidophilic domain and nodular hyperplasia (blue arrow) (H & E., x 400).



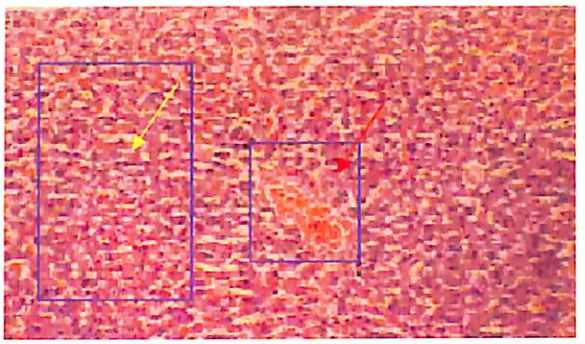
D: Folic acid treated I/R rats showing reduced acidophilic zone and necrosis. Pyknotic condensation and inflammation hepatocytes observed in pericentral area (H& E., x 400).



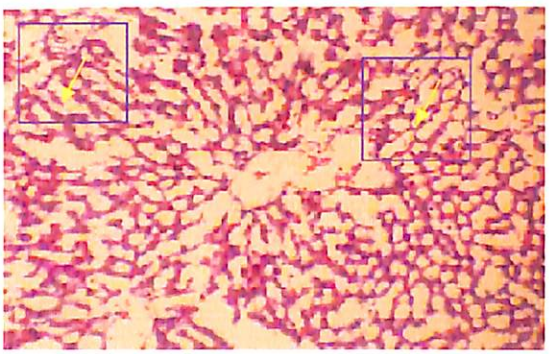
E: Folic acid treated I/R rats treated showing the centrilobular necrosis (Blue square) and dilated sinusoids (H& E., x 200).



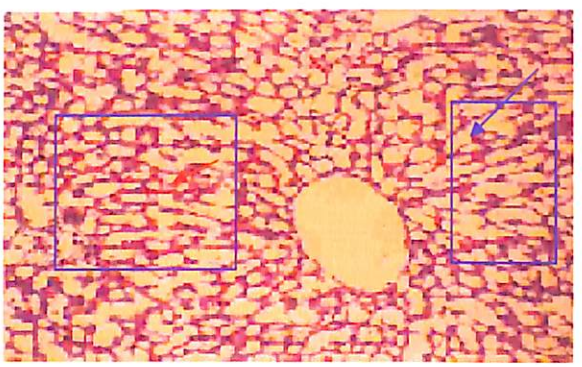
F: Amlodipine treated I/R rats showing acidophilic domain to adjacent hepatic vein (H& E., x 400).



G: Amlodipine treated I/R rats showing cirrhotic nodules, sinusoidal dilation, deposition of erythrocytes (red arrow) and coagulative necrosis (yellow arrow) (H& E., x 200).



H: L- arginine treated rats showing mild inflammation (yellow arrow), dilated centrilobular region (H& E., x 200).



I: L- arginine treated rats showing inflammation, small hepatocytes between nodules (blue arrow) and slightly more eoisinophilic (red arrow) than the cells of the nodules. Portal tracts were in the centers of nodules (H& E., x 200).

Figure: 8.2. (A-E). Histopathological changes of liver in sham operated control group and in treated groups.

8.3.2. Electron microscopy studies (Ultra structural changes)

In sham operated control rat, hepatocytes were found to have prominent nucleus and number of mitochondria. Other organelles such as rough and smooth endoplasmic reticulum, mitochondria and glycogen particle are well preserved. (Figure 8.3 . A to C).

After 1 h ischemia followed by 3 h reperfusion in I/R group, mitochondria were severely swollen and had reduction in the number of cristae. Smooth endoplasmic reticulum increased, glycogen granules decreased, nucleus was not well marked and more secondary lysosomes were observed. There was wide distribution of febrile material throughout the liver with the distended space of disse. Electron micrograph showed sinusoids containing fibrin aggregates and structures resembling de-granulated platelets appearing to block the lumen of the sinusoid. (Figure 8.3. D to F).

In folic acid treated group, hepatocytes were with prominent nucleus, some distended space disse which also contain fibrin and peripheral cytoplasm where golgi complex composed of parallel cisternae of smooth membrane associated with lysosomes and an autophagy vacuole containing a partially digested mitochondrion. (Figure 7.2. G to H).

In amlodipine treated group, hepatocytes were with prominent nucleus with an extended smooth endoplasmic reticulum, composed of numerous profiles of smooth tubules and a few α – glycogen particles dispersed within the network of the reticulum of numerous small mitochondria. (Figure 8.2 I to J).

In L- arginine treated Group V, hepatocytes were with prominent nucleus and sinusoids were open and enlarged. The endothelial cells, which line them, were partly loosened. The segments of cell surfaces, which normally remain in close contact, was dismantled and lie in continuity with the lumen of the sinusoids. Adhesion was maintained in numerous narrow segments (Figure 8.2. K to L).

8.3.3. Transmission Electron microscopy (Cell surface characteristics)

Apoptosis was evaluated based on some distinct morphological features on cell surface such as bulging of cell wall with numerous budding in tissue surface (apoptotic bodies). Formation of apoptotic bodies and budding was evident in ischemic followed by reperfusion injured rat as compared to all other groups (Figure. 7.3 C to E). In folic acid (Figure 7.3 F to G) and amlodipine treated group (Figure 7.3 H to I) the apoptotic body and budding formation in liver surface decreased as compared to I/R group. L- Arginine

treated group (Figure 7.3 J to K) showed maximum reduction of apoptotic plug and apoptotic bodies as compared to all other treated group.

Increasing orders of apoptosis appeared in following order:

Group I (Sham operated rats) >Group V (L- arginine treated) >Group IV (Amlodipine treated) >Group III (Folic acid treated > Group II (I/R Rats).

8.4. Discussion

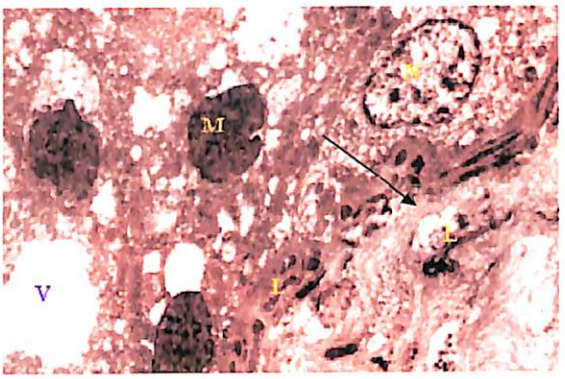
Apoptosis of hepatocytes and sinusoidal endothelial cells are probable cause of sinusoids congestion contributing to hepatic I/R injury, which causes hepatic failure (Ericsson, 1969; Van As et al., 2002). The pathogenesis of I/R injury are closely related to hepatocyte necrosis as well as apoptosis. In I/R injury of liver, shrunken necrosis, apoptosis corpuscular and piece-meal necrosis were the characteristic histological manifestation of apoptotic hepatocytes (Jaeschke and Lemasters, 2003).

The present study showed that reperfusion of ischemic liver caused hepato- cellular necrosis and sinusoidal congestion; after 1 h ischemia followed by 3h reperfusion in vehicle treated I/R rats were characterized by several pyknotic condensation of the periportal area with progressive injury in midzolam and pericentral area, round apoptotic bodies and stellate AB's apoptotic bodies associated with cytoplasmic nuclear degeneration besides stellate AB's approximately 100 times more were in I/R rats as compared to sham operated control rat. (Table 8.1).

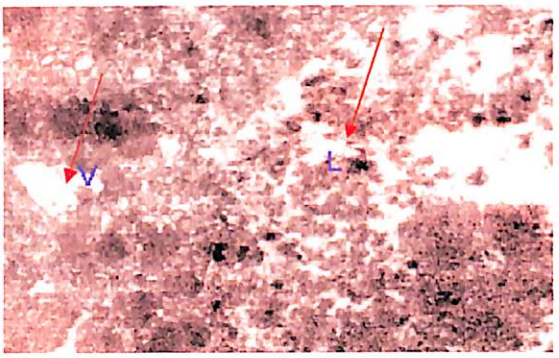
Normal ultra structure by TEM studies were seen in the sham operated controlled group whereas after 1 h ischemia followed by 3 h reperfusion, mitochondria of hepatocytes were severely swollen, glycogen granules and number of cristae were reduced, smooth endoplasmic reticulum increased & nucleus were not well marked as well as more secondary lysosomes were observed in I/R group.

Further cell surface of hepatocytes were ruffled, blebbed and condensed corresponding to cell apoptosis which may be due to deprivation of oxygen in ischemic phase besides parenchymal border lines were destroyed by accumulation of free radicals and mitochondria were digested by activation of Lysomes (Figure 8.2. D and E).

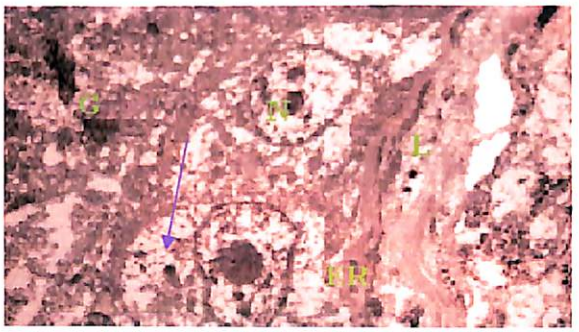
In the present study after administration of L- arginine – rats show only minor patchy spots of mild necrosis in various areas of liver tissue with dilated sinusoids and dilated central



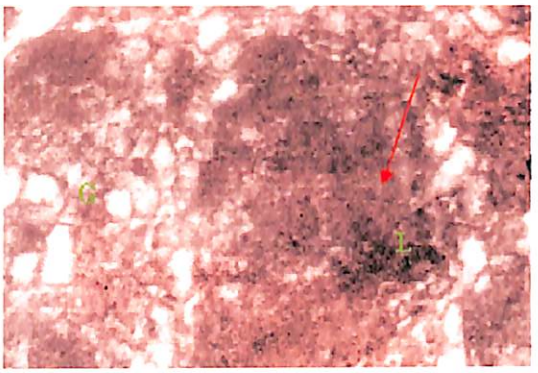
A: Sham operated control rats showing the normal architecture of hepatocytes mitochondria (M); glycogen (g); nucleus (N), golgi complex (G); lysosomes (L); (x 7000).



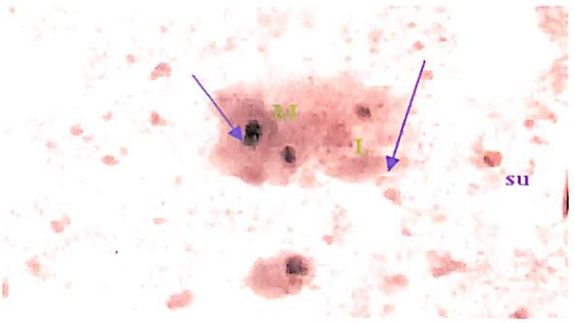
B: Sham operated control rats showing non – autolytic lysomes (L) and large vacuoles (V) in hepatocytes (x 10,000).



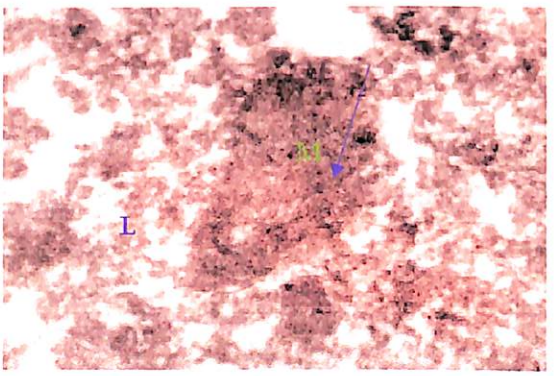
C: Sham operated control rats showing short segment of smooth cisterina of the endoplasmic reticulum (ER) (x 10,000).



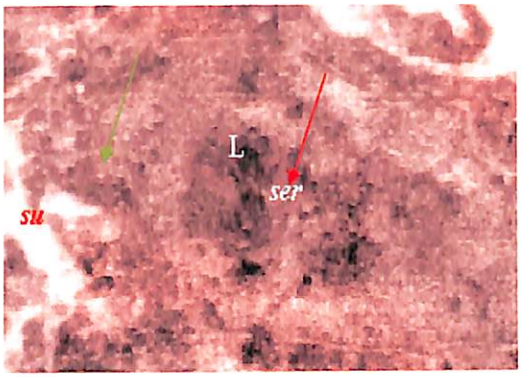
D: I/R rats showing lysomes with digested mitochondria (red arrow). Lysomes in auto- phagocytes process to mitochondria destroyed Glycogen particles of endoplasmic reticulum. (x 12,000).



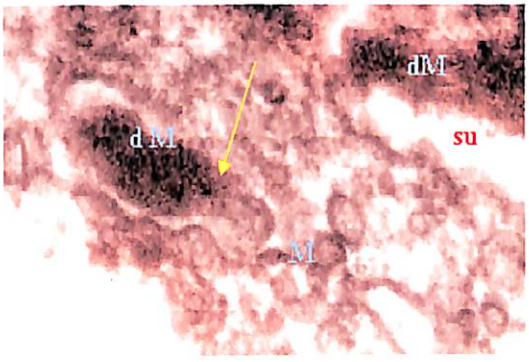
E: I/R rats showing digested mitochondrion (M) lysomes (L) (blue arrow). Wide open and enlarged sinusoids (sn) (x 8,000).



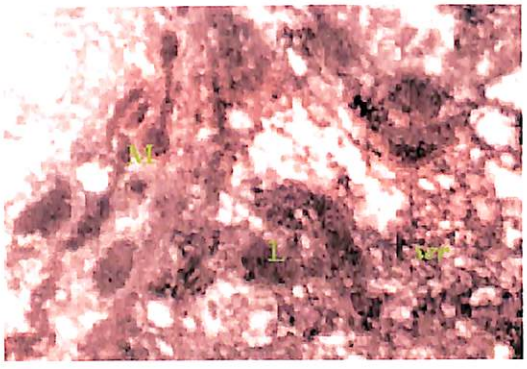
F: I/R rats showing severe degeneration of normal architecture of hepatocytes. Diffuse out Lysomes (L) and were severely swelled mitochondria (M). (x 8,000).



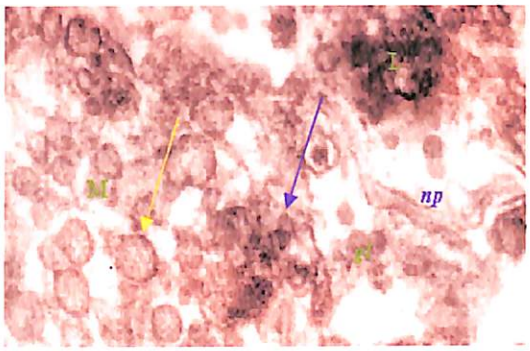
G: Folic acid treated rats showing extended smooth endoplasmic reticulum (ER) (green arrow) composed of numerous profiles of smooth tubules (*ser*) (red arrow). Decreased sinusoids dilation as compared to I/R rats (x 8000).



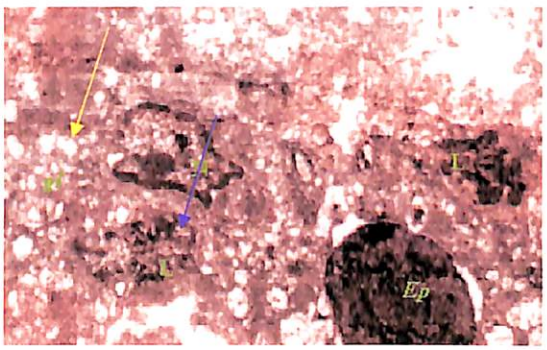
H: Folic acid treated rats showing few mitochondria ere partially digested by lysomes (*d*M) (yellow arrow). Increased mitochondrion was as compared to I/R rats (x 12,000).



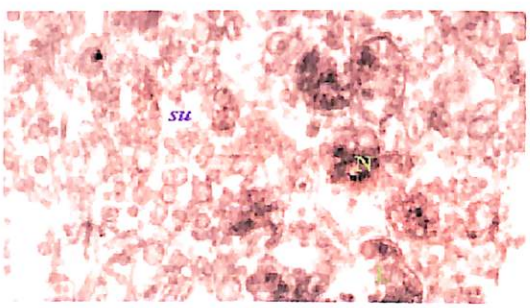
I: Amlodipine treated rats showing secondary lysosomes (L) but no autophagy mitochondria. Numerous smooth tubules (ser) (x 10,000).



J: Amlodipine treated rats showing opens nuclear pores (np)(red arrow), increased number of glycogen (gl) particles and well-defined mitochondria. Mitochondria well preserved with no swelling (yellow arrow). Lysomes (L) (blue arrow) in phagocytes phase.

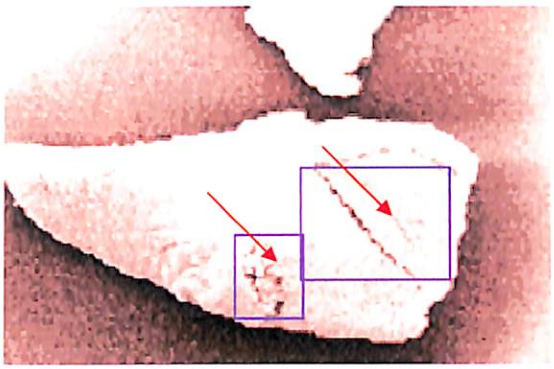


K: L- arginine treated rats showing marked ergatoplasm (mixed with mitochondria and glycogen) (Ep) (red arrow). Increased glycogens (gl,) (yellow marked) content. Autophagy lysomes (L) (blue arrow) present behind of nucleus (N) (x 8,000).

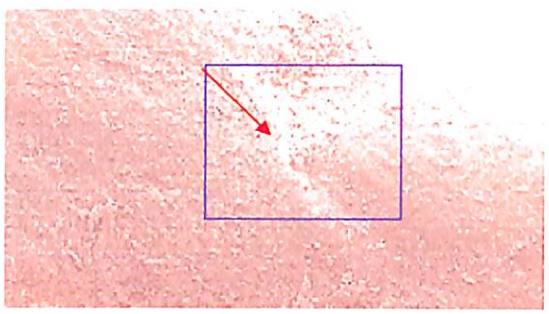


L: L- arginine treated rats showing constricted sinusoids (*su*) and well-marked nucleus (x 8, 000).

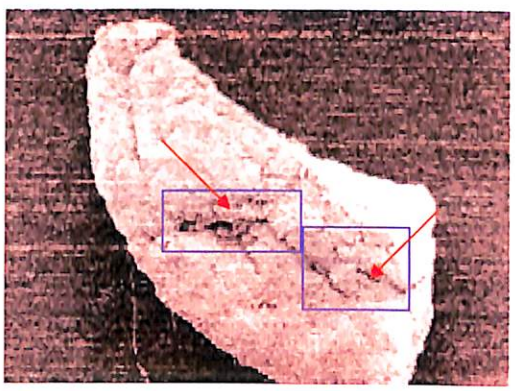
Figure: 8.3. (A-L). TEM photomicrograph of ultra structure of hepatocytes of folic acid, amlodipine and L- arginine treated I/R.



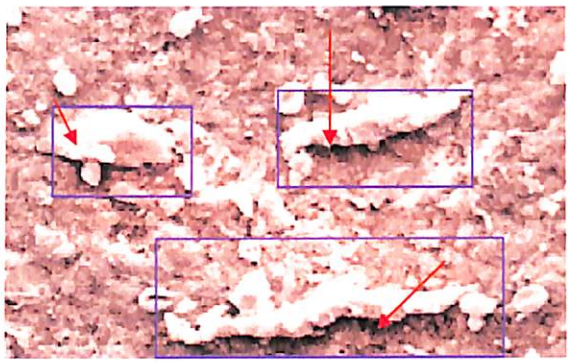
A: Sham operated control rats showing the fissure on liver surface (x 400)



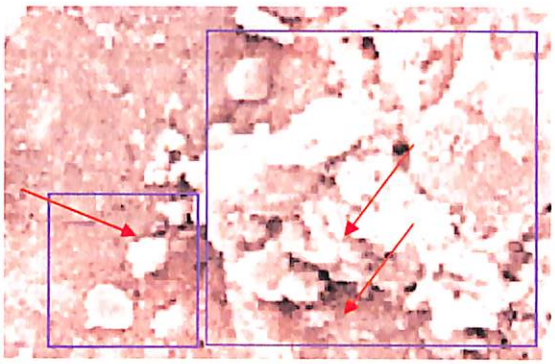
B: Sham operated control rats showing uniform texture on liver surface (x 6000).



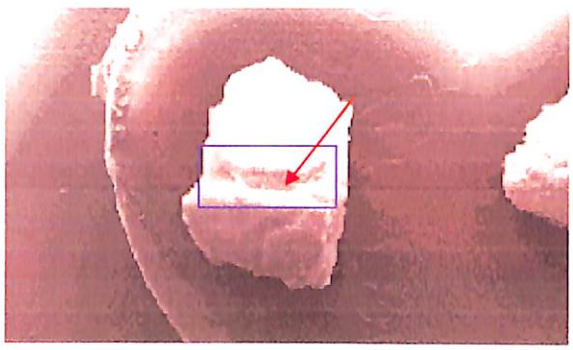
C: I/R rats showing bulging of cell surface and initiated apoptotic bodies (x 400).



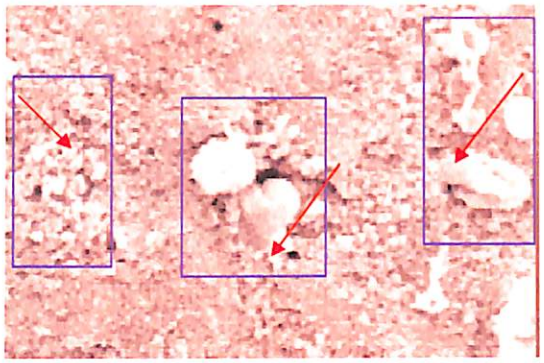
D: I/R rats showing bulging of cell surface and apoptotic bodies (x 6000).



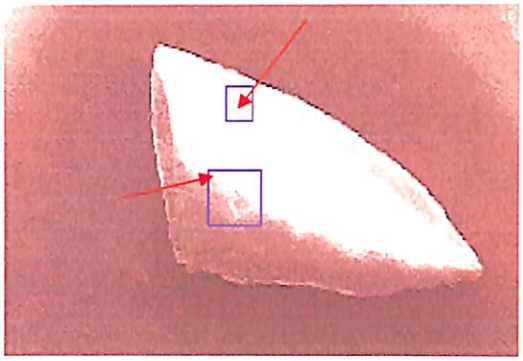
E: I/R rats showing bulging of cell surface and apoptotic bodies at the site of fissure of liver (x 6000).



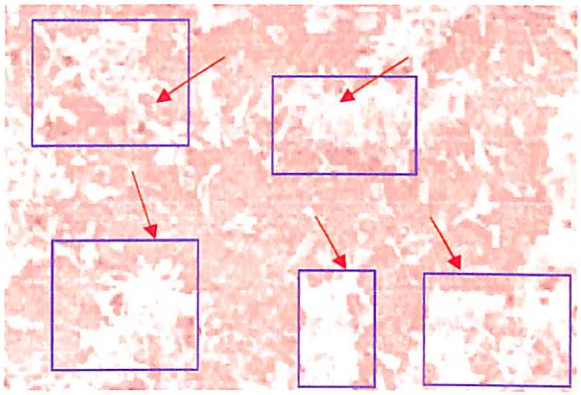
F: Folic acid treated rats showing reduced apoptotic bodies formation at the fissures of liver (x 400).



G: Folic acid treated rats showing distribution apoptotic bodies from fissures of liver and reduced apoptotic bodies formation (x 6000).



H: Amlodipine treated rats showing fissures of liver and reduced surface of belbing lines (x 400).



K: L- arginine treated rats showing distribution of apoptotic bodies form fissures of liver and reduced apoptotic bodies formation (x 6000).

Figure: 8.4: (A- K). Scanning electron Photomicrograph showing formation of apoptotic bodies and apoptotic plug in liver surface after folic acid, amlodipine, L- arginine treatment in I/R rats.

hepatic vein with atrophy and hemorrhage at ischemic and reperfusion site as compared to I/R group which indicated that L- arginine protected the severe degenerative changes of I/R induced hepatocytes injury (Table 8.1 and 8.2 & Figure 8.2 H and I).

TEM studies of L- arginine treated group showed prominent nucleus and mitochondria whereas I/R group showed auto-phagosomes, reduction in the number mitochondria and nuclear condensation indicating that L- arginine significantly protects degenerative histological changes caused by I/R injury (Figure 8.2 K and L). The protective action of L-arginine can be explained as L- arginine is precursor of NO and during ischemia L-arginine restored vasodilatatory effects by generation of NO whereas I/R rats deprived of NO resulted in vasoconstriction leading to ischemic injury.

Earlier reports suggested that during I/R injury Ca^{+2} deposited in mitochondria aggravates the liver injury (Wang et al., 1994). Therefore, Ca^{+2} could be one of the causative agents, which may lead to ischemic injury as well Ca^{2+}channel antagonist, verampril, is reported to be play protective role in hepatic I/R induced injury by restoring microscopical changes caused by I/R injury (Chavez – Cartaya et al., 1996). Therefore, amlodipine a long acting and potent Ca^{+2} antagonist effect of which on hepatic I/R injury are not reported was selected for the study.

The present studies showed that, after 1 h ischemia and 3 h reperfusion, there is accumulation of Ca^{2+} which leads to auto-phagosomes, reduction in the number mitochondria and nuclear condensation (Figure 8.2. A and B) and pre- treatment of rats with amlodipine showed considerable prevention in the ultra structural alteration including disruption of mitochondrial and nuclear fine structures which were characterized by prominent nucleus with an extended smooth endoplasmic reticulum composed of numerous profiles of smooth tubules and a few α – glycogen particles dispersed in the network of the numerous small mitochondria reticulum (Figure 8.2 I and J). Besides, amlodipine reduced nodular hyperplasia, atrophy and karyomegalic nucleus as compared to I/R rats (Figure 8.2 F and G). Both Ca^{2+} accumulation and oxidative stress increase the probability of changes in the mitochondrial permeability together with the opening of the transition pore in mitochondria membrane, which carry the signals for cell death (Siesjo and Bengtsson, 1989). Amlodipine prevents the cellular damage in the membrane by inhibiting calcium channel of hepatocytes. Previous study reported that amlodipine reduced hepatic I/R injury by cytoprotective

effects on parenchymal and non – parenchymal hepatocytes during both preservation and reperfusion (Piratvisuth and Dunne, 1995).

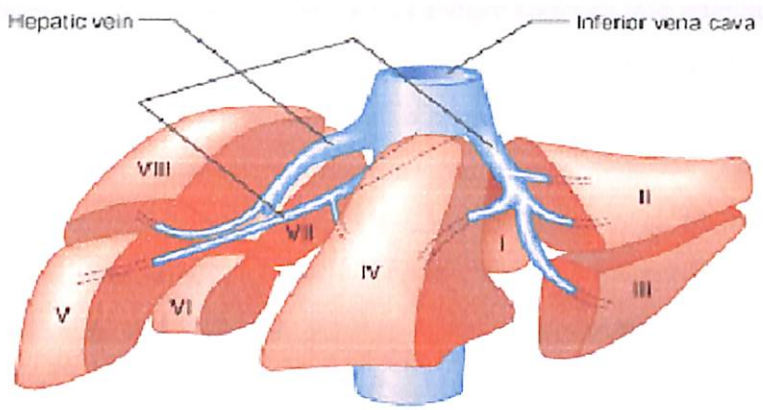


Figure: 8.5. Portal segmentation and circulation in Liver.

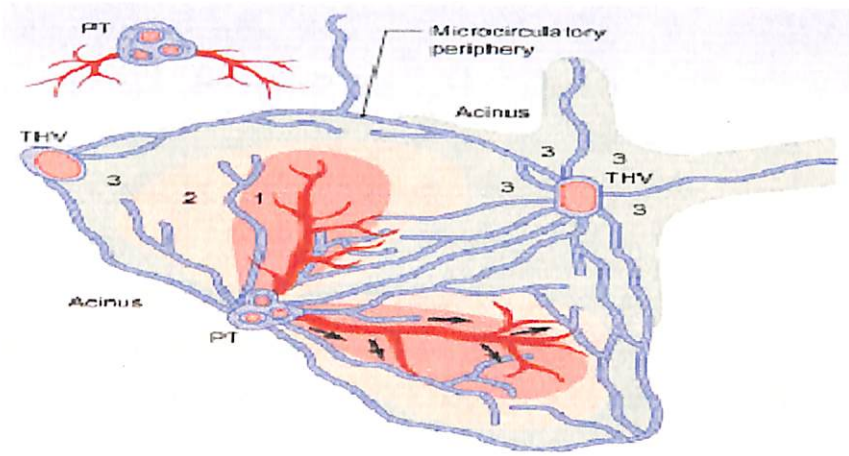


Figure: 8.6. Branches and sub branches of portal vein in hepatic circulation.

Thus, findings in the present studies and earlier work indicate that by inhibiting Ca^{+2} accumulation and prevention of mitochondrial permeability pore amlodipine protects from mitochondrial injury in I/R rats, which gave insight into the changes of cellular level by amlodipine. The role of folic acid which has not been studied sofar showed that its administration in I/R rats attenuated ultastructural changes characterized by minimal disruption in cristae, apoptotic plug and collagen integrity (Figure 8.2 D and E; Figure 8.3. F and G) and also decreased pyknotic nucleus, acidophilic domain, nodular

hyperplasia, steatosis and necrotic cells (Figure 8. 2. D and E) as compared to I/R rats as well as folic acid treated group showed only some focal and slight dilatations in rERs (ribosomal endoplasmic reticulum) and sERs (secondary endoplasmic reticulum) whereas I/R groups showed serious damage viz. irregular lamellar organization, large dilatations visualizations focal breaks and myelin in sERs in many areas (Figure 8.2 G and H). Thus folic acid attenuated histopathological and ultra- structural changes as compared to I/R rats which may be explained on the basis of finding of Blount et al, in 1997 that folate plays a critical role in the *de novo* synthesis of purines and thymidine and influences the cell cycle, DNA stability, and apoptosis in I/R injury by microscopical changes.

8.5. Conclusion

The present studies showed that: -

1. Ca^{2+}homeostasis and cellular changes during I/R. Ca^{2+} homeostasis are regulated by amlodipine. Amlodipine not only antagonizes mitochondrial Ca^{2+} entry but also prevents apoptosis and reduction of ER. However, still molecular mechanisms need conclusive evidence that the Ca^{2+} signaling pathway is utilized by a key endogenous regulator, Bcl-2. The present study provides clue for potential molecular approaches of ischemia followed by reperfusion pathophysiological events.
2. The protective effects of L- arginine against I/R injury on rat hepatocyte might be due to the production of NO and by increased vasodilatation.
3. Folic acid improved the degree of hepatocellular structure and apoptotic cells by regulating salvage pathway of DNA synthesis.

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Chapter - 9

Conclusion

9.0. Conclusion

The present Ph .D work revealed that: -

- Pre - treatment with folic acid prevented ultra structural alteration including disruption of mitochondrial and nuclear fine structure, which indicated that administration of folic acid inhibits DNA damage thereby preventing the cellular damage in the membrane. Further, after 1 h ischemia followed by 3h reperfusion, folic acid reduced TNF- α activation and increased DNA synthesis.

- Protective action of amlodipine, a calcium channel blocker, against the I/R induced liver injury in rats is due to its inhibitory action of Ca^{2+} influx into mitochondria and also probably due to inhibitory action on Cytochrome c and up-regulation of mitochondrial enzymes.

- The enhanced production of NO through administration of L-arginine reduces oxygen free radicals, increases the mitochondrial antioxidants and respiratory marker enzymes, and causes vasodilatation. Subsequently, it decreases the amount of lipid peroxidation by removing substrate for free radical generation by physiological mechanisms. Besides, L –arginine acid protects hepatobiliary function and ultra structure of liver in hepatic ischemic and reperfused induced liver injury.

Chapter - 10

Future Scope of the Work

10.0. Future Scope of Work

The cellular and biochemical data recorded in the present studies suggest four important aspects for successful hepato-protection, (a) NO in hepato-protection by vasodilatory action into hepatic sinusoids, (b) folic acid in the hepato-protection by inhibiting TNF –α and protection from DNA damage, (c) Protection by amlodipine from hepatic injury by inhibiting Ca^{+2} entry in hepatocytes, and (d) all drugs used inhibit ROS-production.

The following would be explored in future research: -

1. Mechanism of folic acid in inhibition of TNFR-I activation.
2. Mechanism of NO production by L- arginine in *invivo* and mechanism involved generation of NOO^{-}.
3. Mechanism of antioxidant entry into the target cells through blood supply.

Appendices

Buffer and Reagents

Sl. No.	Description	Quantity
1.	**Composition of SDS PAGE Gel**	
	Acrylamide	29.2 g
	Bisacrylamide	0.8 g
	Distilled Water q.s	100 ml
2.	**Separating gel buffer**	
	Tris –HCl buffer	1.5 ML^{-1}
	SDS	0.4 %
3.	**Electrophoresis reservoir buffer**	
	Tris–HCl buffer	0.05 ML^{-1}
	Gycine	0.192 ML^{-1}
4.	**Recipe for SDS PAGE Separating (12.5%) Gel**	
	Acrylamide- bisacrylamide Solution	6.25 ml
	Separating gel buffer	3.75 ml
	Distilled water	5.0 ml
	10% APS	50 µl
	TEMED	10 µl
5.	**Recipe for SDS PAGE Stacking (5%) Gel**	
	Acrylamide- bisacrylamide Solution	1.25 ml
	Stacking gel buffer	1.875 ml
	Distilled water	4.375 ml
	10% APS	25 µl
	TEMED	5 µl

Sl. No.	Description	Quantity
6.	**Staining Solution**	
	Commassie blue	0.1 %
	Methanol	60 ml
	Acetic Acid	10 ml
	Water	30 ml
7.	**Destaining Solution**	
	Methanol	30 Part
	Acetic Acid	60 Part
	Water	10 Part
8.	**5 X buffer**	
	Glycine	144 g
	Tris Base	30 g
	SDS 10%	50 ml
	Distilled water q.s	100 ml
9.	**Reagents used for cDNA**	
	RNase inhibitor	1 µl
	Dithiothreitol	0.1 ML^{-1}
	RT buffer (5X)	4.0 µl
	dNTP (30mML-1)	2.0 µl
	M-MuLV reverse Transcriptase	0.5 µl
	Sterile water	1 µl
10.	**Reagents used for PCR amplification**	
	cDNA	2 µl
	PCR Buffer (10x)	6 µl
	dNTP (30mML^{-1})	1 µl
	Forward Primer (100ng μl^{-1})	1 µl
	Reverse Primer (100ng)	1 µl
	Taq DNA polymerase	1Un

Sl. No.	Description	Quantity
11.	**TAE (10x Solution)** pH (8.0)	
	Tris base	48.8 g
	Glacial Acetic Acid	11.4 ml
	EDTA	0.5 M
	Water	1000 ml
12.	**HEPES (1M)**	
	HEPES	4.76 g
	Distilled water	20 ml
13.	**1 (M)Tris**	
	Tris	121.1 g
	Water	800 ml
	Adjusted the pH to the desired value by adding concentrated HCl	
	pH **HCl**	
	7.4 70 ml	
	7.5 60 ml	
	8.2 42 ml	
14.	**Deosyribonucelase triphosphate (dNTPs)**	
	dNTP	100 mML^{-1}.
	Tris base (pH 7.0)	0.05 M L^{-1}
15.	**Reagents for DNA isolation**	
a.	Digestion Buffer	
	Tris- Cl buffer (pH 8.0)	10 mM L^{-1}
	EDTA	25 mML^{-1}
	SDS	0.5%
	proteinase K	0.1mg ml^{-1}
b.	Phenol: Chloroform: Iso-amylalcohol	25:24:1
	TE Buffer	
c.	Tris buffer (pH 8.0)	10 mM
	EDTA	1 mM

Sl No	Description	Quantity
d.	Phosphate Buffer Saline (PBS)	
	$NaHPO_4$	1.15 g
	KH_2PO_4	0.20 g
	KCl	0.20 g
	NaCl	8.0 g
	Distilled water	1000 ml
	pH was adjusted to 7.2 before making final volume	
16.	**Reagents used for submarine agarose gel electrophoresis**	
a.	TAE running buffer	
	Tris buffer pH 7.8	40 mM L^{-1}
	Sodium acetate	5 mM L^{-1}
	EDTA	1 mML^{-1}
b.	6 x Gel loading buffer	
	Sucrose	2.0 g
	Bromophenol blue	12.50 g
	EDTA	1.86 g
	Distilled Water	5.0 ml
17.	**Composition of RPMI Cell Culture medium**	
	RPMI Powder	10.4 g
	HEPES (acidic)	5.36 g
	Na HCO_3	1.68 g
	Gentamicin	100 mg
	0.05ML^{-1} β Mercaptoethanol	1.0 ml
	Distilled water	1000 ml
18.	**Reagents used for DNA estimation**	
	Diphenyl amine	1.5 g
	Conc. H_2SO_4acid.	1.5 ml
	Glacial acetic acid	100 ml

Sl No	Description	Quantity
19.	**Reagents used for Western Blot**	
a.	Electrode Buffer	
	Tris –HCl (pH 7.4)	20mM
	Glycine	150mM
	Methanol	20% v/v
	Distilled Water	100ml
b.	Buffer A	
	Tris –HCl (pH 7.4)	$10mML^{-1}$
	NaCl	$150\ mML^{-1}$
	Distilled water q.s to	100 ml
c.	Buffer B	
	Bovine Serum albumin	0.05%
	Egg albumin	5%
	Buffer A q.s to	100 ml
d.	Buffer C	
	Nonident P-450	0.05%
	Buffer A q.s to	100 ml
20.	**Reagents used for TUNEL assay**	
	Wash buffer	
	Tris – HCl (pH 8.3)	$100mML^{-1}$
	KCl	$50mML^{-1}$
	$MgCl_2$	$15mML^{-1}$
21.	**Lysis Buffer**	
	Tris – HCl (pH 7.4)	100mM
	SDS 10%	10 ml
	Water q.s to	100 ml

Sl No	Description	Quantity
22.	**MOPS Buffer (10X)**	
	MOPS	41.8g
	Sod. Acetate	6.8g
	EDTA 0.5 M	20 ml
	Triple distilled water q.s to	1L

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1. Pronobesh Chattopadhyay, Nevneet Verma, Anurag Verma, Tirath Kamboj, Nazam Ali Khan and Arun Kumar Wahi (2008). L- Arginine protects from Pringle Manoeuvere of Ischemia-reperfusion induced liver injury. Biol. Pharm. Bull., 31(5): 234- 241.
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Paper Presentations

1. P. Chattopadhyay, G. Shukla , P. Chaudhuri , A.K. Sharma, A.K Wahi (2007). Ultra structural analysis of Mitochondrial and nuclear changes and Bcl-2 gene expression of the hepatocytes during Pringle Manoeuvere of Ischemia-reperfusion in Liver transplantation model and Role of Nitric Oxide (NO) donor L-arginine. Abstract, 59 th Indian Pharmaceutical Congress, Varanasi.Pp 309.

2. P. Chattopadhyay F. Anowar, G. Shukla, P. Chaudhary, A.K. Sharma, A.K. Wahi (2007). Effect of third generation calcium channel blocker (CCB) in B-cell lymphoma- 2 family gene mediated programmed cell death in ischemic and reperfusion Liver during liver transplantation. Abstract 59th Indian Pharmaceutical Congress, Varanasi.Pp 448.

3. P. Chattopadhyay, A.K. Wahi (2006). Effect of L-argine in Protection of Liver damage during Liver transplantation and role of Antioxidant enzymes in Ischemic and reperfusion injury to rat liner and Apoptosis' Abstract, 11 th APTI National Conferences, Bangalore (PL: 11) Pp: 21

4. P. Chattopadhya, D.C.P. Singh, A.K.Wahi (2006). Antisence Bcl-2 oligonucleotides blocks liver damage during rat liver Transplantation in ischemia and reperfusion injury of rat liver Abstract, National Seminar sponsored by APTI. IPER Wardha (PH: 6). Pp6.

Brief Biography of Prof. Arun Kumar Wahi

Prof. Arun Kumar Wahi has obtained his B.Pharm., M.Pharm and PhD. degrees from Banaras Hindu University. He has 46 years of teaching and research experience. He retired as Professor from Department of Pharmaceutics, Institute of Technology, Banaras Hindu University, Varanasi and joined as Director, College of Pharmacy, IFTM, Moradabad where, at present, he is Dean, College of Pharmacy. Prof. Wahi has handled several research projects funded by different funding agencies. He has guided over 7 PhD theses and 5 candidates are presently working with him towards PhD. He has published 78 research articles in National and International journals and presented over 23 papers in various National and International conferences.

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Mr. Pronobesh Chattopadhyay did M.Tech in Bioengineering and Biotechnology from Jadavpur University and M. Pharm from Delhi Institute of Pharmaceutical Sciences and Research, Delhi University. He is recipient of All India Council of Technical Education research promotion grant, best research paper award from Kobe Gakuin University, Japan and best poster award in Wardha in National Conference sponsored by Association of Pharmacy Teachers of India. Chattopadhyay's active interest is in molecular and cellular pharmacology. At present he is working as Assistant Professor in College of Pharmacy, IFTM, Moradabad. He has, to his credit, one patent, 22 research papers in International and National journals and 13 paper presentations in various National and International conferences.