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Mechanisms of Hepatocellular Dysfunction and Regeneration: Enzyme Inhibition by Nitroimidazole and Human Liver Regeneration

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1. Introduction

Enzymes so called (Enz'-a-ai-am) are biologically active protein molecules responsible of biochemical reactions in the bacteria, cells and organs. Enzymes regulate the rates of biochemical reactions to maintain the metabolism to keep active physiological actions in the body. High enzyme activities or high rates of reactions cause higher product formation or deposits to initiate disease. Drugs are used as enzyme competitors to normalize the reactions to correct disease. Most of diseases are cured by 'enzyme inhibition'. Enzyme inhibition can be three types: competitive, non-competitive, and uncompetitive. Enzyme inhibition also provides a kind of defense in cells by regulation of metabolism to inhibit or stimulate the biochemical processes. Most of the drugs undergo detoxification and biotransformation in liver to regenerate or formation of new hepatocytes and Kupffer cells. Major biochemical events in liver regeneration are regulated by enzymes in energy metabolism, growth factors and cytokine molecules.

Present chapter describes an example of liver cell enzyme battery to regulate the carbohydrate, lipid and protein metabolism in liver cells, mainly hepatocytes and Kupffer cells in the light of liver damage due to amoebic liver abscess and role of enzyme inhibition in liver regeneration by 2'-nitroimidazole. Liver damage by amoeba is manifested by elevated enzymes in cells. As a result, two major clinical manifestations of *Entamoeba histolytica* infection are amoebic colitis and amoebic liver abscesses. To cure amoebic liver abscess, liver regeneration and amoebic killing by 2'-nitroimidazole therapy is routine in clinical practice. 2'-nitroimidazole acts in liver to perform enzyme inhibition at the level of carbohydrate, lipid, and protein metabolic regulatory steps. Earlier, nitroimidazole derivatives were considered drug of choice in treatment of hepatic hypoxia (low oxygen) conditions in parasitic infections, cancer and recently nitroimidazole derivatives are emerging as hypoxia markers and radiosensitizers in tumor treatment [Sharma 2001, Sharma 2011a, 2011b].

Since 1960, hepatocytes were investigated rich in major enzymes regulating the energy metabolism located in cytoplasm and mitochondria for glycolysis, TCA cycle while drug

metabolizing and redox enzymes are located in lysosomes and microsomes. Other enzymes also participate in defense processes such as respiratory burst, HMP shunt, oxygenase pathways and inflammatory cytokines. Present chapter reviews the ongoing developments with clear and complete information on action of new nitroimidazole derivatives as 'enzyme inhibitors' in liver selective cytotoxicity, oxygen depletion, hepatocellular DNA and enzyme normalization before enzyme can be used in hypoxia monitoring and therapy [Sharma, 2011b]. The paucity of information on nitroimidazole-liver tissue interaction is poor and available data of initial sequential biochemical changes in liver cells is scanty which further leads to detectable hypoxia [Sharma 2011a, 2011b]. It is believed that initially enzyme regulated glucose and calcium hemostasis are the primary targets of hepatic hypoxia followed by enzyme regulated induced metabolic integrity loss leads to apoptosis, regulatory failure in glycolytic, TCA cycle, gluconeogenesis and Ca^{++} mediated cAMP related biodegradation of molecules [Sharma et al.2011c].

The present chapter focuses on hepatic enzyme inhibition by nitroimidazole at different levels of energy metabolism, respiratory burst and drug metabolizing enzymes. In following section, a molecular basis of enzyme inhibition and hepatocellular hypoxia and dysfunction criteria is described in detail. In following section, regulatory behavior of rate limiting enzymes of glycolysis, TCA cycle, phagocytosis is described with examples: glucokinase, phosphofructokinase, pyruvate kinase, lactate dehydrogenase, NADPH cytochrome P450 reductase, phosphodieaterase, lysosomal enzymes in serum, liver biopsy samples. In next section, role of enzymes in liver abscess is described.

1.1 Enzymes in liver abscess

Entamoeba histolytica is a human-specific pathogen and reproducible animal models of intestinal amoebiasis have proved elusive to describe interaction between *E. histolytica* and liver cells. The most common extraintestinal manifestation of disease is amoebic liver-abscess. Amoebic liver-abscesses arise from haematogenous spread (probably via the portal circulation) of amoebic trophozoites that have breached the colonic mucosa.[Thompson et al. 1985, Abuabara et al. 1982, Shandera et al. 1998, Barnes et al. 1987, Adams 1977, Lancet 2003]. Effective nitroimidazole treatment and rapid diagnosis showed the mortality rates to 1–3%. *Entamoeba histolytica* trophozoites can lyse neutrophils *in vitro*, causing them to release toxic substance killer enzymes such as superoxide dismutases, collagenases, elastases and cathepsins [Jarumilinta et al. 1964, Guerrant et al. 1981]. On these lines, author proposed a 'Hepatocellular dysfunction criteria' to make systematic observations in abscess development and step by step method of medical/surgical intervention [Sharma et al.2011]. In previous reports, patient symptoms, leucocytosis without eosinophilia, mild anaemia, raised concentrations of alkaline phosphatase, and a high rate of erythrocyte sedimentation were the most common laboratory findings.[Thompson et al. 1985, Abuabara et al. 1982, Shandera et al. 1998, Barnes et al. 1987, Adams 1977, Lancet 2003].

Amoeba cause amoebic liver abscesses, which are circumscribed regions of dead hepatocytes, liquefied cells and cellular debris surrounded by a rim of connective tissue, some inflammatory cells and few amoebic trophozoites [Sharma et al. 2011]. The adjacent liver parenchyma is usually completely normal. At same sites in liver so called 'acini' fixed sinusoidal phagocytic cells (Kupffer cells) provide defense against any bacteria, virus or foreign drug. Kupffer cells have two intracellular structures filled with enzymes: lysosomes (Lai-zo-somes) and microsomes (Mai-kro-zomes). Lysososmal bags filled with a large

number of lysosomal enzymes acting as destroyers actually lyse and digest the bacteria, virus and toxicants. Microsomal enzymes are active in detoxification of drugs or drug biotransformaton to interact (stimulation or inhibition of enzymes) with metabolic regulation in liver cells. Let us introduce a bit of enzymes in liver cells performing different metabolic regulatory functions in following description. Abscesses occupying large areas of the liver can be cured without drainage, and even by one single dose of nitroimidazole.[Powell et al. 1969, Lasserre et al. 1983, Akgun et al. 1999]. Today, role of ultrasound or percutaneous therapeutic aspiration guided by CT in the treatment of uncomplicated amoebic liver-abscess by surgical drainage is controversial.

Enzyme	Amoeba	Hepatocyte	Kupffer cell
Glucose 6 Pase	++++	++	+
Glucose 6 PDH	++++	++	+
Phoshogluconate DH	+++	+	+
Phosphofructokinase	+++	++	+
Aldolase	++	++	+
Pyruvate kinase	++	+	+
Pyruvate DH	++	++	+
LDH	+	++	+++
Citrate Synthase	++++	+	++
Isocitrate DH	+++	++	++
Succinate DH	++	+	+
Malate DH	+++	++	++
Cytochrome C Oxidase	+	++	++
NADPH Cyt C Reducatase	+	+++	++
NADH Oxidase	+	++	++
Tyrosine Aminotransferase	++	+	++++
Aniline Hydroxylase	++	+	++++
Aminopyrine demethylase	++	+	++++
Acid DNAase	++	+	++++
Glutathione Reductase	++	+	++++
Peroxidase	+++	+	++++
Catalase	++	+	++++
Superoxide dismutase	++	+	++++
Guanase	++	++	+++
Adinosine Deaminase	+	+	+++
Leucine aminopeptidase	++	+	++++
Ca++ ATPase	++	+	++++
5′ Nucleotidase	++	+	++++
Acid Lipase	++	+	++++
Acid Phosphatase	++	+	+++++
Alkaline Phosphatase	+++	+	+++++
Beta glucuronidase	++	++	+++

Source: Sharma R. Effect of Nitroimidazole on isolated liver cells in development of amoebic liver abscess. Ph.D dissertation submitted to Indian Institute of Technology, Delhi, 1995.

Table 1. Carbohydrate metabolizing enzymes in amoeba, hepatocytes and Kupffer cells. Abundance of enzyme activities in cells are shown. Comparative enzyme database of amoeba and isolated liver cells is shown and sketched in Figures 3,5, and 6. Strengths of different enzyme activities in amoeba, liver cells are shown as weak(+), moderate(++), high(+++), extreme(++++ or more).

Characterization of enzymes in amoeba has been a long time quest to explore the possibility of amebic enzyme inhibition by new drugs in drug discovery. Several antiamoebic drugs are in market as potent enzyme inhibitors since last four decades. Author proposed a comparative enzyme database of amoeba and hepatocellular cells in culture (see Table 1 and Figure 1). Of specific mention, cysteine proteinase enzymes are secreted in large quantities by the parasite during immune response and can cleave extracellular matrix proteins, which might facilitate amoebic invasion[Scholze et al. 1988, Keene 1986]. Mainly carbohydrate metabolizing, energy metabolizing enzymes and lyssomal enzymes are abundant and participate in amoebic –host liver cell interaction as shown in Figures 2,3a,3b and 3c. These proteins act as virulence factors in animal models of amoebic liver abscess [Stanley et al.1995, Ankri et al.1999]. A family of six genes encodes *E. histolytica* cysteine proteinases (*ehcp1–6*) but ~90% of the proteinase activity is related to three proteinase enzymes, EhCP1, EhCP2 and EhCP5 [Bruchhaus, et al. 2003]. Although targeted disruption of selected *E. histolytica* genes has not yet been achieved, stable episomal expression of foreign DNA is possible in amoebae by maintaining continuous selective pressure [Hamann et al. 1995, Vines et al. 1995]. This has enabled the investigators to target specific molecules in *E. histolytica* by the episomal expression of antisense mRNA or of genes encoding dominant negative mutants [Ankri et al. 1998]. The antisense approach has been applied to *E. histolytica* cysteine proteinases and episomal expression of an antisense RNA to *ehcp5* could reduce total amoebic proteinase activity by 80–90% [Ankri et al. 1998]. To assess the role of *E. histolytica* cysteine proteinases in amoebiasis, in earlier study, human xenografts in SCID-HU-INT mice were infected with amoebic trophozoites expressing the *ehcp5* antisense RNA(proteinase-deficient amoebae) or amoebic trophozoites containing the same plasmid without the antisense insert (the control group)[Zhang et al. 2000]. Major findings were: 1. no obvious defect was apparent in the ability of cysteine-proteinase deficient amoebae to inhabit and survive within the colonic lumen; 2. post-24 h after infection, cysteine proteinase-deficient amoeba had, in contrast to the control group, failed to induce significant amounts of human IL-1α or IL-8 from infected intestine; 3. gut inflammation was also reduced in human intestine infected with cysteine-proteinase-deficient *E. histolytica* trophozoites; 4. control *E. histolytica* trophozoites damaged the intestinal permeability barrier at 24 h but there was only a minimal increase in intestinal permeability in human intestinal xenografts infected with cysteine-protease-deficient amoeba; 5. histological studies at 24 h, human xenografts infected with control amoebae showed damage to the colonic mucosa, invasion of amoebic trophozoites into submucosal tissues and neutrophil-predominant inflammation; 6. by contrast, xenografts infected with cysteine-proteinase-deficient amoebae showed less mucosal damage, almost no evidence for amoebic invasion into submucosal tissue and little inflammation. Authors speculated that cysteine-proteinase-deficient amoeba might have a defect in their ability to induce gut inflammation and to invade into the submucosal tissues [Zhang et al. 2000]. How amoebic cysteine proteinases contribute to gut inflammation and tissue damage in amoebiasis? Possibly, *Entamoeba histolytica* trophozoites expressing the *ehcp5* antisense RNA might have reduced the phagocytic capabilities or reduced virulence in amoebiasis [Ankri et al.1998]. However, protease-deficient amoebae maintain their ability to lyse target cells, so a defect in cell killing does not underlie the reduced virulence of cysteine proteinase-deficient amoebae [Seydel et al.1997]. In addition, it is unlikely to be a direct effect of cysteine proteases on intestinal epithelial cells, because amoebic lysates rich in cysteine protease activity fail to induce high levels of cytokine production or inflammation when

placed in human intestinal xenografts [Ankri et al.1998]. It suggests that amoebic cysteine proteinases must exert their effects on gut inflammation and tissue damage after an initial step that requires live active trophozoites.

1.2 Entamoeba histolytica and programmed cell death

Other important role of caspase enzymes was explored when *E. histolytica* trophozoites were incubated with common types of mammalian cells. Normally trophozoites kill mammalian cells in a contact dependent manner by two processes: by lytic necrosis [Berninghausen et al.1997] or undergo apoptosis[Ragland et al.1994]. Apoptosis, or programmed cell death, is an ordered system of cell death with an initiator or signaling phase followed by an effector stage that causes cell death by degrading various cellular components. The effector stage involves the activation of caspase enzymes, cysteine proteinases with specificity for aspartate residues that form a cascade, converging on caspase 3. A key biochemical marker of apoptosis is endonuclease cleavage of chromatin between histone bodies, which can be seen as a DNA ladder on separating gels.

Detection of DNA fragmentation *in situ* by the endlabeling of single- or double-stranded DNA breaks with terminal deoxyribonucleotidyltransferase (TUNEL assay) has also been used as a biomarker of apoptosis [Schulte-Hermann et al.1994]. However, *E. histolytica* can induce apoptosis *in vitro* raises doubt if apoptosis is a component of the cell death commonly seen in amoebic liver abscess. To test this fact, amoebae were inoculated into the livers of SCID mice and evidence for apoptosis was sought by gel separation of DNA from infected livers and TUNEL staining of amoebic liver abscesses [Seydel et al.1998]. DNA ladder formation was detected in samples obtained from SCID mice by 1 h after amoebic inoculation. TUNEL staining revealed significant areas of apoptosis within amoebic liver abscesses common in inflammatory cells and hepatocytes close to amoebic trophozoites [Stenley, 2001]. However, some distant liver cells also were TUNEL positive. Thus, in the murine model of amoebic liver abscess, *E. histolytica* induced cellular apoptosis appears to be a significant component of cell death as shown in Fig. 3. Interestingly, *E. histolytica*-induced apoptosis was not blocked by Bcl-2 [Ragland et al.1994] and did not appear to involve either of the two of the major pathways for the induction of apoptosis: ligation of the Fas receptor and ligation of TNFα1 receptor 1 (TNFR1). In both C57Bl/6 MRL-lpr/lpr mice with no Fas receptor, and C57Bl/6.C^{3}H-gld/gld mice with no Fas ligand, apoptosis was detected in amoebic liver abscesses by DNA ladder formation and TUNEL staining [Seydel et al. 1998]. Apoptosis was also present in hepatocytes in amoebic liver abscesses from both TNFR1 knockout mice and the heterozygote controls[Seydel et al. 1998].]. These data indicate that *E. histolytica* causes apoptosis in mouse liver by a mechanism that is independent of Fas–Fas-ligand interactions and TNFR1.

Genetic approaches and new models of amoebic liver abscesses have provided good prospects on the complex interactions between *E. histolytica* and the host liver cells [Hamann et al.1995]. Well-known *E. histolytica* enzymes lyse cells and digest extracellular matrix proteins. Enzymes have capability to induce apoptosis in hepatocytes and inflammatory cells. Enzymes can stimulate, and perhaps enhance, an NF-κB-mediated human liver inflammatory response that contributes to tissue damage. These new enzyme regulated pathways might offer an explanation for some of the clinical and pathological differences

between amoebiasis and amoebic liver abscess. The hepatocyte inhabited apoptosis would predominate in amoebic liver abscesses. If these mechanisms prove to be important in human disease, they will provide new targets in both the host and the parasite for interventions designed to ameliorate or inhibit amoebiasis and amoebic liver abscesses. Recently, author in team established that apoptosis results with hypoxia in liver cells and evaluated nitroimidazole cytotoxicity [Kwon et al. 2009]. In following section, a criterion of hepatocellular hypoxia is described based on liver cell enzymes and cytomorphology.

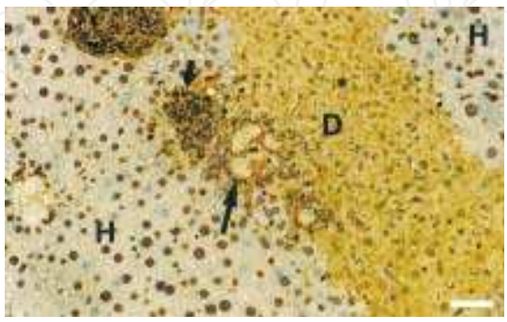


Fig. 1. Entamoeba histolytica trophozoites induce apoptosis in inflammatory cells and hepatocytes in amoebic liver abscesses. Section of liver stained for apoptotic cells with the TUNEL method from an amoebic liver abscess in a C57Bl/6 MRL-lpr/lpr mouse. Amoebic trophozoites (long arrow) are adjacent to a cluster of inflammatory cells (short arrow), which show marked TUNEL staining. TUNEL staining in nuclei and cytoplasm is also evident in a band of dead hepatocytes and inflammatory cells (D). TUNEL-positive nuclei (brown staining) are also visible in hepatocytes in regions (H) flanking the dead cells. Reproduced, with permission, from Ref. Seydel et al. 1998.

2. Liver enzymes and enzyme inhibition in human liver: Hepatocellular criteria

The liver is made of parenchymal hepatocytes and nonparenchymal Kupffer cells as sole targets that exhibit their intracellular biochemical changes as hepatocellular enzyme biomarker profile. Liver cells are rich in enzymes. Enzyme inhibition in liver is characterized by two ways: 1. intact hepatocellular enzymes in hypoxia serum and liver homogenates; 2. Enzyme regulatory behavior by characterizing enzymes in presence of additives added to cultures of isolated liver cells. Additives and drugs are mainly biotransformed and detoxified in liver by a battery of drug metabolizing enzymes: lysosomal and microsomal enzymes. Drugs inhibit enzymes. In following description, readers are introduced with the importance of enzyme inhibition as research tool for two purposes: 1. drug testing and hypoxia disease monitoring (Hepatocellular Hypoxia Criteria) to understand the hypoxia and liver cell interactions with amoeba and nitroimidazole; 2. Enzyme regulatory behavior in presence of additives in isolated liver cells in culture (hepatocytes and Kupffer cells).

2.1 Hepatocellular hypoxia criteria

Author proposed a "Hepatocellular Hypoxia Criteria". 'Hepatocellular hypoxia criteria' assumes that initially liver cells loose metabolic integrity (ATP and NADPH insufficiency from glucose to cause oxygen insufficiency in mitochondria) and undergoes apoptosis

followed by detectable necrosis in liver. Hypothesis was that liver cells undergo a series of changes - hepatocytes undergo metabolic energy loss and oxygen depletion (hypoxia) while Kupffer cells undergo phagocytosis, respiratory burst and proteolysis. Nitroimidazole induces enzyme inhibition in liver cells to normalize the elevated enzymes during metabolic energy loss and oxygen depletion (hypoxia) in liver cell (in mitochondria) and lysosomal enzyme inhibition to combat the phagocytosis. The approach is applicable possibly in design of drugs to treat oxygen starved tumor cells or infected liver lesions and abscesses.

Previous reports on first initial stage of nitroimidazole induced liver cell cytotoxicity indicated the apoptosis, glutathione, DNA interaction, oxygen depletion, lactate inhibition, enhanced NADPH cytochrome reductase and superoxide dismutase enzymes [Ersoz et al.2001, Brezden et al.1997, Noss et al.1988, Sugiyama et al.1993, Berube et al. 1992, Sapora et al.1992, Moselen et al. 1995, Stratford et al.1981, Adams et al.1980, Noss et al. 1989, Mulcahy et al.1989, Widel et al. 1982, Rauth et al. 1981, Chao et al. 1982, Whitmore et al.1986]. Still nitroimidazole structure-function relationship between hypoxia, radiosensitization and cytotoxicity or liver regeneration remain less understood perhaps due to nitroimidazole low sensitivity to clinical investigations and significant enzyme alterations only at the very late necrotic stage [Cowan et al.1994, Melo et al. 2000, Moller et al. 2002, Ballinger et al. 1996, Edwards et al. 1981, Edwards et al.1982, Ballinger et al.2001, Riche et al.2001, Melo et al.2000]. However, nitroimidazole cytotoxicity was reported continuously in last three decades indicating many altered enzymes involved in nitric oxide production, cytokines, oxygen depletion (hypoxia) and immunoactive substance release from both hepatic infections and cancer tumor tissues [Su et al.1999, Rumsey et al.1994, Koch et al.2003, Hodgkiss et al.1997, Sharma 2009a, Sharma et al.2009b]. In following section, author puts an evidence of hepatic metabolic integrity loss (in mitochondria and cytoplasm) and lysosomal stimulation associated with hypoxia in liver. Nitroimidazole is still considered clearly hepatotoxic, renal and neurotoxic drug with several reports of diffused damage of parenchymal and nonparenchymal liver cells as initial stage of hepatic damage observed by electron microscopy and biochemical markers in serum with emphasis of pathophysiology [Mahy et al.2004]. Our previous observation on nitroimidazole induced effects to act against hepatic amoebiasis and amoebic abscess development indicated the possibility of nitroimidazole concentration-dependent effects upon oxygen depletion, nitric oxide production, cytokine synergy and liver cell enzyme alterations as inter-related consequences of liver regeneration or bringing back elevated enzyme levels [Chu et al.2004]. The major players were the energy metabolizing and drug metabolizing lysosomal enzymes in initial liver damage with assumption that liver cell enzyme profile defines the hypoxia sensitivity of nitroimidazole by 'Hepatocellular Hypoxia Criteria'. The novelty of hepatocellular hypoxia criteria was detail cytomorphic-biochemical information extracted from altered enzyme activities during initial cytomorphic changes in hypoxic hepatic cells simultaneously with microstructure changes by electron microscopy to confirm the role of cell organelle in hypoxia development. The criterion can be used to evaluate common liver hypoxia conditions such as hepatic tumors, hepatic infections etc. Several new nitroimidazole derivatives are emerging as potential cancer chemosensitizers, hypoxia markers and hypoxia imaging contrast agents acting as enzyme inhibitors [Gronroos et al.2004, Ziemer et al.2003, Jankovic et al. 2006, Papadopoulou et al.2003, Eschmann et al.2005, Heimbrook et al.1988, Berube et al.1992, Sharma et al. 2009].

Components of Hepatocellular Hypoxia Criteria: The criterion is described a step by step sequence of oxygen depletion in hepatocellular damage: 1. initial loss of metabolic integrity; 2. programmed regulatory failure of cell oxygen and energy metabolism; 3. liver tissue inflammation and immunity loss; 4. necrosis and active death. The purpose of metabolic integrity in hepatocytes is keeping cells intact by maintaining balance between glucose formation and glucose breakdown to maintain energy flow of NADPH and ATP molecules. Sequence: The nitroimidazole induced liver cell cytotocity leads to enhanced glucose breakdown and more demand of ATP and NADPH. With course of time, high energy demand at the cost of cell metabolic resources leads to metabolic integrity loss or energy loss and oxygen deprivation followed by possibility of step by step programmed cell death (only in tumor cells). In normal liver cells, the nitroimidazole cytotoxicity effect cause less damage and cells sustain the effect while struggling oxygen starved infected or tumor cells further loose capability to stay alive (such cells die after nitroimidazole induced infected or tumor cell killing). In normal cells, liver regeneration recovers the damage.

Hepatic hypoxia criterion has 3 major enzyme components: 1. major event of high glycolytic rate is immediate result of high glucose turnover such as glycolysis followed by secondary metabolic cycles viz. tricarboxylic acid, glycogenolysis, gluconeogenesis, pentose phosphate pathways; 2. changes in peripheral biomarkers such as cytokine immunity, altered glutathione reductase, nitric oxide production, super oxide dismutase enzymes; 3. after severe energy loss, changes occur in liver cellular morphology and tissue shrinkage [Sharma et al.2011, Kwon et al. 2009]. The sensitivity of different enzymes as cytomorphic manifestation of hypoxia in liver abscess and nitroimidazole induced liver regeneration is a simple scheme of "Hepatocellular Hypoxia Criteria" in steps is shown in Table 2. In following section, different enzymes are described.

Morphological changes	Clinical changes	Liver biochemical changes
1. Physical examination:		
--	abdominal pain	--
intestinal damage	fever	--
hepatic infiltration	hepatomegaly	liver function tests(elevated)
	⇩	
	Loss of cellular metabolic integrity	
2.Electron microscopy:	hepatomegaly with	altered hepatocyte enzymes of:
Mitochondria(M)	diffused injury	low ATP/ADP; NADPH/NADP
endoplasmic reticulum(ER)		gluconeogenesis
peroxisome (P)		glycogenolytic
lysosome (L)	⇩	lysosomal enzymes
nuclear changes (N)		oxygen flux related
	Hepatocellular enlargement (Apoptosis)	

Table 2. Continued

Morphological changes	Clinical changes	Liver biochemical changes
3. Cellular organelle damage:	poor drug response	slow metabolic disorder:
mitochondria(M)		oxidative phosphorylation
microsomes (MI)		drug metabolizing enzymes
lysosome (L)	⇩	initial phagocytosis
nuclear (N)		DNA fragmentation(beads)
cytosol (C)		glucose/protein/respiratory burst
	Hepatocellular Oxygen insufficiency (inflammation)	
4. Liver pathology changes:	raised diaphragm	
mitochondrial damage	amebic liver scan +ve	stimulation of Kupffer cells
exfoliative ER		hyperplasia of Kupffer cells
anisonucleosis		loss of metabolic control
autophagy & lysosomal irritation	⇩	increased water accumulation
cytosolic granulation		increased molecule imbalance
fatty liver appearance with		increased lipid synthesis
membrane damage		
	Hepatocellular degeneration and necrosis	
5. Hepatocytology:		
Cell proliferation	advancing tumor vascularization	surgical aspirates (altered
Cell debris	tissue growth on ultrasound	proteins, lipids, enzymes)
	⇩	
	Nitroimidazole single dose therapy schedule	
6. Liver cell recovery	negative liver scan/ultrasound	normal liver function test
Tissue shrinkage	Hypoxia monitoring positive	-ve Hypoxia biomarkers
OR	if unchanged or poor recovery	abnormal ELISA,enzymes
	⇩	
	Surgical intervention	

Source: Sharma R. Effect of Nitroimidazole on isolated liver cells in development of amoebic liver abscess. Ph.D dissertation submitted to Indian Institute of Technology, Delhi, 1995.

Table 2. A step by step scheme of "hepatocellular hypoxia criteria" to evaluate liver hypoxia damage in infected hepatitis or hepatic tumors. Different clinical methods suggest composite picture of hepatic hypoxia and associated biochemical and cytomorphic changes.

2.2 Origin of hypoxia and enzyme regulation

Oxygen insufficiency or hypoxia begins with low NADPH and ATP supply from glucose. Glucokinase, aldolase, pyruvate kinase and lactate dehydrogenase possibly serve as initial glycolytic regulatory enzymes for energy flow to generate tricarboxylic acid cycle (TCA) precursors. TCA cycle being as main source of energy molecule synthesis and gluconeogenesis, it serves as sole source of central ATP pool in glucose homeostasis during liver cytototoxicity or regenerated liver cell [Sharma et al.2007, Sharma et al.2010]. The citrate synthase, malate dehydrogenase, isocitrate dehydrogenase and succinate dehydrogenase enzymes serve as TCA cycle regulatory enzymes. These enzymes in hepatocytes characterize the tumor hypoxia and hepatocyte response after nitroimidazole therapy [Horlen et al.2000, Tabak et al.2003, Sapora et al.1992, Moselen et al.1995, Stratford et al.1981]. At the cost of ATP from TCA cycle and oxygen from the cell, oxidative phosphorylation maintains the metabolic integrity and continuous flow of energy. In the state of low NADPH and low oxygen cell undergoes state of metabolic integrity loss and "hypoxia". NADPH dependent cytochrome redox enzymes are biomarker of oxidative phosphorylation status and oxygen insufficiency (hypoxia) in liver. Other glutathione reductase and superoxide dismutase enzymes are biomarkers of hypoxic state of liver cells [Larrey et al.2000, Michaopoulos et al.1997]. Any energy imbalance and oxygen insufficiency (hypoxia) in liver cell are indicated by these enzymes [Michaopoulos et al.1997, Ramirez-Emiliano et al.2007]. The behavior of these enzymes in isolated liver cells is described in following section on isolated liver cell enzymes.

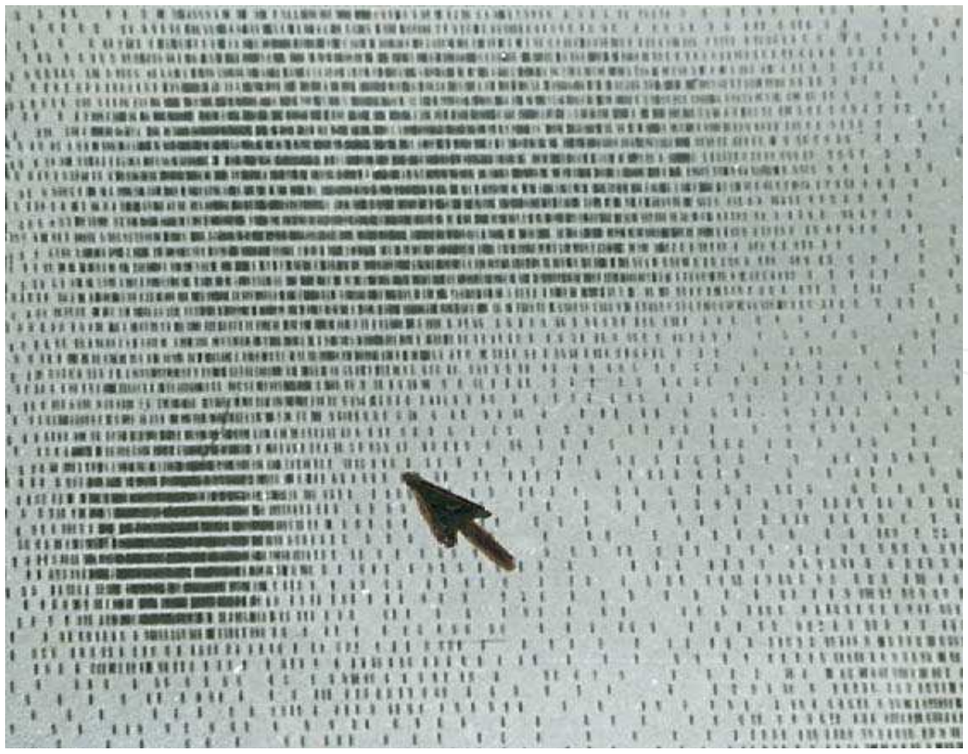


Fig. 2. The liver scan (on left panel) is shown for assessing the position of hepatic hypoxia and associated hepatomegaly and for biopsy collection site as shown with arrow.

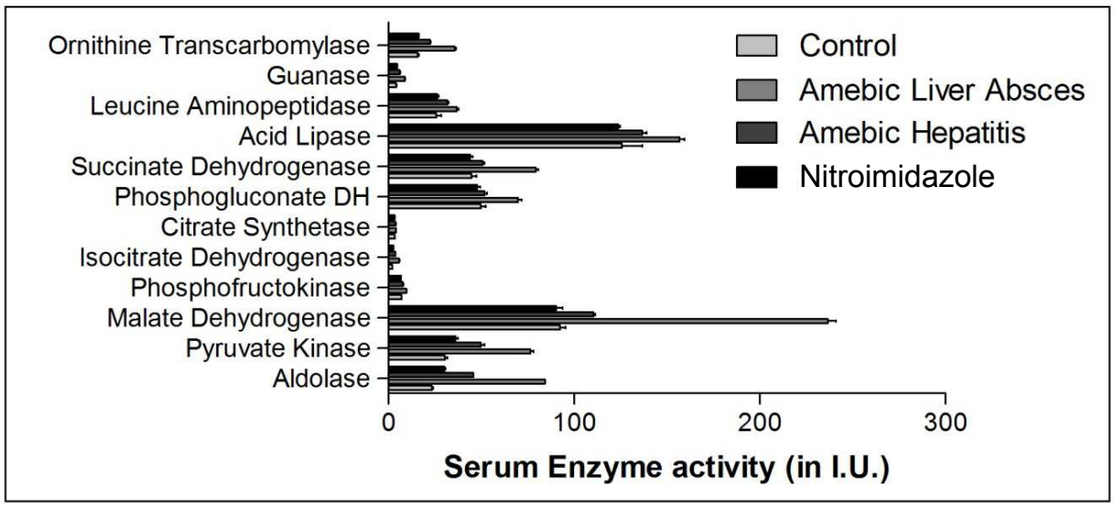


Fig. 3a. The histogram bars show the effect of nitroimidazole on biomarker enzymes and comparison of different enzymes in hepatocytes in control vs nitroimidazole treated subjects

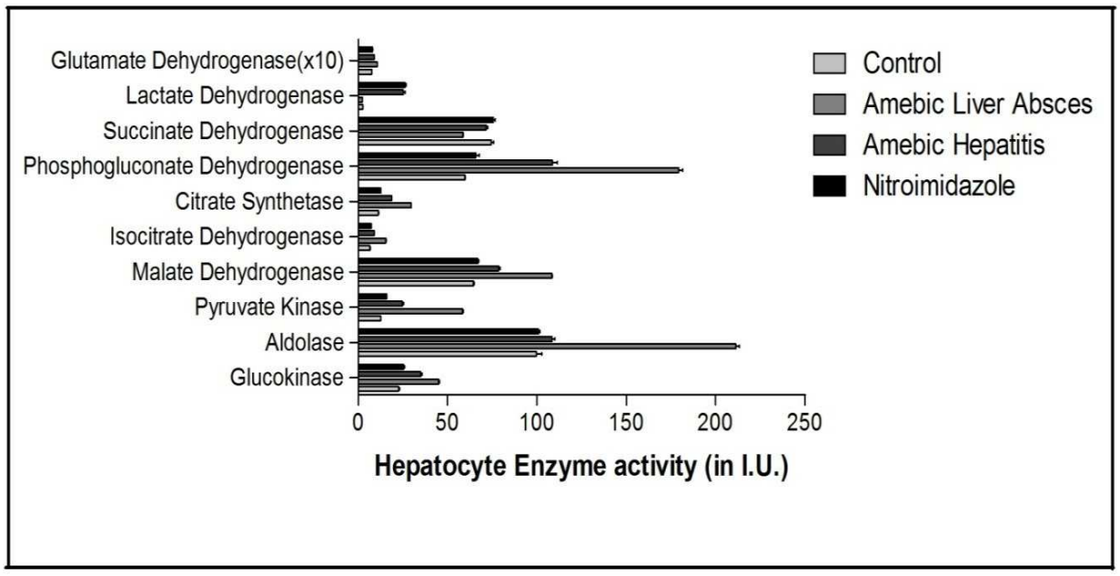


Fig. 3b. The histogram bars show the effect of nitroimidazole on biomarker enzymes and comparison of different enzymes in Kupffer cells in control vs nitroimidazole treated subjects in control vs nitroimidazole treated subjects

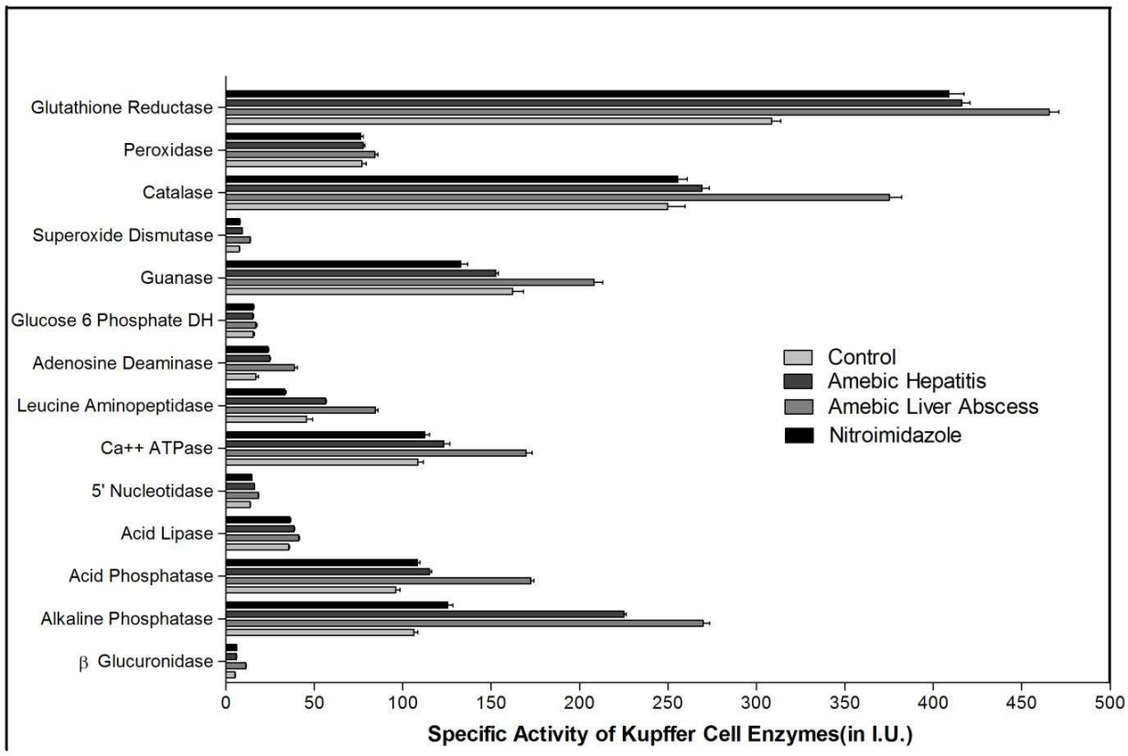


Fig. 3c. The histogram bars show the effect of nitroimidazole on biomarker enzymes and comparison of different enzymes in serum biomarker enzymes.

3. Enzyme inhibition in initial loss of metabolic integrity, glycolysis and ATP in hepatocytes

Enzymes in serum and liver cells from positive control amoebic liver abscess patients exhibited more or less specific characteristic changes in enzymes [Sharma et al.2008]. First goal was to establish elevated enzyme levels in patients and to prove subsequent enzyme inhibition after nitroimidazole therapy. Author reported ten control subjects with 2′-nitroimidazole therapy follow up for their carbohydrate metabolizing enzymes in serum and hepatocellular enzymes in liver biopsy tissues. Proven ten hepatic hypoxia control subjects were studied for hypoxia by enzyme assays [Sharma et al.2008]. The 2′-nitroimidazole treated paired ten subjects were studied for hypoxia related enzyme inhibition using enzyme assays and hepatocellular cytomorphology by electron microscopy. Out of ten positive control subjects, nine patients showed elevated carbohydrate metabolizing and lysosomal enzyme levels in serum: Glucokinase (in 80% samples), aldolase (in 80% samples), phosphofructokinase (in 80% samples), malate dehydrogenase (in 75% samples), isocitrate dehydrogenase (ICDH) (in 60% patients) were elevated while succinate dehydrogenase and lactate dehydrogenase (LDH) levels remained unaltered. Lysosomal β-glucuronidase, alkaline phosphatase, acid phosphatase enzymes showed enhanced levels in the all patient serum samples. In ten control liver biopsies, the isolated hepatocytes and Kupffer cell preparations showed altered liver cell enzyme levels. Hepatocytes showed reduced glucokinase (in 80%), LDH (in 80%), and higher content of

aldolase (in 80%), pyruvate kinase (in 100%), malate dehydrogenase (in 80%), ICDH (in 80%), citrate dehydrogenase (in 70%), phosphogluconate dehydrogenase (in 80%). Kupffer cells showed higher enzyme levels of β-glucuroronidase (in 80%), leucine aminopeptidase (in 70%), acid phosphatase (in 80%) and aryl sulphatase (in 88%). In these 10 biopsy samples from subjects, the electron microscopy cytomorphology observations showed swollen bizarre mitochondria, proliferative endoplasmic reticulum, and anisonucleosis. In 2'-nitronidazole clinical trial patients, after 2'-Nitroimidazole effect in liver cell damage was manifested as enzyme inhibition in liver cells as shown in Figure 3[Sharma 2008]. Nitroimidazole treated patients shows altered enzymes: glucokinase levels were more or less normal in both serum and liver biopsy (100%); phosphofructokinase levels were nonspecific in nitroimidazole treated subjects or normal (80%); elevated lactate dehydrogenase levels were reversed to normal. In nitroimidazole treated subjects the elevated enzyme levels due to amoebic abscess brought back to normal after nitroimidazole treatment due to enzyme inhibitory effect of nitroimidazole: isocitrate dehydrogenase enzyme normal levels (80 %); the citrate synthase normal levels (80%); phosphogluconate dehydrogenase normal levels (80 %). In nitroimidazole treated subjects, the succinate dehydrogenase levels were normal (60 %) in both. The enzymes are shown in Figure 3 and Tables 2-4.

3.1 Enzyme inhibition in Kupffer cell lysosomal enzymes and Hypoxia

In nitroimidazole treated patients showed elevated enzymes brought back to normal: β-glucuronidase normal levels (50 %) in Kupffer cells and normal in serum (60 %); acid phosphatase normal levels in both; leucine aminopeptidase levels were normal in serum (80 %) and remained high in Kupffer cells (60%); Guanase levels were normal in both Kupffer cells and serum (80%). The enzymes are shown in Figure 2 and Table 3.

Kupffer cell hyperplasia was observed with swollen lysosomal contents as shown in Figure 4. Altered glycolytic enzymes in cytosol, TCA cycle enzymes in mitochondria, lysosomes and increased synthesis of enzymes by endoplasmic reticulum showed correlation with clear liver cell degeneration of microbodies. After nitroimidazole treatment, observations of both electron microscopy and biochemical parameters suggested the reversed hepatocellular changes towards normal recovery or liver regeneration. In following section, I describe the outcome of nitroimidazole effect in liver regeneration and enzyme inhibition as defense.

The enzyme biomarkers could be analyzed in serum and liver cells (hepatocytes and Kupffer cells) as clinically significant indicator of hepatic damage in disease or cytotoxicity of drug. We assume that initially metabolic integrity loss leads to over-secretion of liver cell enzymes including lysosomal enzymes. Soon after, the ultrastructural changes in liver cells become evident by electron microscopy suggestive of acute organelle degeneration. Ultrastructural hepatocyte cytotoxicity was associated with nitroimidazole overdosage (normal dosage is 2 × 3 gm one time in amoebic hepatitis and thrice in amoebic abscess). The regenerative changes were consistently observed after nitroimidazole therapy to reverse liver damage. Few pathology reports are available to show nitroimidazole cytotoxicity and no electron microscopy study is available to support asymptomatic inflammatory or unequivocal diffuse parenchymal injury exhibiting diffused sinusoidal and portal

infiltration events [Tabak et al.2003, Larrey et al.2000]. Biomarker liver cell enzymes with electron microscopy put evidence of amoebic cytotoxicity in liver biopsy samples and liver regeneration after nitroimidazole treatment by 'hepatic hypoxia criteria'. Degenerative changes of hepatocytes suggested possible necrosis.

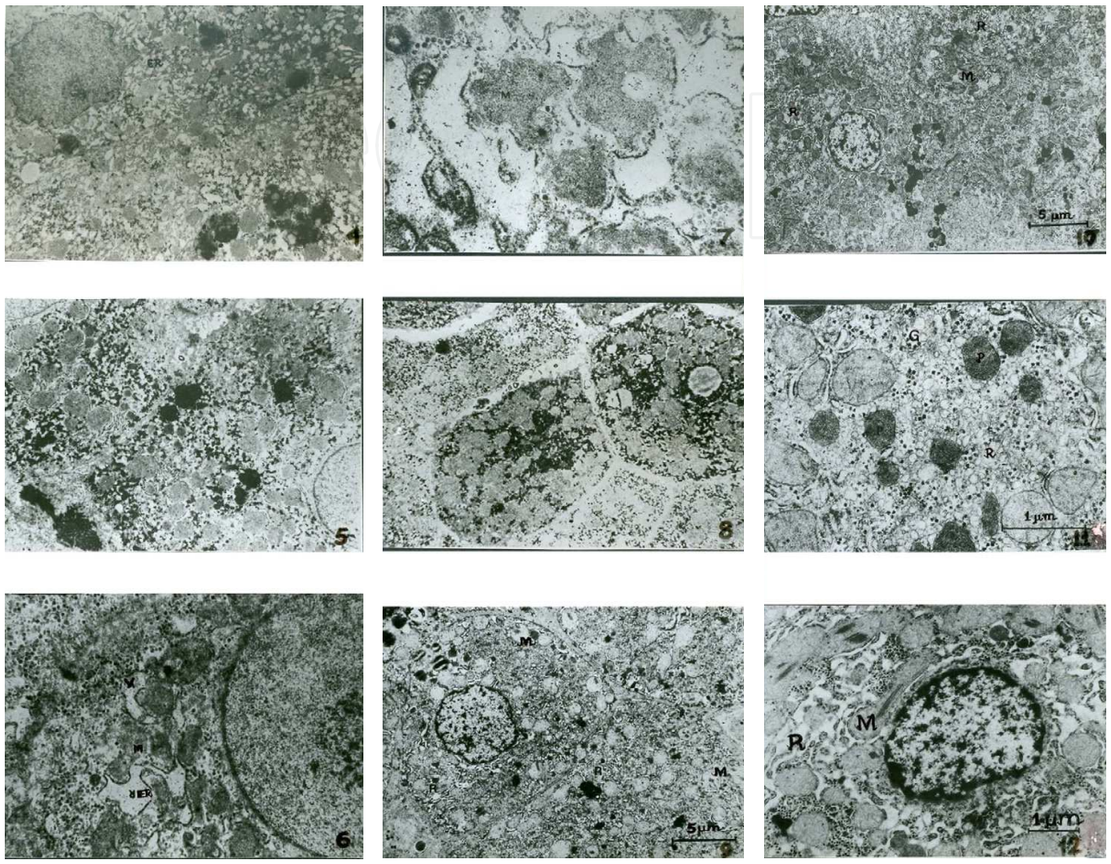


Fig. 4. Ultrastructural changes are shown in hepatocyte organelles during hypoxia: exfoliation of endoplasmic reticulum (top on left); anisonucleosis (mid and bottom on left); intercellular junction gaps (top and mid panels in center); nuclear inclusions (bottom on center); swollen and bizarre mitochondria(top on right); inclusions in peroxisome (mid on right); mitochondrial atrophy with lipid vesicles (bottom on right). Hepatocyte mitochondria became swollen, bizarre with dense matrix and showed destorted cristae, endoplasmic reticulum dilated vesicles, giant nuclei with diffuse proliferation of endoplasmic reticulum and clear anisonucleosis features. The ultrastructure of liver cells showed characteristic organelle changes in nitroimidazole treated liver.

Evidence of hepatic regeneration and nitroimidazole cytotoxicity (hypoxia induced changes) was characterized previously in terms of cytokine synergy, unusual degree of anisonucleosis, nitric oxide production and the presence of giant nucleus in hepatocytes after liver regenerative therapy [Michalopoulous et al.1997]. Endoplasmic reticulum showed a diffuse and intense proliferative activity in these liver cells with normal appearance of mitochondria as earlier reported [Ramirez-Emiliano et al.2007, Das et al.1999]. However, intramitochondrial inclusion bodies were absent while they were very prominent features

[Das et al.1999]. The cause of the ultrastructural changes after nitroimidazole cytotoxicity were supported by initial enzyme alterations reflecting loss of metabolic integrity probably induced by free radical formation from nitroimidazole [Das et al.1999]. Since ultrastructural changes in liver were completely reversible after nitroimidazole therapy within 7-9 days, it is quite reasonable that pathogenesis of diffused hepatocyte damage was due to nitroimidazole breakdown products.

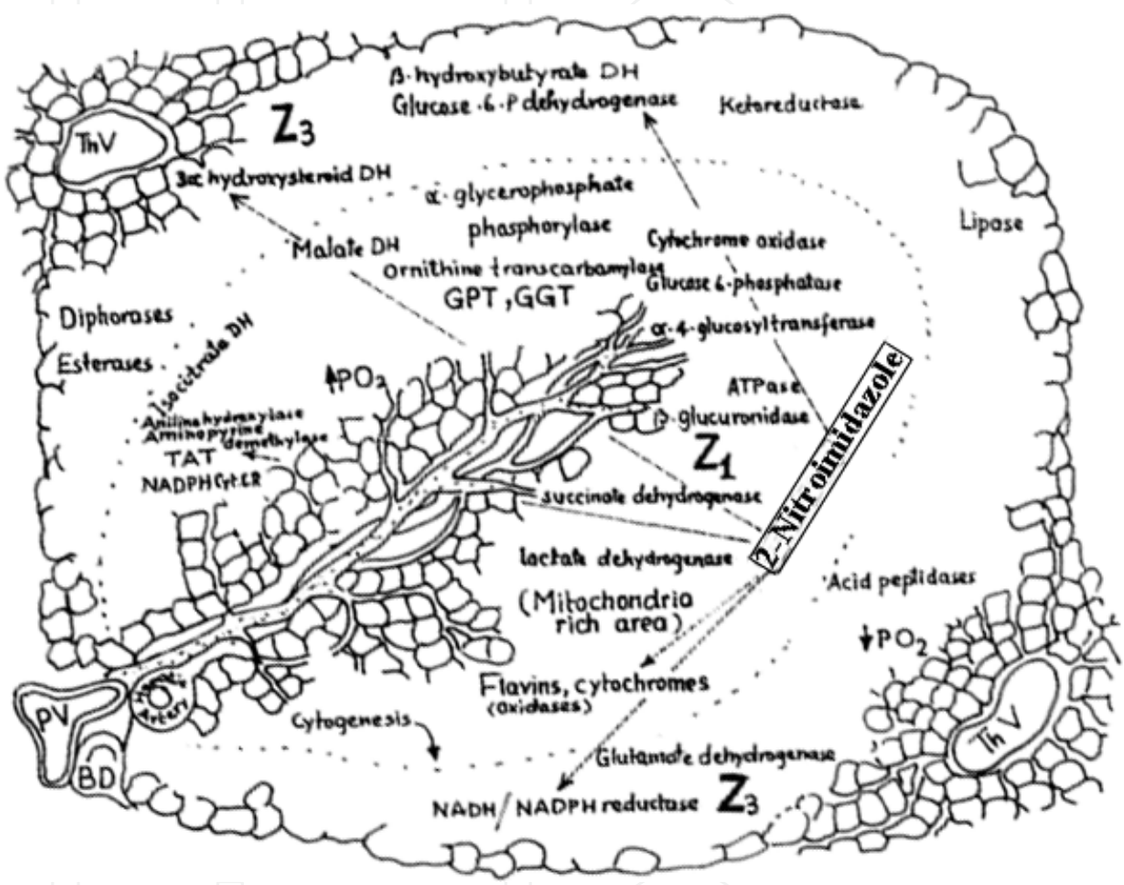


Fig. 5. The sketch of nitroimidazole induced intracellular enzyme inhibition changes indicating the points of metabolism and metabolic control during hypoxia and subsequent liver recovery after nitroimidazole treatment. The enzymes distribution in organelle and enzyme location in different hepatic sites explains the liver damage and enzymatic basis of hepatocellular hypoxia criteria [reproduced from Sharma R. 1990. Ph.D dissertation].

As indicated in previous section, 'Hepatocellular hypoxia criteria' is a sequence of events in hepatic cell injury at molecular level. The initial loss of hepatocellular metabolic integrity leads to hepatic injury. Glucose-energy metabolic integrity loss and nitric oxide formation with Ca^{++} homeostasis are main initial determinants of hypoxia [Ramirez-Emiliano et al.2007]. In following section, present chapter extends the enzyme regulatory behavior during glucose metabolizing pathways viz. glycolysis, TCA cycle and gluconeogenesis to establish the value of enzyme inhibition in liver regeneration. In isolated liver cells, metabolic alterations of energy metabolism pathways explain the events in hepatitis and

hepatic tumors as best correlated respective enzyme dysfunctions and ultrastructural changes observed in cells in biopsy. The following description is broad explanation of different enzymes secreted from hepatocytes as a result of nitroimidazole induced cytotoxicity and oxygen depletion. From biochemical stand point, different regulatory enzymes are discussed as biochemical events of hypoxia development as shown in Figures 3a,3b,3c and 5.

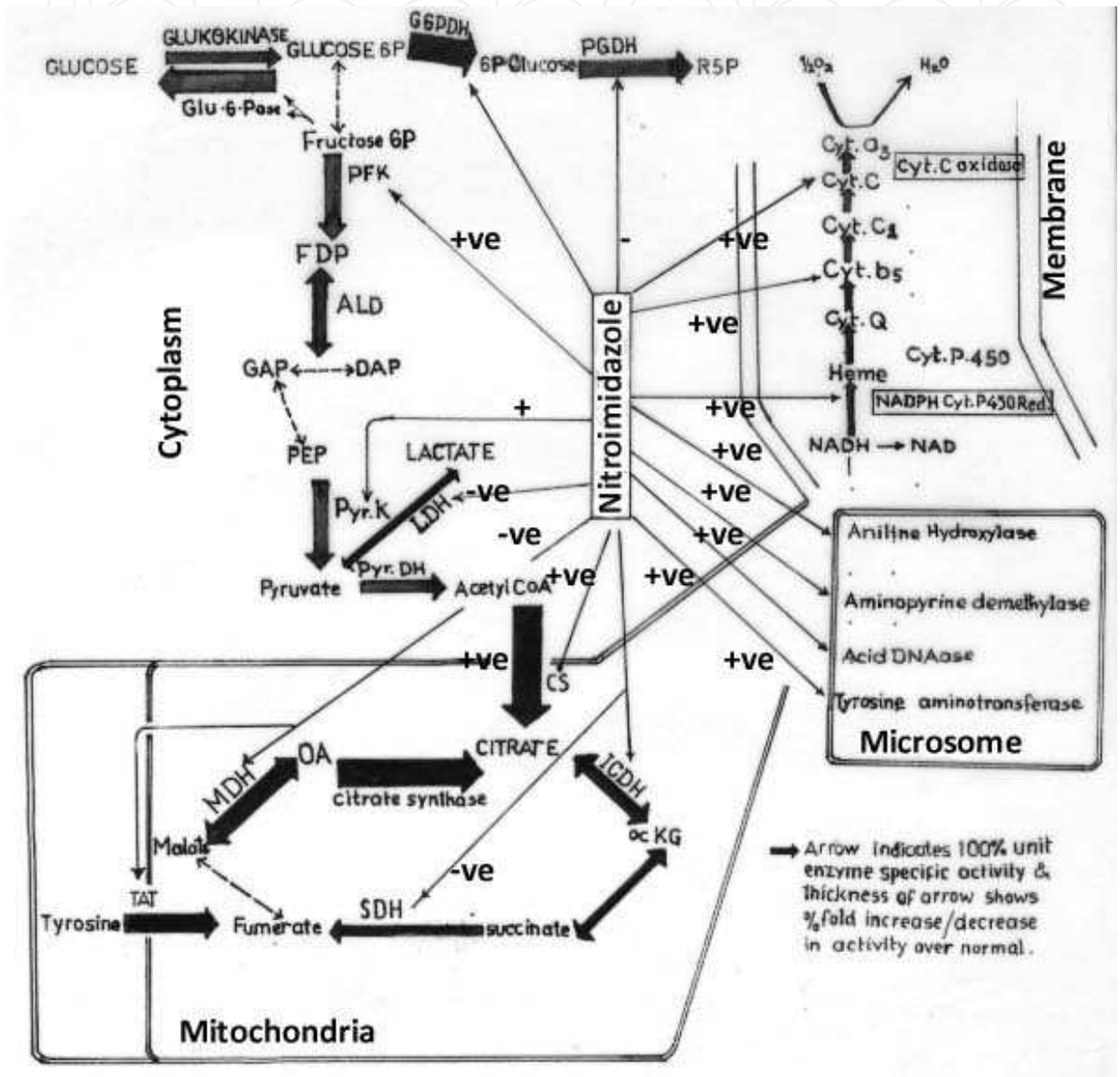


Fig. 6. The sketch of relative biomarker enzyme changes (thickness of arrow) in liver hypoxia. The +ve sign denotes the relative increase in enzyme activity and –ve sign denotes the decrease in enzyme activity in liver cells in different organelles due to ameba induced hypoxia. The figure sketch also represents a relative enzyme activities in sequence of metabolic steps as 'hepatocellular hypoxia criteria'. Notice the liver regenerative or enzyme inhibitory action of nitroimidazole at different enzyme reactions is shown [Reproduced from Sharma R. 1995. Ph.D dissertation].

3.2 Role of enzymes in energy metabolic integrity in hypoxia

Hexokinase, a rate limiting enzyme in cytosol for glucose turnover was estimated due to high glucose conversion into glucose 6P showed high enzyme secretion from hepatocytes. Aldolase, rate controlling enzyme which splits fructose-1, 6 Diphosphate into two 3-phosphoglyceric acid and dehydroxyacetone phosphate, showed high enzyme secretion from heptocytes. Phosphofructokinase, rate controlling enzyme which phosphorylates fructose 6P into fructose 1, 6 DiP, enhanced as its more demand in hepatocellular glucose turnover which probably induced higher secretion of phosphofructokinase. Pyruvate kinase, transferring phosphoryl group from phosphoenolpyruvate to ADP with pyruvate formation, showed high enzyme secretion from hepatocytes. It may be attributed due to high concentration of glycolytic intermediates as terminal step was blocked or due to 2, 3 Di-Phosphoglycerate regulated abnormal oxygen dissociation. Malate dehydrogenase and isocitrate dehydrogenase enzymes, catalyzing malate oxidation into oxaloacetate and oxidative decarboxylation of isocitrate into α-ketoglutarate respectively both require NAD^+. Both enzymes were elevated in damaged cells due to reducing equivalent low ratio of $NADH/NAD^+$ pushing in forward direction. Succinate dehydrogenase, oxidizing succinate to fumerate using FAD, showed decreased enzyme levels due to low iron sulphur proteins resulting with lowered electron transport system in inner mitochondrial membrane i.e. supply of electrons to molecular oxygen by electron transfer from $FADH_2$ to Fe^{+++} (SDH) in amoebic recruited liver cells. Citrate synthase, synthesizing citrate from oxaloacetate and acetyl CoA by aldol condensation followed by hydrolysis, showed elevated levels due to rapid turnover of oxaloacetate and acetyl CoA molecules during cytolysis. Moreover, high TCA cycle activity in hepatocyte during liver damage conditions was described earlier [Sharma 2010]. In serum, the enzymes exhibit their significance but aldolase, pyruvate kinase and LDH, MDH, ICDH observed as distinguishing the diffused injury or abscess formation [Virk et al.1989]. Phosphogluconate dehydrogenase elevated levels may be attributed due to ribulose 5P formation and transaldolase and transketolase control, for phosphogluconate pathway. Nitroimidazole induced cytotoxicity perhaps have insignificant impact on ATP supply. In following section, different enzyme regulatory behaviors of nitroimidazole induced enzyme inhibition are described during liver cell regeneration. A strategy was developed to isolate hepatocytes and Kupffer cells in monoaxenic cultures from excised liver samples. Mixing different inhibitors or additives in liver cell cultures stimulate the enzyme regulatory behavior in presence of specific growth factors, metabolite analogues.

3.3 Role of lysosomal enzymes in hepatocellular damage

In previous section, role of secretory lysosomal enzymes was described in defense of liver cells in context of intestinal amoebiasis and amoebic liver abscess. Conceptually, lysosomal hydrolases cause foreign body damage by several reactions including redox reaction, phosphorylation reaction, glucuronidation reaction, hydroxylation reaction, dehydroxylation reaction, nucleation reaction, terminal breakdown reaction, trans-carbamylation reaction. Other studies also reported molecular mechanisms of enzymes in pathogenesis [Das et al.1999], liver cell damage [Virk et al.1989, Virk et al.1988], phagocytosis [Sharma 2009], cyclooxygenase-2 expression [Sanchez-Ramirez et al.2000].

4. Enzyme inhibition properties and enzyme regulatory behavior in isolated liver cells

Isolated liver cells with characteristic enzyme properties are research tools. In our previous study, the hepatocyte yield from livers was 2.5×10^8 per mg liver. The cytosol fraction showed the removal of all cell organelle from supernatant and resultant supernatant as cytosol was free of any remnant by microscopic observation by trypan blue exclusion. Isolation of human Kupffer cells was developed by a modified pronase-collagenase enzymatic isolation and purity of Kupffer cells by using biomarker enzyme assay. Earlier reports used similar practice of enzymatic digestion and enzymatic assays as indicators of cell viability and yield [Hendriks et al.1990, Neaud et al.1995, McCuskey et al.1990]. A new rapid method was used for the isolation and fractionation of both rat and human Kupffer cells without the need of liver perfusion techniques. The study used rat livers or small human liver wedge biopsies obtained peroperatively and incubated with pronase under continuous pH registration. Kupffer cells were subsequently separated from other nonparenchymal cells by Nycodenz gradient centrifugation and purified by counterflow centrifugal elutriation [Neaud et al.1995]. Identification of Kupffer cells was achieved on the basis of ultrastructural analyses and immunophenotyping [Neaud et al.1995]. The fractionation of Kupffer cells gave good yield comparable with other studies [Hendriks et al.1990, Neaud et al.1995, McCuskey et al.1990]. For details of protocol of liver cell isolation, see the Appendix in the end of this chapter. Human liver cell enzymes in isolated liver cells (hepatocytes and Kupffer cells in cultures) showed characteristic regulatory behavior in presence of additives.

Liver cell hypoxia is represented as a state of oxygen depletion in cell. Initially liver cell gets its ATP and NADPH supply from glycolysis and TCA cycle. Consequently, electron transfer chain (oxidative phosphorylation) through series of cytochrome redox reactions converts available cell oxygen to water to produce high energy metabolites in the cell (metabolic integrity). Infected liver cells show loss of metabolic integrity and less available oxygen (oxygen starved or hypoxia). Nitroimidazole was established two decades ago as potential oxygen quenching drug in most of the infected and tumor cells. "Nitroimidazole oxygen quenching action" makes oxygen depleted or starved cells that further undergo worse to die and leaving normal cells fully functional that can be observed as potential tumor hypoxia therapy and antiparasitic treatment by nitroimidazole.

In following section, enzyme inhibition characteristics in energy metabolism regulation including glycolysis, TCA cycle regulatory enzyme behavior and drug metabolizing proteolytic lysosomal enzymes induced by additives in liver cell cultures are described.

4.1 Glucokinase

The actinomycin D showed non-significant inhibitory change in glucokinase enzyme activity as shown in Figure 7 at the top histogram bars. The nitroimidazole showed enhanced glucokinase activity and reversed the inhibitory effect of actinomycin D. The glucokinase activity in hepatic abscess hepatocytes did not show any significant effect of nitroimidazole. Overall actinomycin D alone did not show any change in glucokinase enzyme activity in hepatic abscess hepatocytes or nitroimidazole treated hepatocytes.

The addition of triamcinolone with insulin in combination showed significant increase in glucokinase activity in all hepatocyte groups. It pointed out to the enhancement in hormone dependent glucokinase enzyme synthesis in hepatocytes and enzyme activity showed highest enzyme synthesis rate in hepatic abscess recruited hepatocytes while nitroimidazole treated hepatocytes showed lesser increase in hormone dependent glucokinase in comparison with hepatocytes as shown in Figure 7. The increase in enzyme activity or enhanced glucokinase enzyme synthesis in hepatocytes showed that glucokinase enzyme behavior was hormone dependent. Different concentration of insulin and progesterone hormones further indicated the regulatory behavior of glucokinase enzyme as hormone dependent.

The addition of progesterone hormone 0.1 µgm/ml in hepatocyte cultures showed maximum enhanced glucokinase enzyme activity and nitroimidazole treated hepatocytes showed less enhanced glucokinase activity but more than control hepatocyte enzyme activity as shown in histogram bars (see Figure 7).

In control group, glucokinase enzyme activity in untreated hepatocytes did not show any chnage in their glucokinase enzyme activity in presence of nitroimidazole additive. The yield of hepatocytes and glucokinase enzyme activities from hepatic abscess liver biopsy were similar as yield of cells and glucokinase enzyme activities in untreated and nitroimidazole treated hepatocytes. So, it was clear that addition of actinomycin-D and insulin with triamicilone both enhanced the glucokinase enzyme synthesis in hepatocytes as shown in Figure 7 histograms bars.

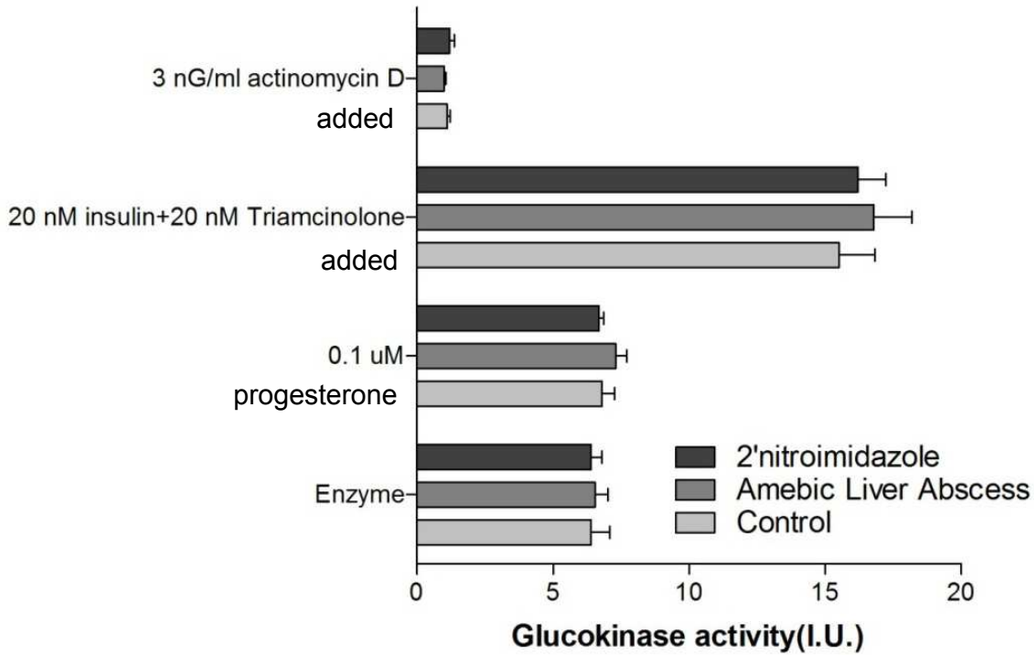


Fig. 7. The nitroimidazole effect on glucokinase enzyme activity in guinea pig normal and infected hepatocytes is shown in presence of different enzyme regulatory additives actinomycin D, insulin-triamcilone. Notice the enhanced effect of insulin-triamicilone on glucokinase enzyme (P value <0.0001; r^2 0.9872) in nitroimidazole treated vs liver abscess biopsy heaptocytes.

4.2 Pyruvate kinase

The altered behavior of pyruvate kinase regulation in isolated hepatocytes from nitroimidazole and progesterone induced cultures showed characteristic sigmoid behavior in normal group but towards linear relationship at increased pH of hepatocyte culture media. In other words, a pH increase alters the enzyme velocity in same proportion with ATP concentration changes in linear manner in liver cell medium. At high ATP concentration, enzyme synthesis in nitroimidazole stimulated cells remained significantly elevated while at pH 7.0-7.4 cells did not show sudden enzyme elevation and showed a fall in enzyme activity as observed in normal hepatocytes (see Figure 8). The higher ADP/ATP ratio kept the enzyme synthesis high at pH 7.4 in progesterone while nitroimidazole stimulated the hepatocyte cells in cultures (see Figure 8).

The intricacy was reported as ATP interacts with H^+ and Mg^{++} to give complex distribution of many ionized and metal complex species. However, Mg^{++} free ADP itself has three subspecies viz. ADP^{--}, $ADPK^{--}$, $ADPH^-$ whose relative proportions vary with change in pH. Out of which ADP^- happens to be true substrate of pyruvate kinase which presumably was utilized by hepatocytes stimulated by nitroimidazole. It further suggested the binding of ADP to unbound and fully bound enzyme conformations involving different groups within active site. The binding of ADP and ADP free conformation of enzyme did not involve groups that ionized within applied pH range in nitroimidazole and progesterone stimulated hepatocytes but binding of ADP to the fully bound enzyme conformation (cooperativity) involved the ionizable groups with high pK values (at pH 6.6-7.4). Moreover, phosphoenol pyruvate bound enzyme ionization has been previously reported to promote cooperative binding of ADP in diseased cell. In present study, ADP binding becomes less cooperative by stimulated hepatocytes. It is possible that enzyme may have two active conformations. So, allosteric interactions actually might have altered the mode of ADP binding in normal cells while they were non-effective in pyruvate kinase enzyme of stimulated hepatocytes. Pyruvate kinase active site conformation perhaps seems to change the ADP binding.

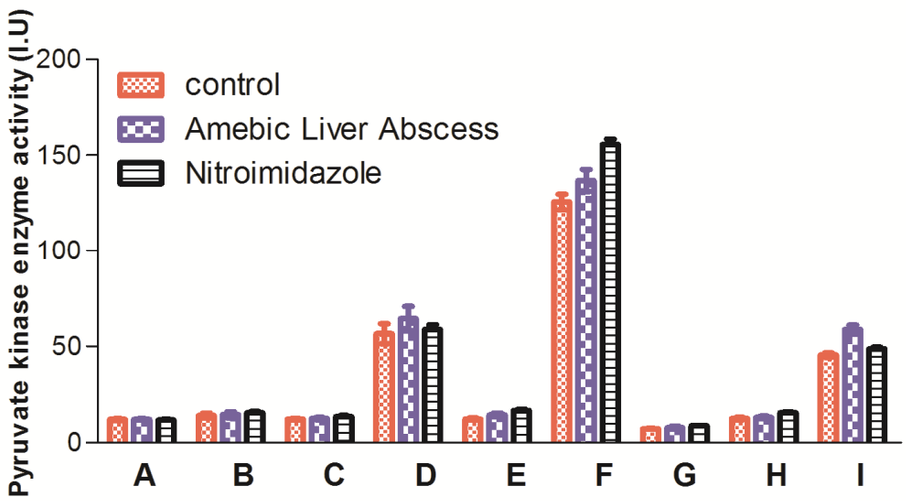


Fig. 8a. The pyruvate kinase enzyme activity in control, amoebic liver abscess and nitroimidazole treated hepatocytes is shown in presence of different enzyme regulatory additives (A-I).

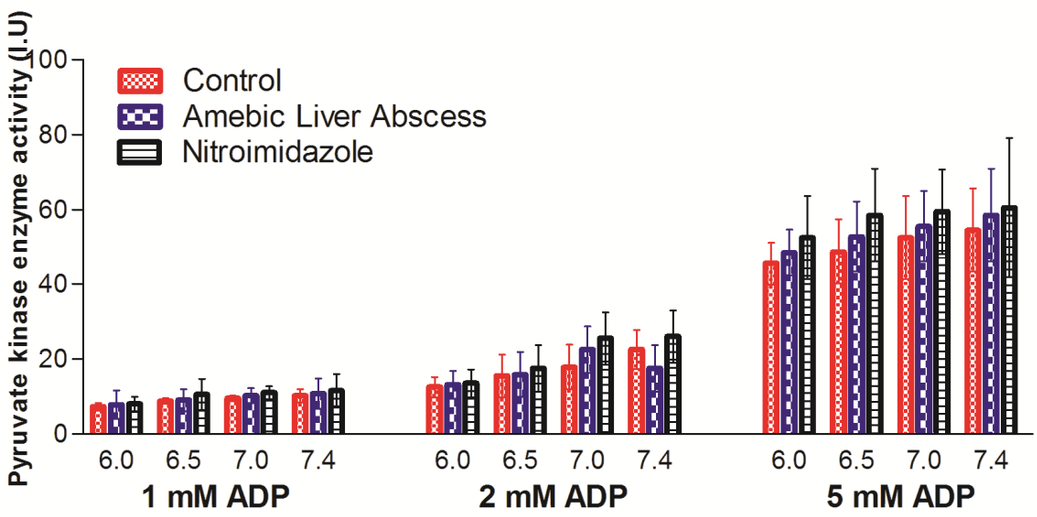


Fig. 8b. The pyruvate kinase enzyme activity in control, amoebic liver abscess and nitroimidazole treated hepatocytes is shown in presence of different enzyme regulatory ADP additives (2-5 mM ADP).

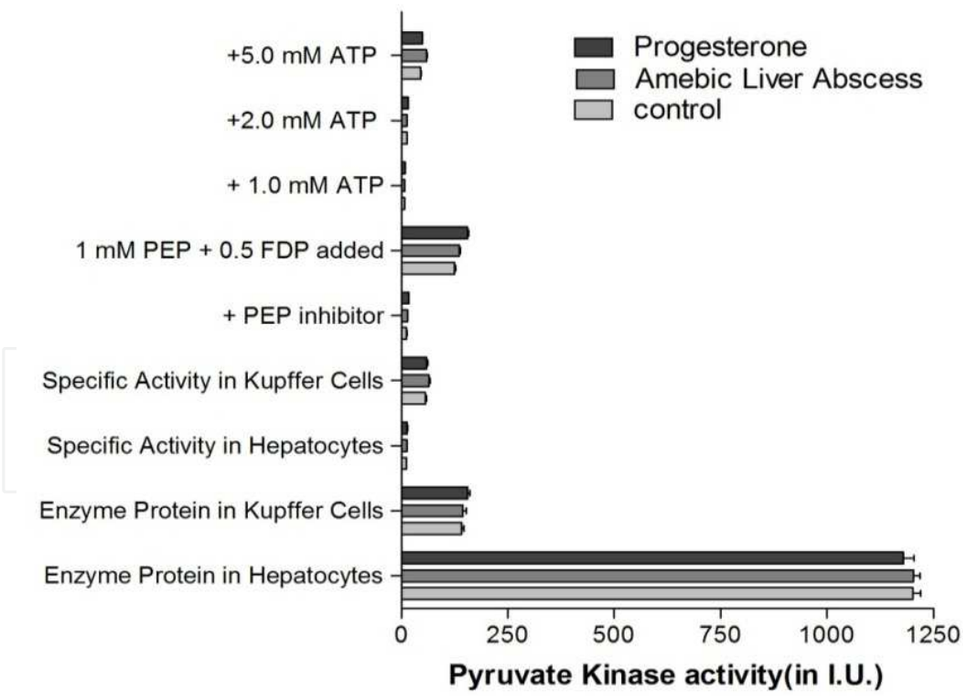


Fig. 8c. The pyruvate kinase enzyme activity in control, amoebic liver abscess and progesterone treated liver cells is shown in presence of different enzyme regulatory additives (ATP, PEP+FDP,PEP inhibitors).

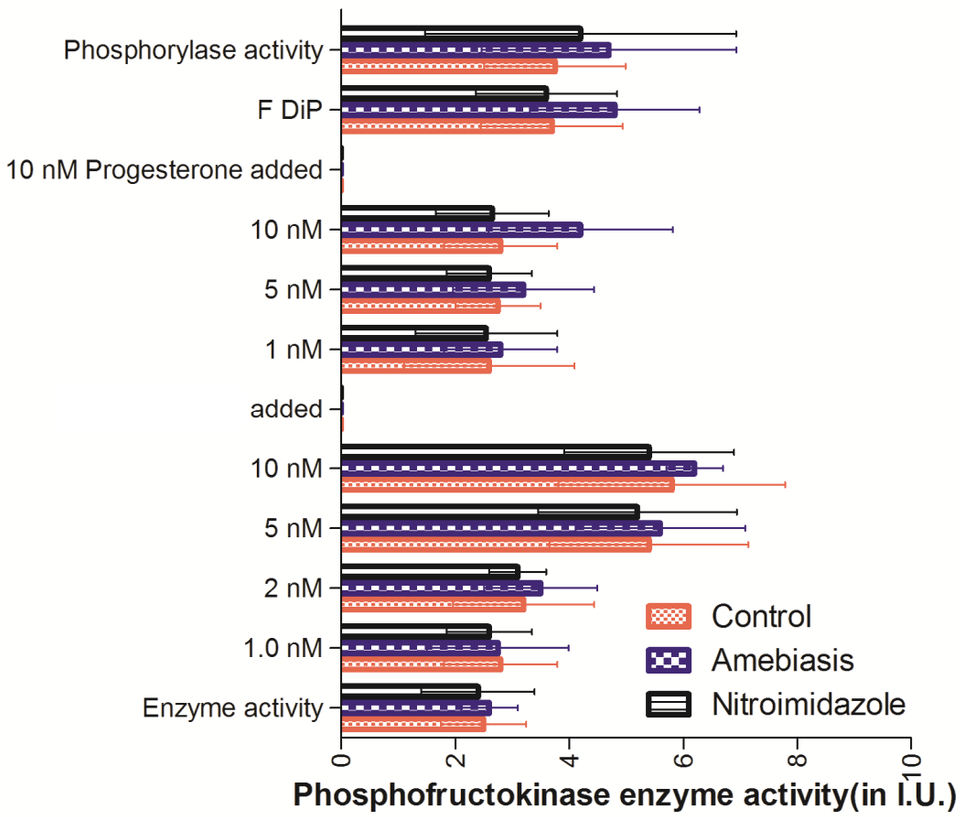


Fig. 9. The effect of nitroimidazole on phosphofructokinase enzyme activity of isolated hepatocytes from control, amebiasis and nitroimidazole groups. Hepatocytes from each group were sedimented and homogenized in Kreb Hanseleit buffer pH 8.0 containing 2.5 mM dithiothretiol, 0.1 mM EDTA and 50 mM NaF for 90 seconds followed by centrifugation at 27000 g for 10 minutes. One unit enzyme activity catalyzed 1 micromole fructose 6 phosphate to fructose 1,6 DiP per minute per mg enzyme protein.

4.3 Phosphofructokinase

Phosphofructokinase enzyme regulation in isolated hepatocytes cultures added with amoebic trophozoites, nitroimidazole and progesterone exhibited specific regulatory behavior. Addition of fructose 1,6 Diphosphate additive exhibited a stimulation of phosphofructokinase activity (see Figure 9). The effect of progesterone was seen as best response at 10 nM in hepatocytes of all groups while fructose 1,6 diphosphate showed maximum effect at concentration of 10 nM on the hepatocytes in presence of trophozoites added in hepatocyte cultures. However, hepatocyte cultures in other groups did not show any alteration (see Figure 9). Similarly, progesterone addition to hepatocytes in presence of trophozoites exhibited maximum fructose 1,6 diphosphate in the medium. The addition of progesterone concentration was within the physiological range (see Figure 9). Progesterone was presumed to effect enzyme activity by influencing intracellular level of various allosteric effectors of enzyme. Fructose 1,6 diphosphate acts as potent activator of enzyme activity. The phosphofructkinase enzyme activity was reported as cyclic AMP dependent

[Pilkis et al.1979, Sanchez-Martinez et al.2000]. However, nitroimidazole added hepatocytes did not respond or responded slow after progesterone addition.

4.4 Phosphodiesterase

Cyclic AMP dependent phosphodiesterase regulation over prostaglandin E_2 and $F_{2\alpha}$ synthesis was stimulated in Kupffer cells added with trophozoits and nitroimidazole. Phosphodiesterase activity in Kupffer cells was dependent upon calcium ion concentration which was regulatory factor for cyclic AMP pool and prostaglandin production. In amoebic trophozoite added cultures, cells exhibited insignificant elevated enzyme activity while nitroimidazole addition did not alter the enzyme activity as shown in Figure 10. The enzyme behavior was similar in nature for its dependence on cyclic AMP secretion, prostaglandin synthesis and calcium ion. However, tropozoites stimulated more cyclic AMP secretion and prostaglandins. It provides the probable mechanism of trophozoite stimulated macrophagal action. Earlier calmodulin dependent adenylate cyclase and phosphodiesterase regulation for cyclic AMP pool have been described [Hidi et al.2000]. Moreover, the stimulated prostaglandin synthesis was described as calmodulin dependent cyclic AMP regulated mechanism [Hidi et al.2000]. Thus it seems that amoebic trophozoite addition with hepatocytes regulates intracellular calcium ion concentration which controls prostaglandin synthesis via phospholipid and arachidonic acid precursors in hepatocytes. Thus synthesized prostaglandins regulate the cyclic AMP intracellular levels. The similar mechanism has been described as "self-limiting" mechanism [Martina et al.2006].

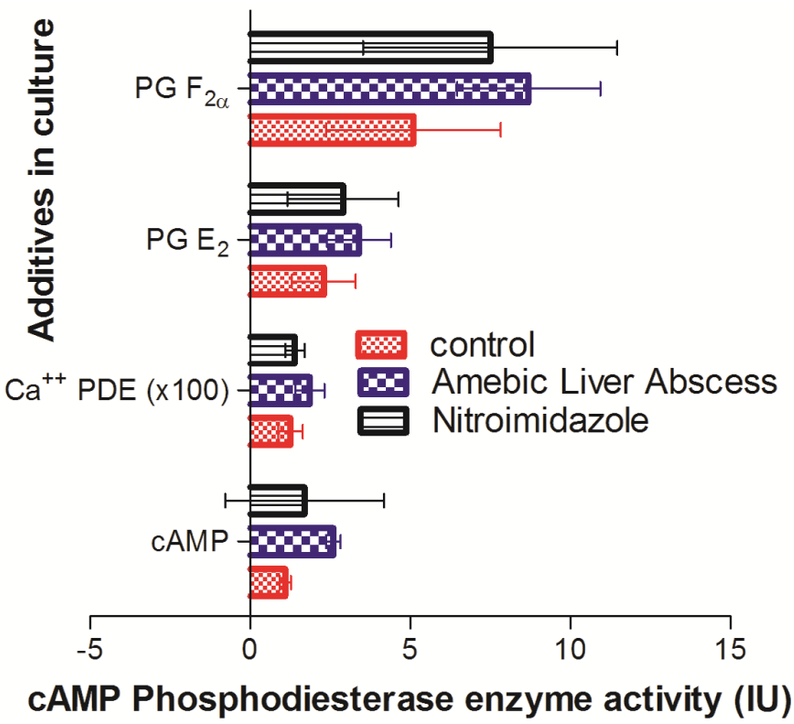


Fig. 10. Cyclic AMP dependent phosphodisesterase activity and secretion of prostaglandin E_2 and $F_{2\alpha}$ in isolated kupffer cells from different groups. The phosphodiesterase enzyme activity in amebic liver abscess group showed highest enzyme activity after additives were added.

4.5 Enzyme inhibition in respiratory burst of liver cells

An initial evidence of liver cell survival at different oxygen pressures is shown in Figure 11 to highlight respiratory burst of enzymes. The inhibited enzymes related with cell respiration are presumably considered responsible of induced respiratory burst (hypoxia) in liver cells. Respiratory burst process including enzyme alterations (superoxide dismutase, glutathione reductase, NADPH oxidase enzyme activity) was suggested as initial Kupffer cell stimulation or respiratory burst by additives and followed by energy and oxygen depletion as possible cause of hypoxia and cell viability loss as important findings in following section.

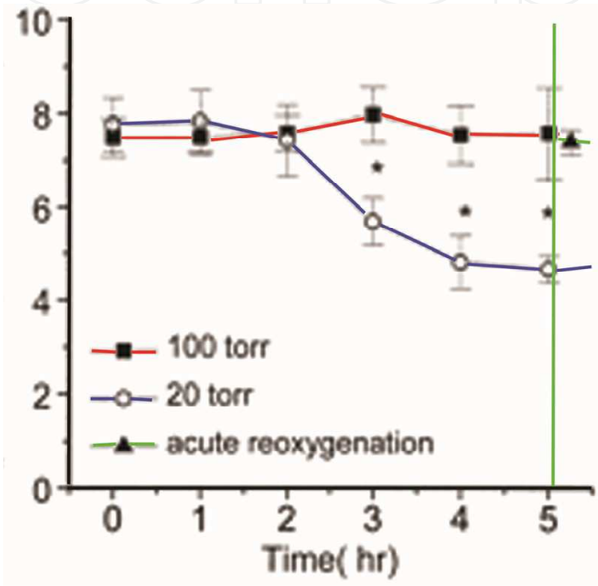


Fig. 11. Effect of oxygen supply (shown in different pressures) to liver cells. Notice the low oxygen supply depletes the oxygen content in cells while high oxygen supply keeps cells in in good survival. For long survival, reoxygenation of cells is needed(see green vertical line).

4.6 Superoxide dismutase enzyme activity in respiratory burst of Kupffer cells

The superoxide dismutase enzyme activity in isolated cells was comparable and showed minimal difference in both control and additive triggered Kupffer cells. Addition of sodium cyanide concentration showed small effect on elevated superoxide dismutase activities in both control and nitroimidazole triggered Kupffer cells. However, Kupffer cells showed elevated respiratory burst activity significantly high after adding 10 nM sodium cyanide in the cell culture medium as shown in Figure 12. However, the response of Kupffer cells with sodium cyanide addition was more in trophozoites added cell cultures at 10 mM sodium cyanide concentration. Zymosan stimulation on superoxide production was insignificantly high in the Kupffer cells in both groups. Earlier stimulation of zymosan induced superoxide anion production in Kupffer cells by sodium cyanide was reported to show the effect of cyanides on cytosolic superoxide dismutase enzyme activity [D'Alessandro et al.2011, Shahhosseini et al.2006]. Moreover, zymosan stimulated superoxide production was reported in Kupffer cells as reaction of cytochrome C reduction [Duan et al.2004].

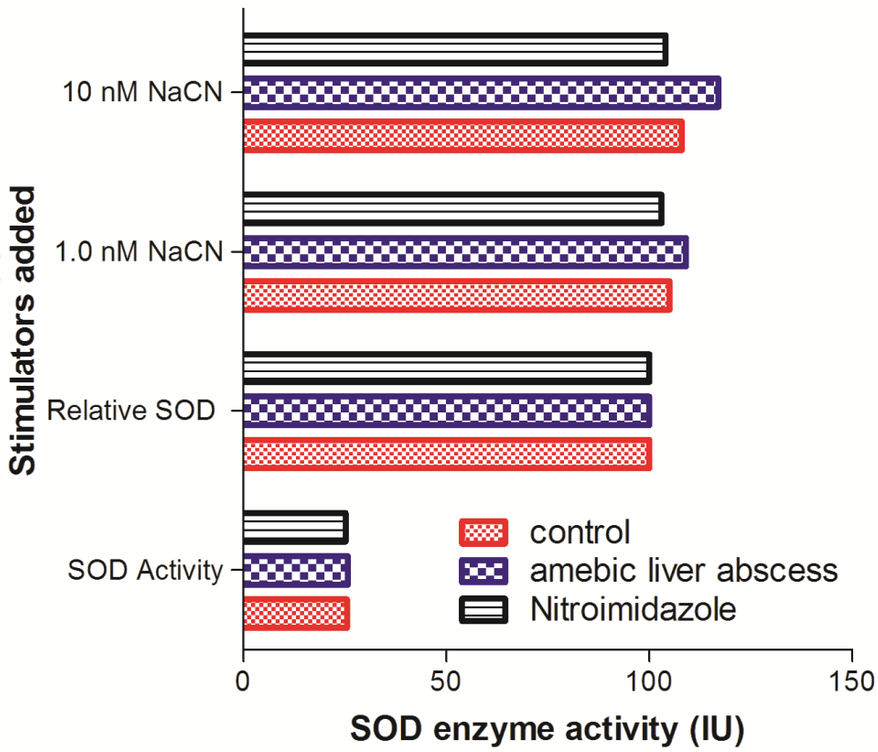


Fig. 12. The enzyme activity was estimated in Kupffer cells cultures added with sheep erythrocytes, trophozoites, nitroimidazole. The Kupffer cells were maintained for 48 hours or more to determine superoxide anion production in presence of 1.0 mM and 1.0 mM sodium cyanide additive added in culture medium. In brief, 3 x 10^5 Kupffer cells were suspended in Kreb's Henseleit buffer containing 5 mM glucose, 3 mM calcium chloride pH 7.4 in presence of 50 µM ferricytochrome in total 1.0 ml reaction mixture and cells were incubated for 15 minutes in presence of different sodium cyanide additives. The reaction was started by addition of zymosan 500 µg per ml to the medium after 10 minutes at 37 °C supernatant was taken, centrifuged at 8000 x g in cold for two minutes and assayed for cytochrome C reductase enzyme activity. Ref. Sharma, R. et al. 2010.

The O_2 superoxide anion radical production as result of respiratory burst activity showed proportionate elevated enzyme activity in isolated Kupffer cells from cultures added with trophozoites and nitroimidazole additives. The active role of reactive oxygen species and superoxide dismutase activity in cultured Kupffer cell damage during hypoxia is shown in Figure 12. Previous study supported these observations on cellular damage and cytotoxicity from reactive oxygen species from superoxide anion acting as a scavenger [Sharma et al.2010]. Another possibility of Kupffer cell toxicity by additives may be associated with cytokines, specifically TNF-α which was associated with the cytotoxicity [Sharma et al.2007]. Previous study also reported a decreased hepatocyte cell viability associated with altered cAMP phosphodiesterase and HMP shunt activity as a result of hypoxia [Carre et al.2010]. Authors suggested that the oxygen insufficiency is possibly a consequence of energy depletion and increased oxygen reactive species [Keeling et al.1982]. Other reports support this view of vasodilatory sensitive ATP depletion in Kupffer cells [Ana et al.2011]. The reactive oxygen species production also seems as another cause of hypoxia as suggested by

the nature of superoxide dismutase as additive cyanide sensitive enzyme. We observed a good reason of association between superoxide anion scavenger, glutathione reductase and NADPH oxidase as cause of cell viability loss leading further to Kupffer cell damage. In this direction, ample evidence supports the possibility of activation of HMP shunt due to depleted ATP and NADH in the Kupffer cells [Grahm, 2000; Spolarics et al. 1991].

4.7 HMP shunt pathway in respiratory burst

Hexose monophosphate shunt activation during respiratory burst in isolated Kupffer cells exposed with amoebic trophozoites and nitroimidazole showed enhanced enzyme activities as shown in Figures 13A-13C. HMP shunt activation is measured in three ways: 1. HMP shunt activity in presence of additives such as latex, sheep erythrocytes, trophozoites; 2. HMP shunt activity as radiolabeled glucose conversion to carbon dioxide; 3. HMP shunt activity as enzyme inhibition.

a. The HMP shunt activity was maximum and it elevated in Kupffer cells after incubation with sheep erythrocytes and trophozoites while Kupffer cells exposed with inert latex particles did not show any activation or change in HMP shunt activity. Sheep erythrocytes and trophozoites both stimulated glucose metabolism via hexose monophosphate shunt activity. Perhaps latex particles being biologically inert nature could not change the cellular activity at all. So, latex particles were phagocytosed without altered HMP shunt activity. It suggested that HMP shunt activity may be a triggering event in Kupffer cell activation dependent not solely upon intracellular fate of ingested material or particles. However, other additives such as zymosan, erythrocytes and trophozoites also showed same response. Previous study reported similar response of zymosan stimulated and iodoacetate inhibited Kupffer cell glucose-6-phosphate dehydrogenase and phosphogluconate dehydrogenase enzymes.[Sharma et al.2010]
b. The HMP shunt activity as [^{14}C] glucose conversion to [^{14}C]-CO_2 liberated product indicated the active inflammation elicited by macrophage response of Kupffer cells [Schorlemmer et al. 1999]. The HMP shunt activity in control and triggered Kupffer cells was measured by [^{14}C]CO_2 release in dpm from labeled [^{14}C] glucose per minute per mg protein in presence of different effectors known as quenching the hexose monophosphates in glycolysis. The HMP shunt activity was similar with no difference (P value > 0.001) in control compared with triggered Kuffer cells with effector and inert latex particles did not alter the HMP shunt activity. The HMP shunt activity was significantly enhanced in triggered Kupffer cells in presence of trophozoites (P value < 0.001) but the HMP shunt enhancement was lesser in presence of erythrocytes. It indicated the dependance of HMP shunt activity on nature of effector either inert or surface active. The latex was inert, erythrocytes were nonvirulent and trophozoits were virulent as a result HMP shunt activity was enhanced in the order of latex < erythrocytes < trophozoits added in the medium of Kupffer cells.
c. The trophozoites further activated the Kupffer cell glucose-6-phosphate and phosphogluconate dehydrogenase enzymes in association of HMP shunt activity. In following section, enzyme inhibition in liver cells is reported in control, hypoxic cells in different groups. Hexose monophosphate shunt activation was enhanced during respiratory burst in isolated Kupffer cells in amoebic trophozoits and nitroimidazole added cultures. In the cultures added with trophozoits and nitroimidazole both, Kupffer cells exhibited significantly elevated HMP shunt activation. The HMP shunt

activity was highly elevated in all groups cultures incubated with sheep erythrocytes and amoebic trophozoits while cells with latex particles did not show any activation. Perhaps latex particles being biologically inert could not change the cellular sinusoidal function activity. Sheep erythrocytes and trophozoits perhaps stimulated the glucose metabolism via hexose monophosphate pathway but latex particles were phagocytosed without altered shunt activity. It suggested that HMP shunt may be a triggering event in Kupffer cell activation dependent not upon intracelllular fate of ingested material or particles [Knook et al. 1980]. The trophozoits added Kupffer cells showed direct interaction with increased glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase enzyme activities as described as a result of enhanced hexose monophosphate shunt activity. Earlier zymosan stimulated and iodoacetate additives inhibited Kupffer cell glucose 6 phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities have been reported [Heuff et al. 1994].

- **Inhibition of Glutathione Reductase enzyme activity in respiratory burst:** The glutathione reductase GSH specific enzyme activity was measured in hypoxic hepatocytes in presence of different GSH enzyme inhibitors such as DEM, antimycin, BSO as additive effectors. To demonstrate the dependence of enzyme activity on exposure time of inhibitors, the hypoxic hepatocytes were incubated in presence of effectors and enzyme activity was measured in medium. At 0 hour, the enzyme specific activity was same in hepatocytes in all groups. After 24 hours, the activity in hepatocytes was inhibited maximum by actinomycin while DEM and BSO inhibited the enzyme at similar extent and hypoxic hepatocytes exhibited no difference in enzyme activities (P value > 0.001) (see Figure 14). After 48 hours, the GSH enzyme activity was inhibited maximum by addition of BSO and minimum by antimycin in cell cultures. However, the behavior pattern of enzyme inhibition by DEM and BSO was similar. After 72 hours, the inhibition pattern of GSH enzyme activitity by inhibitors was same as after 48 hours. After 96 hours, all inhibitors showed maximal inhibition and hypoxic hepatocytes showed same specific activities in all groups. Antimycin inhibitor exhibited lesser inhibition activity of hypoxic GSH enzyme initially during 24-48 hours.
- **Inhibition of NADH oxidase in respiratory burst:** The respiratory burst activity was described as increased activity in liver macrophages during hepatic cell damage showing increased oxygen consumption, stimulation of HMP shunt and elevated superoxide production with activated membrane NADPH oxidase enzyme as one of the mode of phagocytosis [Dusting et al. 2005]. Moreover, our experiments suggested the specific roles of additives on respiratory burst biomarkers: 1. The increased sodium cyanide concentration showed stimulation of superoxide dismutase activity; 2. The live erythrocytes and trophozoites showed HMP shunt stimulation while inert latex particles showed no effect on HMP shunt activity suggesting the association of additive nature as determinant of the HMP shunt activity or trio-enzyme inhibition; 3. The glutathione plays a critical role in surviving Kupffer cells during hypoxia and indicates the stimulated reactive oxygen species in respiratory burst activity [Bhatnagar et al.1981]. All these respiratory burst biomarker activities showed dependence on the nature of additives in corroboration with earlier reports [Kumar et al.2002; Forman et al. 2002; Ding et al. 1988]. In present chapter, both HMP shunt activity and respiratory burst activity (superoxide dismutase, glutathione reductase, NADPH oxidase enzyme activity) are outlines for suggested initial Kupffer cell stimulation by additives and later followed by energy and oxygen depletion as possible cause of hypoxia and cell viability

loss as important finding. However, association of Kupffer cell stimulation and hypoxia is not conclusive and needs further investigation.

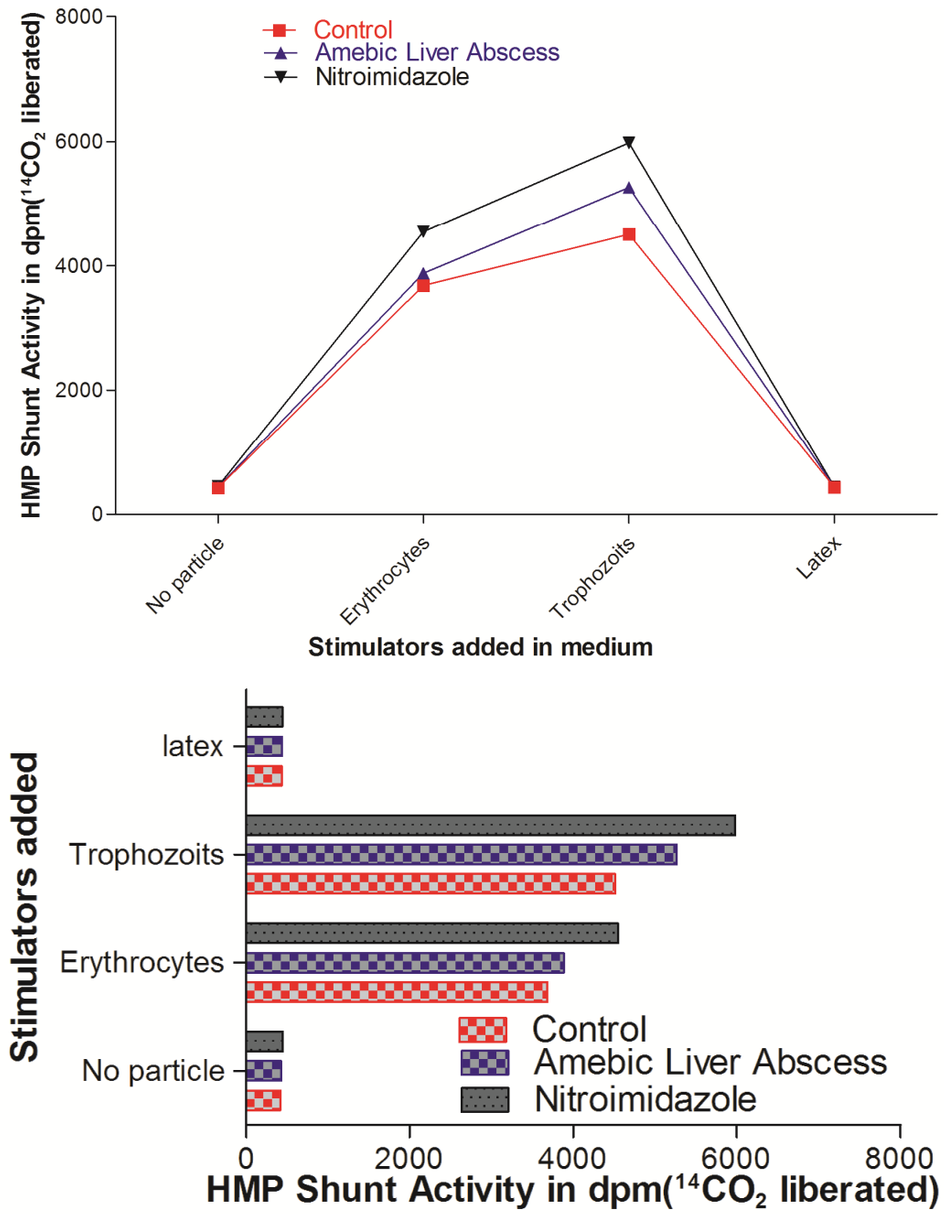


Fig. 13. Hexose monophosphate shunt activity is shown in isolated Kupffer cells from ameba infected and nitroimidazole treated cells in presence of erythrocytes, trophozoits and latex additives (see panel on top). In 3.0 ml medium, 1 micro Curie ^{14}C glucose was added and simultaneously particles added with Kupffer cells in ratio 5:1(particle:cell) for erythrocytes, 2:1 for amebic trophozoits and 40:1 for latex. After one hour, 2 ml of cell free medium soaked by filter paper wetted with 100 µL of 10% KOH in counter well which further was added with 200 µL 1.0 N H_2SO_4 and after 15 minutes filter paper strip elute in tritosol were measured for scientillation counts. The trophozoits showed maximum activity while inert latex beads did not show any change in activity(see panel at bottom).Ref. Sharma, R. et al. 2010.

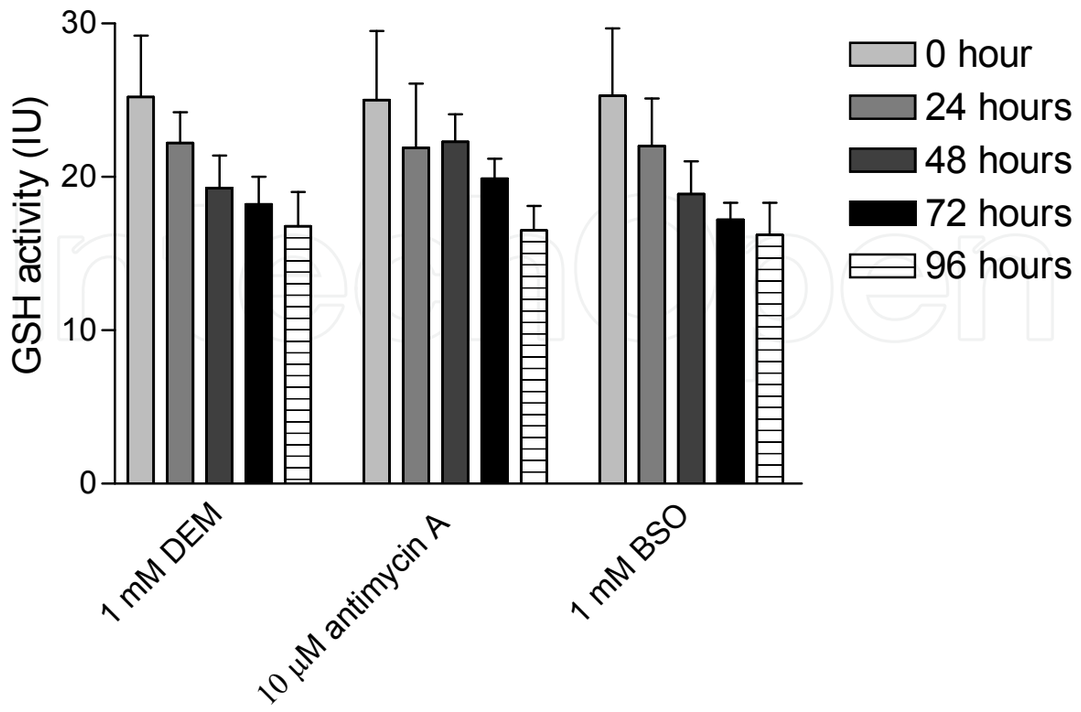


Fig. 14. Effect of added stimulators on Glutathione reductase enzyme activity in hypoxic hepatocytes in culture. Ref. Sharma, R. et al. 2010.

4.8 Cytochrome oxidase

Cytochrome C oxidase was determined based upon oxidation of ferrocytochrome C by decrease in absorbance at 550 nm on spectrophotometer. In 3 ml reaction mixture containing 66 mM potassium phosphate buffer pH 7.4. 20µM reduced cytochrome C and 20µl mitochondrial protein reaction was followed at 30°C for two minutes. Reading was recorded after two minutes for absorbance of completely oxidised cytochrome C by adding 0.05 M potassium ferricyanide. The activity of cytochrome C oxidase was expressed in n moles of cytochrome C oxidase/mg protein/min which is equal to concentration of cytochrome C x K divided by concentration of protein. For calculation of cytochrome C concentration a millimolar extinction coefficient of 19.1 was employed for reduced vs oxidized cytochrome C at 550 nm as described by Emmelot et al.1964.

4.9 Inhibition of NADPH cytochrome P450 reductase

Cytochrome P 450: Cytochrome P 450 was measured by spectrophotometer in absence or presence of carbon monoxide and determined by the method [Guengerich et al.2009]. In brief, 0.1 ml microsomal fraction in two cuvettes test and standard which mere added 1,5 mg sodium dithionite and carbon monoxide passed in only test sample cuvette for 25 seconds and same repeated again and ΔA recorded at 450 nm for cytochrome P 450 content in n moles per mg protein calculated by extinction coefficient 91 mM/cm path length.

4.10 Inhibition of adenylate cyclase

Adenylate cyclase regulatory properties: Adenylate cyclase enzyme in isolated hepatocytes has been reported as tool of regulatory studies of hormones, enzymes in the presence of Gs bound receptors [Small et al. 2000]. Membrane adenylate cyclase activity is defined due to its multi-pass transmembrane protein made of two cytosolic segments as shown in Figure 15. These segments play active roles in association of $G_{s\alpha}$-GTP. The GTP induced $G_{s\alpha}$ binding with adenylate cyclase can be a key mechanism of adenylate cyclase stimulation or inhibition during progesterone bound α-adrenergic receptor [Sunahara et al. 1997]. The possible mechanism of adenylate cyclase activation or inhibition by progesterone is shown in Figure 15. The membrane adenylate cyclase activity can be an indicator of membrane viability. However, the enzyme activity depends on the type and nature of effecter. The behavior of liver cells in culture in the presence of effectors may indicate the regulatory effect of stimulators *in vivo*. However, in cultures, hepatocytes and Kupffer cells may likely experience different physiological environment different from *in vivo*. In controlled liver cell culture conditions of medium, the liver cells showed adenylate cyclase stimulation in a specific manner by effectors such as progesterone, nitroimidazole, GTP, GITP, and prostaglandins [Aoshiba et al. 1997]. The receptors for prostaglandins interact with $G_{s\alpha}$ subunits. $G_{s\alpha}$-GTP complex binds with α-adrenalgic receptor made of seven subunits as shown in Figure 5. Binding of receptor with $G_{s\alpha}$-GTP protein inhibits adenylate cyclase activity and cAMP formation. The present study highlights the behavior of liver cells and the characteristic features of their cyclic AMP dependent adenylate cyclase regulatory system in culture.

Progesterone modulation of adenylate cyclase activity: Progesterone modulates the activity of adenylate cyclase and adenylate cyclase response is hormone sensitive [Gilman et al. 1984]. The progesterone stimulates the receptor activating the adenylate cyclase by stimulatory $G_{s\alpha}$-GTP complex resulting with formation of cAMP. Hepatocytes with progesterone added in medium exhibited the enhanced enzyme activity. The addition of GTP stimulator did not change the adenylate cyclase activity. In contrast, GITP stimulated cells exhibited an apparent adenylate cyclase activation. Unlike the adenylate cyclase enzyme activity in the presence of different effectors in stimulated hepatocytes, the rates of cyclic AMP production was delayed in control hepatocytes. However, progesterone addition to hepatocytes did not exhibit significant delay in GITP stimulated adenylate cyclase activity. Thus reason can be speculated that progesterone binds with stimulatory receptors on hepatocytes which lead to increase in intracellular cyclic AMP accumulation through adenylate cyclase activation via guinine nucleotide regulated mechanism [Cohen-Tannoudji et al. 1991]. The Kupffer cells also showed similar guanine regulatory enzyme properties.

The progesterone response of adenylate cyclase activity is a two phase process [Ko, et al.1999; Martin et al. 1987]. First, progesterone receptor and catalytic subunit (C complex) of adenylate cyclase occupy the place on membrane followed by $G_{s\alpha}$ protein interaction with enzyme to make G protein C complex [Ko et al. 1999]. However, the addition of GITP stimulated the adenylate cyclase to a greater extent in hepatocytes more than pre-exposed hepatocytes to progesterone. Recently, G protein-enzyme complex formation by GTP/GITP stimulation has been reviewed [Niu et al. 2003].

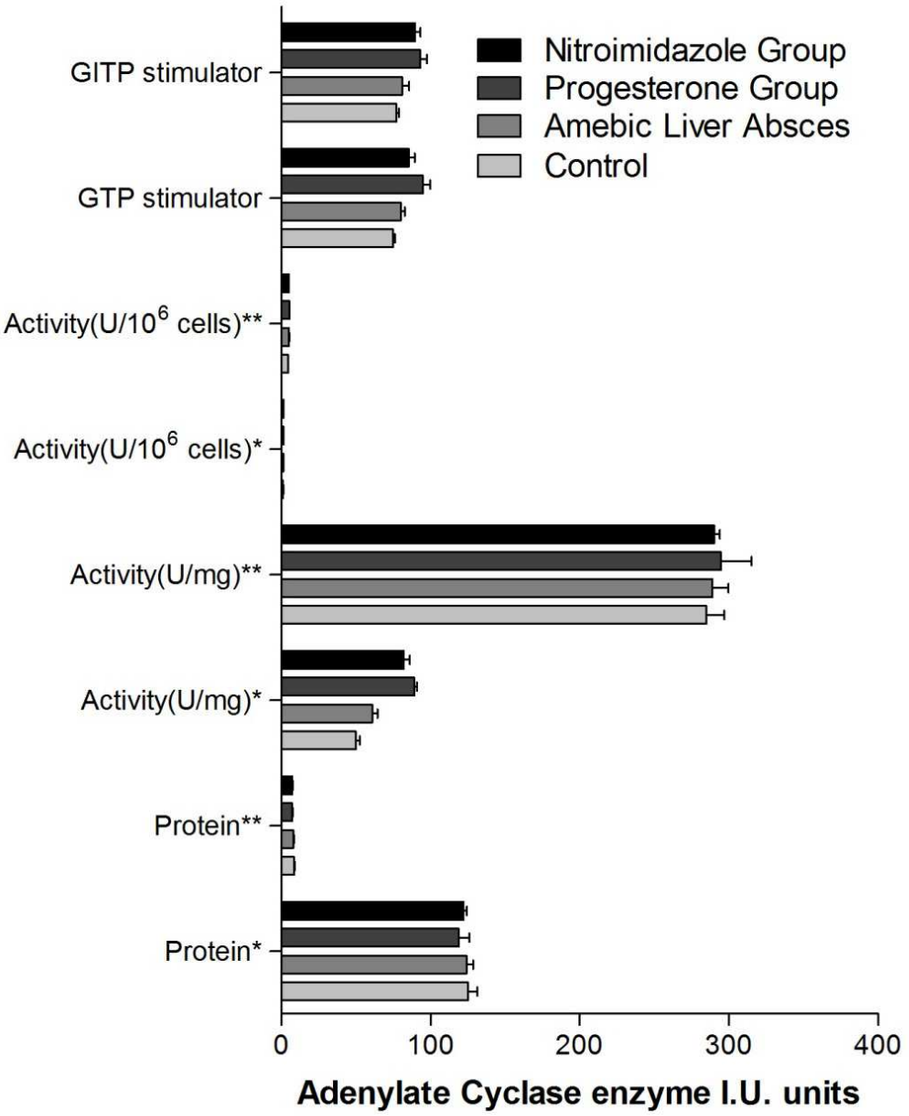


Fig. 15. Adenylate cyclase enzyme activity is shown in control, nitroimidazole, progesterone and amoebic liver abscess recruited liver cells in presence of GITP/GTP stimulators, protein* additives. Ref. Sharma, R. et al. 2010.

Nitroimidazole stimulation of adenylate cyclase activity: Nitroimidazole is recently emerged as anti-tumor and radiosensitizer compound. It is used in tumor imaging, therapy and anti-amoebic applications. Nitroimidazole has phenolic rings and phenolic side chains as shown in Figure 6. So, it is obvious that the side chains interfere with the dephosphorylation of ATP by phosphodiesterase enzyme. It appears to result with inhibition of hepatocyte adenylate cyclase catalytic subunit function in the presence of nitroimidazole. The cytotoxicity of nitroimidazole is poorly studied and reported. However, its anti-amoebic effect has been proven to decrease hepatic and intestinal infection rate. The nitroimidazole is widely reported as single dose in treatment of amoebic liver abscess. Moreover, nitroimidazole induced cytotoxic effect on human hepatocytes are still not known. DNA strand breakage,

energy regulatory enzymes, phagocytosis, and immune response have been reported as nitroimidazole induced cytotoxic effects in animals [Zheng et al. 1997; Whitaker et al. 1992; Singh et al. 1991]. The role of nitroimidzole is understood to restore energy regulatory process involving adenylate cyclase, phosphodiesterase, cAMP formation in hepatocytes and reported as hormone and GTP specific in action. The nitroimidazole derivatives inhibited anterior pituitary cell function apparently by their direct effect on the catalytic subunit of the adenylate cyclase holoenzyme [Stalla et al. 1989]. The nonparenchymal Kuffer cells play a significant role of phagocytosis sensitive to prostaglandins. Recently, nitroimidazole derivatives have been identified as anticancer potential agents. Still the role of imidazoles as anticancer agent is not established [Anderson et al. 2006]. The indicated study the association of imidazole ring structure with the cyclic AMP dependent adenylate cyclase catalytic subunit regulatory mechanism in liver cells.

5. Inhibition of drug metabolizing enzymes in isolated liver cells

Drug metabolizing enzymes are abundant in microsomes and lysosomes. Most of these enzymes are elevated or secreted at the sites of inflammation, disease conditions and in presence of foreign bodies. An elevated enzyme in cells and secretion from lysosomes or microscomes action is in defense by 'suicide action' of microbody against disease or foreign bodies. Drugs inhibit these enzymes or bring back any disease or inflammation induced effect on enzyme activity in liver cell. Characteristic inhibition of enzyme activities in different groups by nitroimidazole are shown in Figure 15. Recently, we reported the enzyme inhibition by nitroimidazole in detail [Sharma 2011a, Sharma 2011b]. In general, lysosomal enzymes are peptidases, act at acidic pH, and participate as terminal scavengers of harmful anions, radicals and peptides. For details, read section 2.

Nitroimidazole inhibits the liver enzymes by making adduct for glyoxyl-glutamine metabolism in liver cells as author described in detail [Sharma, et al. 2011].

6. Phagocytosis

Although the clearance and distribution of ligand molecules in circulation represent the function of hepatic sinusoidal cells, these mechanisms reveal a network that is more intricate than would at first seem, since several receptors are common to not only one type of cell, but also to two or three types of cells in the liver. In the case of latex particles in which their uptake by a particular cell type seems to be determined by their size, sinusoidal endothelial cells are able to internalize particles up to 0.23 microns under physiologic conditions, in vivo, and larger particles are taken up by Kupffer cells. However, when the phagocytic function of Kupffer cells were impaired by frog virus or alcohol, the endothelial cells were reported to take up particles larger than 1 micron in diameter after the injection of an excess amount of latex particles. Endothelial cells would thus constitute a second line of defense in the liver in that they remove foreign materials from the blood when Kupffer cell phagocytic function is totally disturbed [Schoremmer et al. 1990]. This potential role may not, however, be fully expressed under physiologic conditions when Kupffer cells are active in clearing foreign substance from the circulation. The functions of liver sinusoidal cells are varied and complex and these cells can be regarded as "a sinusoidal cell unit." This cellular interaction must be taken into account for any quantitative analysis [Kampas et al. 1999].

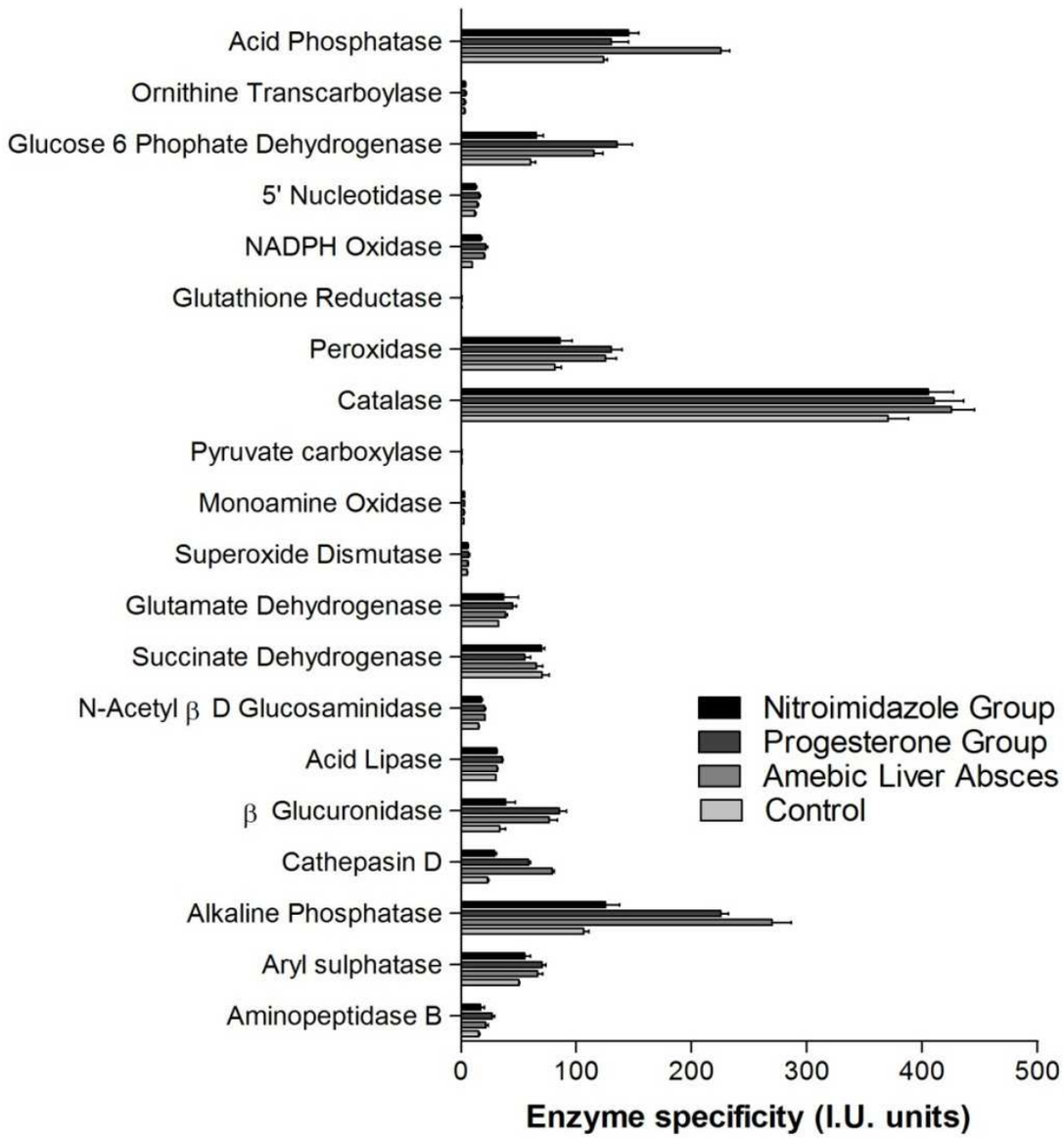


Fig. 16. Lysosomal and microsomal enzymes in isolated nonparenchymal liver cells are shown in control, nitroimidazole, progesterone, amoebic liver abscess recruited livers. Note inhibitory action of nitroimidazole on liver cells to bring normal activities in comparison to amoebic liver abcess recruited cells. Ref. Sharma, R. et al. 2009.

- **Enzyme inhibition in phagocytosis**: In our experiments, liver cell cultures were mixed with zymosan (7:1 particle cell ratio); erythrocytes (5:1 particle cell ratio); amebic trophozoits (2:1 particle ratio), latex (20:1 particle cell ratio) and nitroimidazole was added 1 μM/10^6 cells. Kupffer cell phagocytic activation in isolated Kupffer cells from human livers (added with amoebic trophozoits) was elevated 2-3 fold over normal Kupffer cells. Kupffer cells incubated with zymosan and trophozoits exhibited maximum phagocytic activity while with latex and sheep erythrocytes these exhibited minimum and insignificantly increased activity respectively. Increased phagocytic activity was time dependent which was exhibited by β-glucuronidase, lactate dehydrogenase and plasminogen activator activity. Phagocytosis of zymosan and sheep erythrocytes triggered the immediate release of β glucuronidase, stimulated synthesis of cellular lactate dehydrogenase and induced delayed production with secretion of plasminogen activator. Inhibition of these enzymes as nitroimidazole response is shown in Figures 16-18, in detail. Major findings were: 1. effect of nitroimidazole and latex were minimum. Similar observations were reported in earlier study [Kamps et al. 1999, Bhatnagar et al. 1981, Kuttner et al. 1982]; 2. nitroimidazole was biotransformed into water soluble products and cleared by drug metabolizing enzyme system of Kupffer cells; 3. latex particles being inert confirmed the independence of Kupffer cell activation from intracellular fate of ingested material; 4. amoebic trophozoits with their toxins or specific membrane surface signals stimulate Kupffer cells for phagocytosis resulting early release of lysosomal hydrolases than the release by other particles [Virk et al 1989, Virk et al. 1988]. However, increased lysosomal enzymes in Kupffer cells incubated with amoebic trophozoits were reported as a consequence of phagocytic activation [Froh et al. 2003]. Recently, these phagocytic activation changes due to nitroimidazole have been reported as associated with prostaglandin synthesis and their release [Spolarics et al. 1997].
- The glucuronidase activity showed the enhanced enzyme activity trend in all additives but different enzyme enhancement for different additives. In control cells, the activity enhanced during 24-36 hours in incubation. The erythrocytes showed most vulnerability to Kupffer cells in initial 24 hours. Zymosan beads showed slow response to phagocytosis while latex remained inert to Kupffer cells. The nitroimidazole showed normal enzyme pattern. It was interesting that maximum rate of phagocytosis was observed during 24 and 36 hours except erythrocytes.
- Lactate dehydrogenase enzyme activity showed the enhanced enzyme activity trend in all additives except latex additive but different enzyme enhancement for different additives. In control cells, the activity enhanced during 12-36 hours in incubation. The erythrocytes showed most vulnerability to Kupffer cells in initial 24 hours. Zymosan beads showed maximum response to phagocytosis while latex remained inert to Kupffer cells. The nitroimidazole showed inhibitory enzyme activity in Kupffer cells but after 24 hours the response was stimulatory to enzyme activity (delayed enhanced enzyme stimulation as a result phagocytosis). It was interesting that maximum rate of phagocytosis was observed during 48 hours. However, the leaked out LDH activity represented the probable phagocytic activity of Kupffer cells relationship with time. Nitroimidazole addition in incubation medium perhaps showed the minimizing amoebic virulence [Sharma 2010]. Earlier lysosomal enzyme release with increased LDH cellular levels has been reported [Koppele et al. 1991].

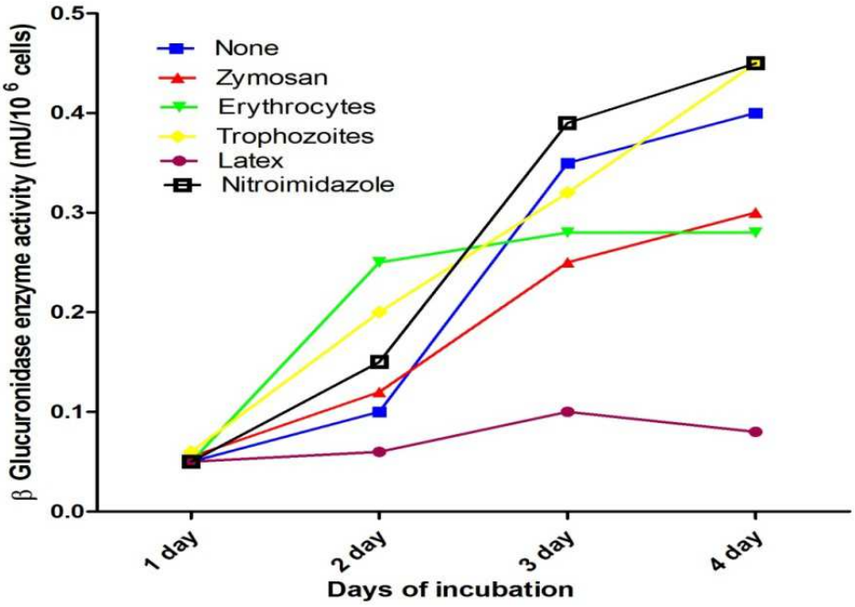


Fig. 17. Phagocytic activity of Kupffer cells is shown as β glucuronidase enzyme activity of cells after adding zymosan, erythrocytes, entamoeba histolytica trophozoits, latex beads additives, and nitroimidazole as drug in cultures of Kupffer cells from liver biopsy samples. The enzyme activity was measured after 1, 2, 3, 4 days of incubation. Each point value represents the average of 3 readings of observations. Ref Sharma,R. et al. 2009.

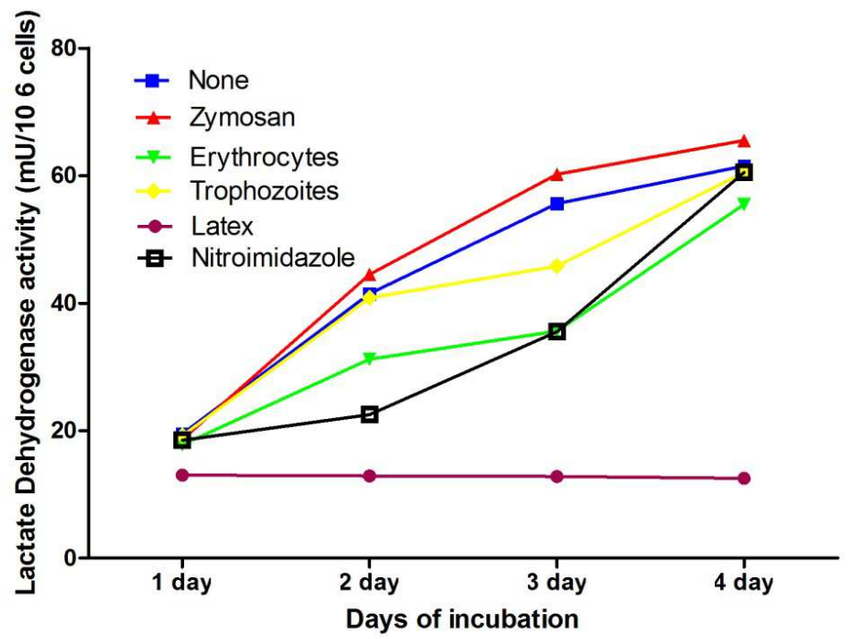


Fig. 18. Phagocytic activity of Kupffer cells is shown as lactate dehydrogenase enzyme activity of cells after adding zymosan, erythrocytes, entamoeba histolytica trophozoits, latex beads additives, and nitroimidazole as drug in cultures of Kupffer cells from liver biopsy samples. The enzyme activity was measured after 1, 2, 3, 4 days of incubation. Each point value represents the average of 3 readings of observations. Ref Sharma,R. et al. 2009.

- The plasminogen activator induced lysis of Kupffer cells showed the enhanced plasminogen activity only after 36 hours. Initially the activity trend in all additives was same and immune to any additive added. In control cells, the plasminogen activity remained unaltered while kupffer cells showed immunity to zymosan and latex additives throughout the incubation period. However Kupffer cells showed sharp enhancement of plasminogen activity after 36 hours during 36-48 hours in incubation. The trophozoits showed maximum effect on plasminogen activator in Kupffer cells after 36 hours. Plasminogen activator induction has been reported dependent on de novo enzyme synthesis in stimulated macrophage by endotoxins and asbestos [Nagaoka et al. 2003].

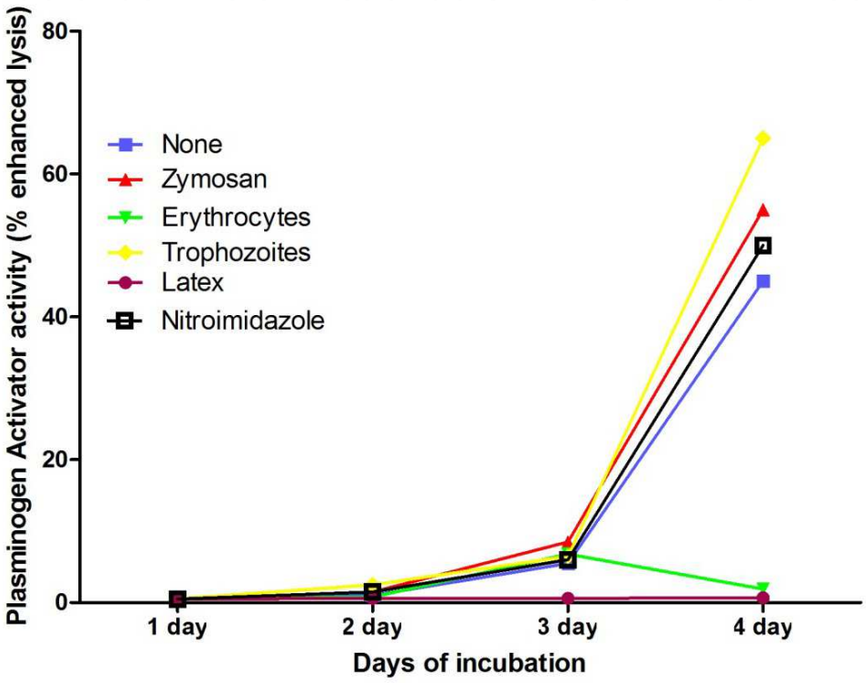


Fig. 19. Phagocytic activity of Kupffer cells is shown as plasminogen activator activity of cells after adding zymosan, erythrocytes, entamoeba histolytica trophozoits, latex beads additives, and nitroimidazole as drug in cultures of Kupffer cells from liver biopsy samples. The enzyme activity was measured after 1, 2, 3, 4 days of incubation. Each point value represents the average of 3 readings of observations. Each point on graph represents the activity as extent of cell lysis in percent increase over basal value of 100. Ref Sharma et al. 2009.

7. DNA synthesis in Kupffer cells

Stimulated DNA synthesis is considered as defense mode. From physiological point of view, it is true that drastic change in enzymatic pattern in liver regeneration permits DNA synthesis. Major enzyme players are DNA polymerase and DNase (allosteric complex enzyme) in mitosis. We present five sequence steps of events: deoxyribonucleotides, ribonucleotides, DNA, RNA and proteins (see Figure 20) [Bresnick et al.1970].

- DNase exposes 3′-OH end groups thus increases capacity of chromosomal DNA in vivo. DNA polymerase plays a passive role in DNA synthesis in presence of high

chromosomal DNA and deoxyribonucleotides. Priming DNase action on native and denatured DNA initiates DNA synthesis. Earlier study reported that arginase enzyme acts strong DNA synthase inhibitor perhaps due to lack of neosynthesized histone (arginine-rich histone) by reducing priming state of chromosomal DNA. DNA sysnthesis by means of additive or replicative DNA polymerase needs 3′-OH roup and polymerization proceeds from 5′-phosphate end to the growing 3′-OH end.

- Increased RNA synthesis also increases ribonucleotide pool, rise in RNA polymerase activity, increase in polyamine synthesis and s-RNA methylation. The temporal relationship between kinetic changes in the DNA channel and the RNA channel seem to indicate that RNA channel is primer of DNA channel. In other words, cyclic AMP level may initiate both DNA and RNA synthesis beginning at the nucleotide level under hormonal regulation. Best example of elevated RNA sysnthesis in liver regeneration is elevated orotate pyrophosphorylase/decarboxylase to keep CTP/UTP pool in presence of UTP/CDP coenzymes for glycogenesis and lipogenesis respectively. UMP stimulated orotate-tRNA labeling around 18s-28s RNA complex step is preceded by an increase in tRNA methylation by tRNA Methylase enzyme. For its action, short chain of monocistronic mRNA on 18s component acts as primer for synthesis of constitutive RNA and export protein synthesis. Nobel Prize was awarded in 2009 on resolving the intricacies of ribosomal subunit interplay and consequent role of DNA/RNA Polymerase enzyme reactions in protein synthesis during drug induced regeneration [Schmeing et al.2009]. However, other enzymes RNase activity, cataionic polyamine play role in stabilization of ribosomal RNA (labeled methionine to spermidine) in protein synthesis. Increased polymine synthesis enhances ornithine decarboxylase to increase amino acid pool in presence of cAMP induced prostaglandins mediated growth hormone. Moreover, growth hormone stimulates thymidine kinase in liver. Author describes DNA synthesis in isolated Kupffer cells during liver regeneration after nitroimidazole treatment in amoebic liver abscess in following section.
- Kupffer cells isolated from human amoebic liver abscess liver-biopsies and progesterone treated Kupffer cell in cultures were induced for DNA synthesis by adding nitroimidazole. This offered a simple model to study the mechanism of cell DNA induction and interdependence between two cell populations. Earlier such interactions between cells were reported regulated by thymidine kinase (thymidine pathway) and ribonucleotide reductase (thymidylate pathway)[Bresnick et al.1970]. Conceptually, immediately after disease in regeneration, liver cell gets confronted a challenge of increased protein synthesis for cell division involving metabolic enzymes leading to DNA synthesis (binding of ribonucleotides with deoxyribonucleotides). Two pathways play role in this process by Thymidine pathway and thymidylate pathway. Thymidylate pathway for DNA synthesis needs dTTP supply, thymidine kinase, TDP kinase to keep TMP pool. Basically, a repressor molecule (thymidine) inhibits transcription of thymidine kinase by allosteric regulation. Thymidine kinase is a TTP insensitive dimeric form (one catalytic site and other allosteric site) means K_m for enzyme decreases with elevated ATP concentration (ATP positive effector and TTP negative effector). dimeric form and shows sigmoid behavior of thymidine kinase [Barroso et al. 2005]. Activation of thymidine kinase also depends on cyclic AMP activated protein kinase. On other side, ribonucleotise reductase reduces CDP in presence of dCMP deaminase to make dUMP (a substrate for thymidylate synthesis) by thymidylate sysnthetase in presence of N^5,N^{10} hydroxymethyl-FH_4. dCMP is good enzyme inhibitor of thymidylate sysnthetase and activator of folate reductase to keep net high TMP pool. Ribonucleotide synthesis is regulated by

ribonucleotide reductase. Increase in CTP build up dCTP, purine deoxyribonucleotides and TTP pools[Cheung et al.1970].

- Several types of growth factors have been reported as regulatory substances participating in liver regeneration process. Stimulation of DNA synthesis in Kupffer cells by amoeba trophozoites is shown here as response induced by colony stimulating factor. Since measured increase of radio label uptake by increasing amoeba trophozoite concentration in medium reflected an increase of cell number entering into S phase rather than higher rate of [^{3}H] TdR incorporation seen in all the cells, it was possible that different Kupffer cells normal or pretreated with amoeba (from these different liver cell populations) may vary in susceptibility to colony stimulating factor (CSF). High concentrations of amoeba caused a reduction of thymidine uptake and remarkably reduction was measured on whole cover slip scintillation counts. It reflected lower incorporation of [^{3}H] TdR into each DNA synthesizing cells. This phenomenon may be due to reduced capacity of Kupffer cells uptake as a result of DNA synthesis but it requires further inhibitor study or presence of CSF. Thus higher concentration of amoebae trophozoites caused a reduced Kupffer cell uptake capacity during CSF production.

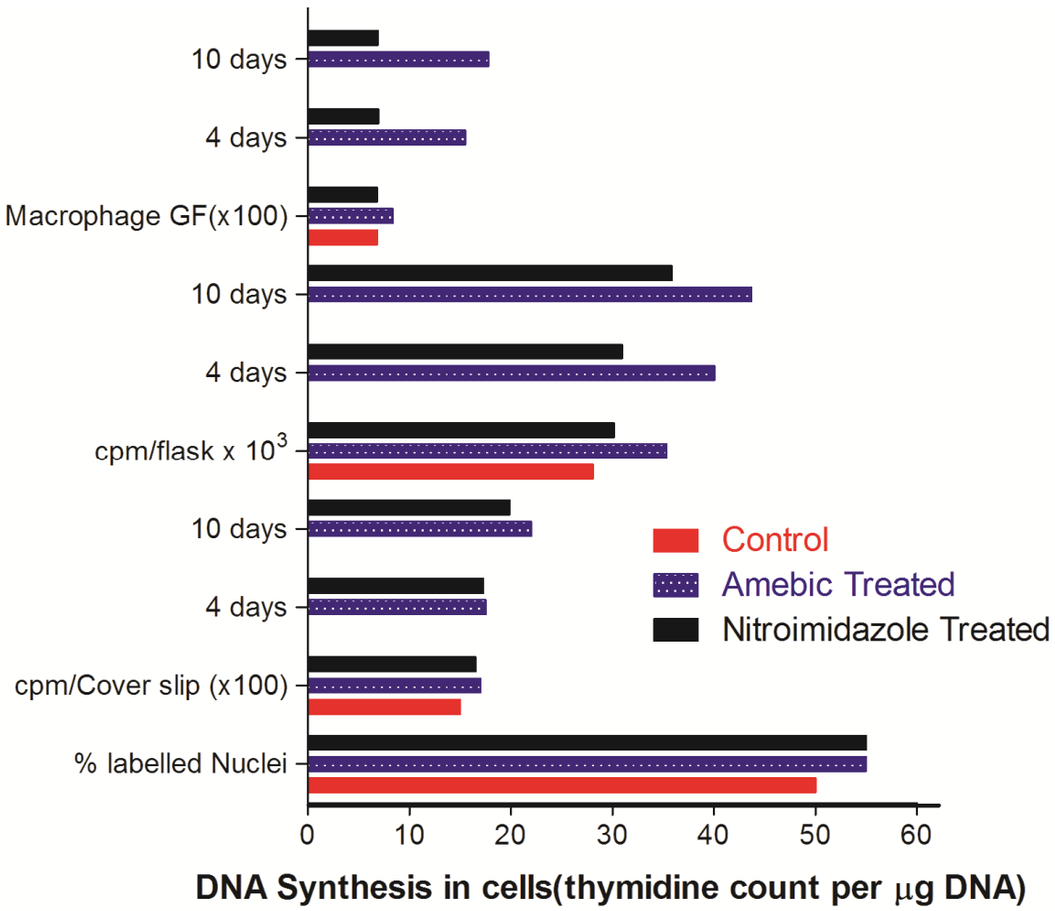


Fig. 20. DNA synthesis in liver cells in culture medium is shown in presence of macrophage growth factor measured as cpm/flask and cpm/cover slip during 4-10 days. Notice the nitroimidazole induced inhibition of DNA synthesis in amebic treated cells.Source: Sharma, R.(1990) Ph.D dissertation.

8. Inhibition of liver lysosomal enzymes in protein metaboilism

Isolated lysosomal preparations from L[1-^{14}C] leucine (25 µCi per 100 gm wt) intraperitonially injected human Kupffer cell cultures exhibited percent degradation at pH 3.0, 4.0,5.0, 6.0,7.0, 8.0 and 9.0 at temperatures 10 °C, 20 °C, 30 °C, 40 °C, 50 °C and 60 °C, in presence of amoebic trophozoits after 15 minutes(short lived) and 15 hours(long-lived) addition as shown in Figures 21. Major players in this liver regeneration process are ribosomes (sitting on endoplasmic reticulum), GTP (ribotide pool) and coenzymes for ribotide polymerase enzymes (a balance in thymidine kinase and thymidylate synthetase). Other enzyme inhibition is LDH-M type gets converted to LDH-H type or lactate → pyruvate (accelerated glycolysis). In simultaneous other set of experiment, Kupffer cell lysosomes in presence of leupeptin, pepstatin inhibitors and nitroimidazole in 100 µg, 100 µg and 50 mM per ml concentration respectively showed aryl sulphatase enzyme activity changes (see Figures 21,22) in the presence of 0.3 M sucrose at 37 °C for 60 minutes at pH 5.5 in incubation mixture of Kupffer cell lysosomes and additives. Proteolysis rate and degradation of short and long lived proteins in isolated lysosomes from the Kupffer cell

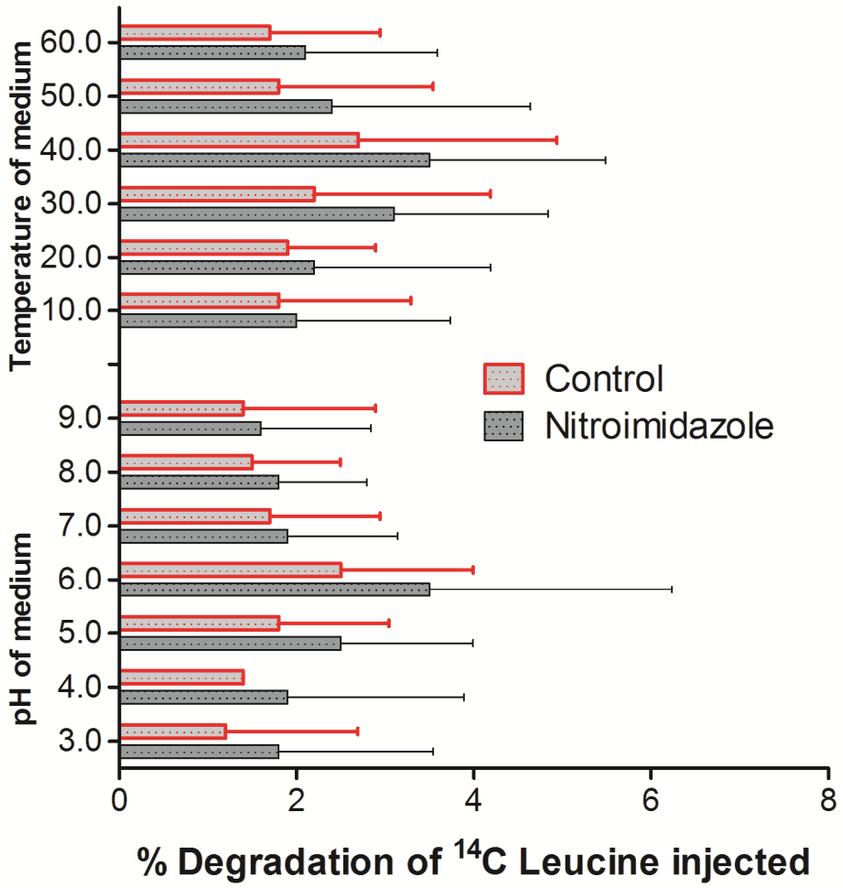


Fig. 21. Figure shows the effect of of radiolabeled [^{14}C]-Leucine in Kupffer cultures represented by aryl sulphatase enzyme activity in normal and ALA recruited cells at different pH and temperatures. Notice the low enzyme activity in normal cells while maximum leucine degradation was at pH 6 and 40 °C.

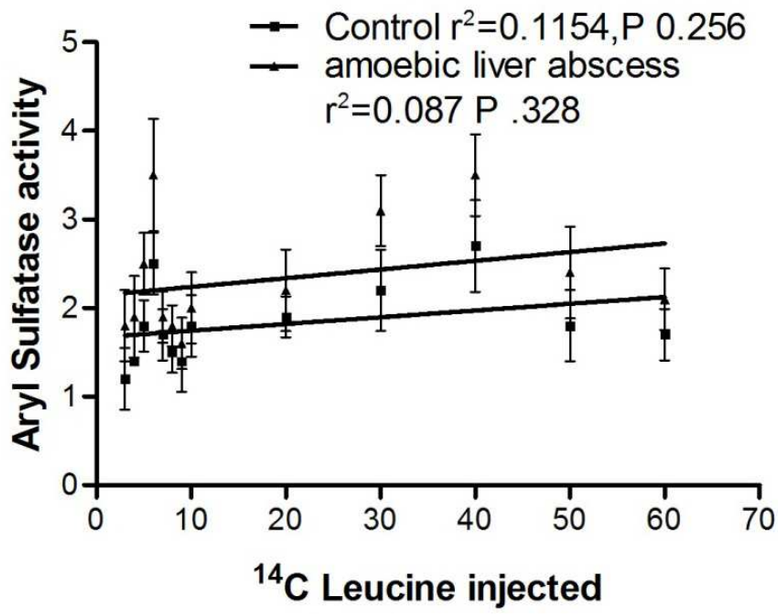


Fig. 22. Figure shows the effect of radiolabeled [^{14}C]-Leucine in Kupffer cultures represented by aryl sulphatase enzyme activity in normal and ALA recruited cells. Notice the threshold of leucine turnover at 40 IU in terms of enzyme activity. Control cells showed slow % protein degradation and low enzyme concentration. Source. Sharma 1990. Ph.D dissertation.

cultures added with trophozoits, nitroimidazole, exhibited increased protein degradation. Lysosome as proteolytic component was shown to contribute for protein degradation as earlier various reports indicated protein size dependence [Neff et al.1990]. Proteolytic rate in isolated lysosomes increased several fold with decreased intralysosomal pH. However, leakage of hydrolytic enzymes (elevated orotate phosphorylase/decarboxylase) may cause degradation of extra lysosomal orotate substrates. Thus incubation time should be short. Degradation of short and long-lived proteins in isolated lysosomes demonstrates that after 15 minutes of [^{14}C] leucine labeling, these lysosomes were active in degradation of short time labeled proteins. Since transit time for lysosomal enzymes from endoplasmic reticulum to lysosomes was around 2-3 hours [Zhu et al. 1994]. So after, 15 minutes some other proteins may be thought to enter lysosomes. The degradation rate in isolated lysosomes was sometimes faster for short time labeling because of short lived proteins as cytosolic or secretory in nature. On the other hand, membrane proteins had half lives in range of few days. So these long labeled proteins were degraded at different rates within lysosomes. Moreover short lived proteins were reported more susceptible to digestion by lysosomal proteases. The effect of proteolytic inhibitors on protein breakdown in isolated lysosomes for distinguishing lysosomal from extra lysosomal protein degradation was dependent upon cell type, culture medium, labeling schedule and inhibitors used. Inhibitors of lysosomal enzymes impede their supposed target of action viz. the lysosomes. But complete inhibition of proteolysis could not be possible due to lysosomal and nonlysosomal proteolysis. Pepstatin inhibited only selective aspartic proteinase cathepsin D, leupeptin inhibited thiol proteinase. Similarly nitroimidazole either intralysosomal pH and restored proteinases. Moreover, cathepsin D inhibition by pepstatin, cathepsin B, H and L inhibition by leupeptin are reported commonly [Katunuma, 2011]. Thus lysosomes were active in degradation of both short and long lived proteins during basal state.

Infected Kupffer cell lysosomes exhibited more pronounced elevated lysosomal degradation at pH 5.0 at temperature range 30 °C-40 °C as shown in Figure 23.

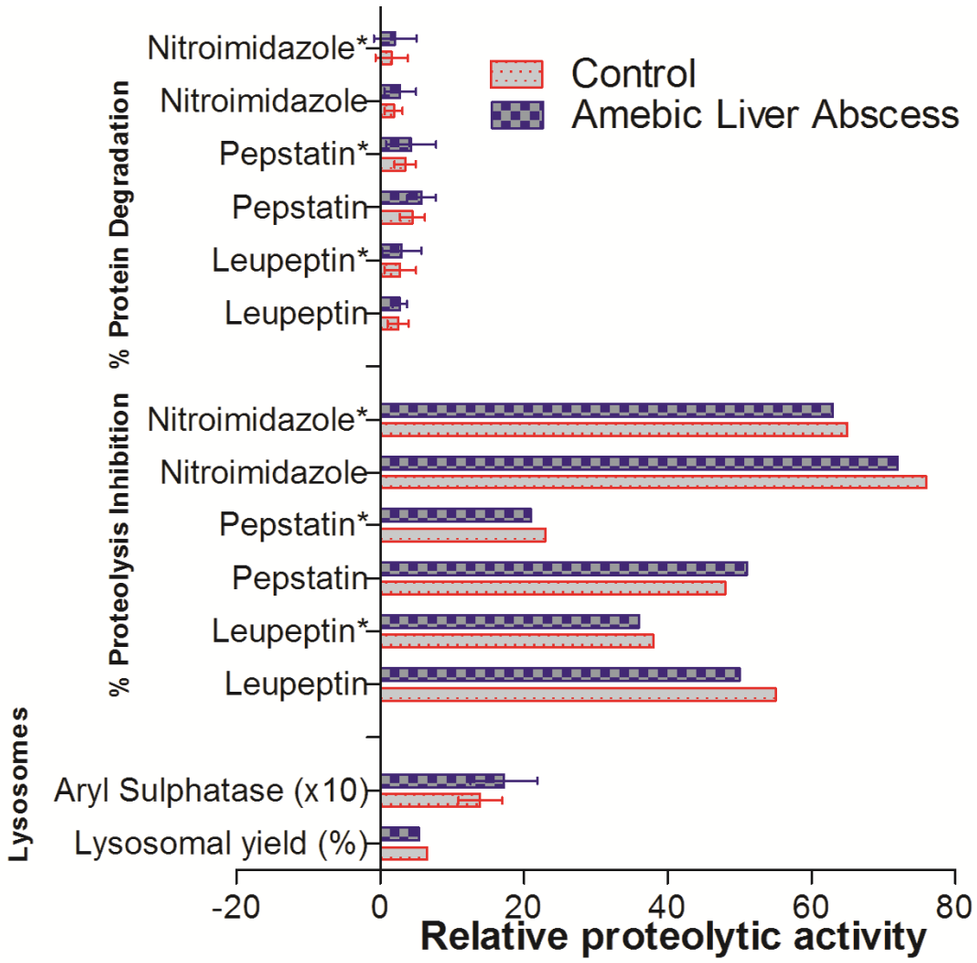


Fig. 23. Figure shows a comparison of % proteolysis inhibition and % protein degradation in presence of nitroimidazole, pepstatin and leupeptin. Proteolysis is represented in terms of arylsulphatase enzyme activity. Notice the size of peptide length makes difference shown by * for long duration and short duration of reaction in each additive. Control cells showed slow % protein degradation and low enzyme concentration. Source. Sharma R 1990,Ph.D dissertation.

9. Liver regeneration and role of lysosomal enzymes

During metabolic integrity loss and resultant hypoxia state, other factors in liver cell signal the alarm to produce nitric oxide, release of cytokines (IFN-α with IL-1β or TNF-α). These changes also trigger the sequence of Kupffer cell stimulation and lysosomal action simultaneously. As a result, lysosomal enzymes, growth factor, plasminogen stimulating factor consistently participate or stimulate the Kupffer cell suicidal phagocytic action (hepatic necrosis) by nitroimidazole analogs and previously reviewed [Michalopoulos, 1990; Thurman, 2000]. In drug induced hepatitis, liver lysosomal enzymes have been reported elevated as stimulation of hepatocellular defense apart from initiating respiratory burst and chemotaxis in liver cells

[Vician et al.2009]. Our study showed the associated electron microscopic observations that Kupffer cells accumulate around the site of hypoxia and exhibited hyperplasia condition showing degenerated nucleus, enlarged lysosomal vesicles [Sharma, 2009]. The enlarged lysosomal vesicles were further correlated by elevated lysosomal enzyme levels in serum. In liver biopsies, these lysosomal enzymes were significantly enhanced. Acid phosphatase, leucine aminopeptidase enzymes catalyzing phosphorylation and amino-peptidization, seem to be secreted more and suggestive of active protein degradation. β-glucuronidase and aryl sulphatase high enzyme secretion was suggestive of continuous breakdown of aryl substituted and β-glucuronidation reactions during cytolysis and proteolysis [Knook et al. 1981]. No previous data is available on liver cell enzymes estimated in liver cells isolated from liver biopsy of nitroimidazole treated livers in hypoxia. Our observations of enzyme inhibition in hypoxic liver cells are very valuable to design a nitroimidazole based radiosensitizer or drug for parasites, myocardial ischemia, tuberculosis, neurotoxicosis etc. Liver cell enzymes may extend the better biochemical explanation of complex hypoxia state at molecular level. However, several scattered studies on serum choline esterase, alkaline phosphatase, glucose-6P-dehydrogenase, ornithine carbamoyl transferase, cyclooxygenase-2, lactate dehydrogenase enzymes have been reported significant in hepatic hypoxia or hepatic damage evaluation with addition of new members of enzymes [Carlson et al. 1976; Koppele et al. 1991]. Enzyme inhibition in liver cells is significant information to design new liver cell metabolic drug stimulators in detoxification, evaluation of hypoxia imaging probes, and cultivation of type of monoaxenic liver cell cultures (hepatocytes and Kupffer cells) with specific enzyme profile.

There appear two main reasons of enzyme level hepatic cell recovery by nitroimidazole. First, altered enzyme changes were recovered by nitroimidazole therapy as drug inhibits the enzymes participating in energy status in hepatocytes and reduces Kupffer cell macrophagal activity with reduced hepatic enzyme secretion to bring back enzyme levels to normal. Second, regenerating hepatocytes may also regulate the normal cell recovery process and initialize the signaling Kupffer cells to store enough lysosomal enzymes.

10. Role of enzyme inhibition in liver transplantation and tissue engineering

Liver regeneration after the enzyme inhibition or loss of hepatic tissue is a fundamental parameter of liver response to injury. Enzyme inhibition phenomenon is recognized as specific external stimuli involving sequential changes in gene expression, growth factor production, and morphologic structure. Author sums up an account on different enzymes in following description:

- Angiotensin Converting enzyme inhibition by lisinopril degrades bradykinin and subsequently induces liver regeneration in rats. During this enzyme inhibition process (liver regeneration) induces 3.4 S DNA polymerase activity in both cytoplasmic fraction and nuclear fractions as constant while 6 S to 8 S DNA polymerase activities in the cytoplasmic fraction increased 6- to 7-fold [Ramalho et al.2001; Chang et al.1972]. The activity of histidine decarboxylase was markedly increased in regenerating liver tissues put. Ornithine decarboxylase is an enzyme catalyze in polyamine synthesis. Inhibition of ornithine decarboxylase, histidine decarboxylase, and other amino acid decarboxylases, was reported in regenerating rat liver and several rat tumors.[Russell, et al.1969]. Amine synthesis in rapidly growing tissues: ornithine decarboxylase activity

in regenerating rat liver and various tumors. Apart from enzyme inhibition, many growth factors and cytokines such as most notably hepatocyte growth factor, epidermal growth factor, transforming growth factor-α, interleukin-6, tumor necrosis factor-α, insulin, and norepinephrine, appear to play important roles in this enzyme inhibition and liver response. Many reviews attempted to integrate in last three decades and looked toward clues to the nature of triggering mechanisms in liver and cellular response. [Michalopoulos et al. 1997]. The major events are signaling cascades involving growth factors, cytokines, matrix remodeling, and several feedbacks of stimulation and inhibition of growth related signals. Liver manages to restore any lost mass and adjust its size to that of the organism, while at the same time providing full support for body homeostasis during the entire regenerative process [Michalopoulos et al. 2007].

- Recently, role of tissue engineering concept showed several advantages in liver transplantation. However, several factors are significant in regeneration: matrices design, cell mass up to whole-organ equivalents, optimized microarchitecture, cell-adhesion peptides, growth factors and extracellular matrix molecules to the polymeric scaffold, hepatic enzymes in microenvironment [Mooney et al. 1992]. The use of enzyme characterization is very helpful in hepatocyte culture to test biocompatibility required for the retention of tissue-specific gene expression [Kim et al. 2000; Voytik-Harbin et al. 1997]. Liver cell transplantation into polymeric matrices (cell-matrix-constructs) is emerging as routine in hospitals to meet absolute requirement of the transplanted hepatocytes for hepatotrophic factors that liver constantly receives through its portal circulation [Starzl et al. 1973]. Apart from cellular therapies, two experimental approaches are worth mentioning for successful clinical translation. The first "cell sheet" technology developed by Sasagawa et al.,2007. Ott et al. reported perfusion decellularization to generate whole organ scaffolds [Ott et al. 2008].
- In previous report, investigators developed a similar perfusion decellularization method for the liver [Baptista et al.2009] for organ bioengineering. These bioscaffolds preserve their tissue microarchitecture and an intact vascular network that can be readily used as a route for recellularization (intact enzymes of regulatory metabolic reactions) by perfusion of culture medium containing different liver cell populations. Significant enzyme inhibition assays are used as biomarkers of liver cell viability. In following example, a bioengineered liver (the regenerated hepatic tissue) is shown clearly visible by the naked eye after 7 days in the bioreactor (Fig. 24A). Regenerated tissue was highly cellular (Fig. 24B) and displays biliary duct structures positive for cytokeratin 19 (Fig. 24C), as well as clusters of hepatocytes expressing cytochrome P450 3A and albumin (Fig. 24D, E, respectively). Expression of endothelial cell nitric oxide synthase (eNOS) enzyme in seeded human endothelial umbilical vein cells (hUVECs) were also observed coating and spreading from vascular structures (Fig. 24F). In other approach, Uygun et al. 2010 decellularized rat livers and repopulated them with rat primary hepatocytes, showing promising hepatic function and the ability of heterotopically transplanting these bioengineered livers into animals for up to eight hours [Uygun et al. 2010]. Bioengineered livers displayed some functions of a native human liver (albumin and urea secretion, CYP450 enzyme expression, etc) and also exhibited an endothelial vascular network that prevented platelet adhesion and aggregation, critical for blood vessel patency after transplantation. Hence, this liver regeneration technology with proper active enzyme functions has the potential to translate in the future into the bioengineering of human size

livers to offer readily available liver for drug discovery applications and for liver transplantation, overcoming organ shortage.

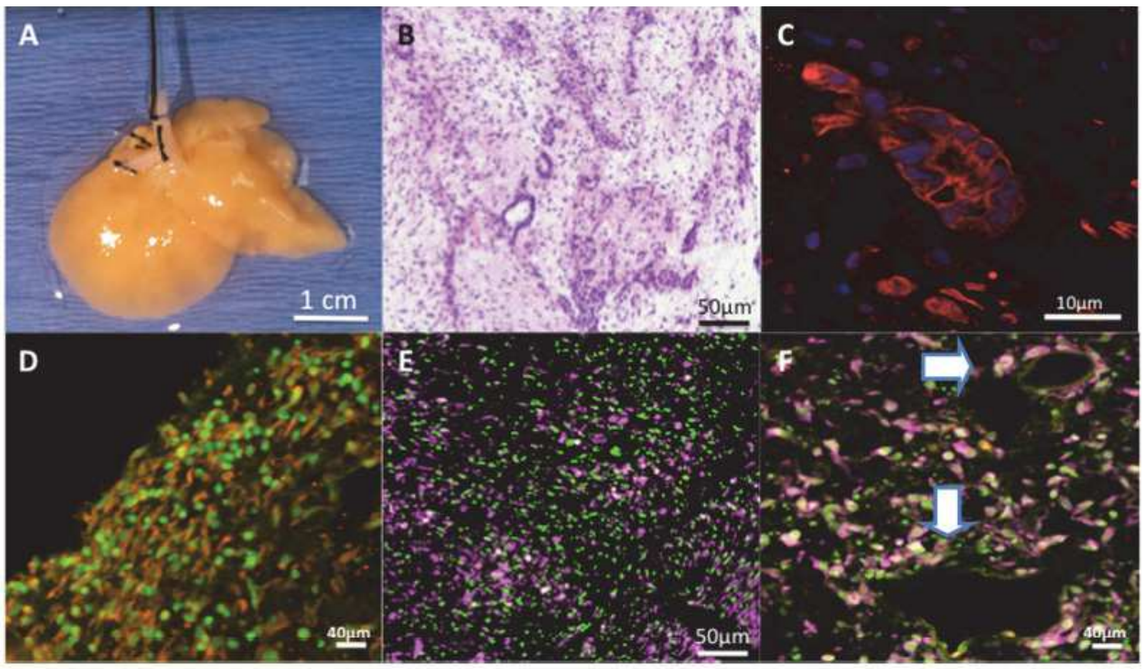


Fig. 24. Human bioengineered livers are highly cellular and display some of the functions observed in native hepatic tissue. (A) Macroscopic appearance of a seeded liver bioscaffold 7 days after seeding with primary human fetal liver and endothelial cells. (B) H&E showing broad recellularization of the bioscaffold with the formation of biliary ductal structures. (C) Immunofluorescence staining for cytokeratin 19 (red) showing a biliary duct formed within the bioscaffold with a visible lumen. (D) Immunofluorescence staining of cytochrome P450 3A (orange) and (E) albumin (purple) showing groups of hepatocytic lineage cells expressing these more mature hepatic markers. (F) Immunofluorescence staining of eNOS (purple) showing hUVECs coating and spreading from vascular structures (arrows). All nuclei stained with YO-PRO1 (green) or DAPI (blue). Reproduced with permission from reference Baptista et al. 2010.

11. Challenges, limitations in enzyme inhibition

There are two major challenges. First challenge was to get enough biopsy to estimate several enzymes. Second challenge was to choose significant enzymes as representative of hepatocellular criteria. The main limitation was that the 'hepatocellular hypoxia criteria' was used in small number of human subjects. The biopsy samples for electron microscopy observations needs more thoroughly controlled experiments. Other limitation was that the enzyme estimations in serum and biopsy samples may not be perfect representative samples and it needs to establish the measurable and actual enzyme activities in cells. The most crucial issue is that hypoxia is a combination of sequence of several metabolic and subphysiological reactions in cells. Moreover, other inflammatory cytokines, apoptosis, nitric oxide production and phagocytosis are associated changes during hypoxia. It further needs tracer technique to track the details of hypoxia in cell.

12. Current developments and future prospects

Nitroimidazoles are rediscovered antiamoebic drugs having great potential of radiosensitizers in diagnosis and treatment of tumors, tuberculosis, myocardial infarction, and hypoxia. Nitroimidazole cytotoxicity was recently capitalized in hypoxic tumor detection and tumor killing. Nitroimidazoles act as smart enzyme inhibitors on one hand and play as enzyme modulators. Liver enzyme inhibition is unique for developing drugs in drug discovery at molecular level. The major challenge is to explore enzyme inhibitor that selectively and specifically catalyzes enzyme inhibition in only abnormal or diseased liver cells leaving safe normal cells in acute stage of liver damage such as hepatic coma, threatening hepatitis, hepatic cancer. The success of enzyme inhibition mainly depends on intracellular physiological conditions such as pH, temperature, concentration of active enzyme or inhibitor, coenzyme, co-factor, enzyme substrate structural-functional conformation state, type of biochemical reaction such as coupled, cascade or individual enzyme reaction and finally regulatory enzyme behavior to control metabolism. Currently, art is developing in liver transplantation using cultured cells to replace diseased or non-functional liver cells from diseased liver. Tissue engineering has opened the new opportunity of using conditioned monoaxenic and monoclonal liver cells in both experimental and pre-clinical or clinical therapeutics in both industries and academics. Recently, gene therapy in liver was a great success and it will be a boom in medical science of detoxification or drug metabolism if and when clinically accepted. Battery of enzymes in hepatocytes and rate limiting enzyme regulatory behavior in presence of additives or inhibitors will certainly enhance better understanding of disease development and new drug pharmaceutical action in drug discovery. Still today, liver cells remain as major research tool to discover drugs in therapeutics and search of transplantable disease resistant liver cells because of liver as sole organ responsible of bile formation, detoxification and drug metabolism. Cirrhosis is a serious health hazard that solely requires functional liver. In future, it remains to see if molecular biology will assist in silencing genes ON or OFF responsible of enzyme stimulation or inhibition in liver as method of disease therapy and cure. The major discoveries will be discovery and better understanding of new growth factors, colony stimulating factors affecting enzyme reactions in hepatocytes and Kupffer cells as major engineered cancer resistant or cirrhosis resistant liver cells. In future, chip technology or silica or polymer coated enzyme estimation techniques may be more reliable in small sample collections or in liver cells with high degree of accurate enzyme estimation.

13. Conclusion

The 2′nitroimidazole is both antiaparasitic drug and radiosensitizer hypoxia marker in tumor therapy. It shows hepatocellular cytotoxicity. The ‘hepatocellular hypoxia criterion’ distinguishes the nitroimidazole induced hepatocellular oxygen depletion and associated organelle changes by enzyme levels in serum, biopsy samples and cell organelles by electron microscopy. Initially, glucose regulation leads to metabolic integrity loss. Later, it may be oxygen insufficiency and slow cell death. The 2′-nitroimidazole is drug of choice in hepatic infections and its derivatives are emerging choice of tumor treatment. Its action may be evaluated rapidly by enzyme biochemical estimations without time consuming drug monitoring and therapeutic assay techniques. Entamoeba histolytica, an intestinal

protozoan parasite can proliferate, lyse and destroy human liver cells. Within the last decade, new animal models of E. histolytica infection showed amoebic trophozoites cause intestinal disease and liver abscess. Recent studies have expanded our understanding of a large number of enzyme inhibition reactions in hepatic regeneration after liver abscess is treated by nitroimidazole as remarkable drug to kill parasites. Still interactions between E. histolytica and human intestine; and between E. histolytica and hepatocytes or Kupffer cells are not fully known. In present chapter, a scheme of enzyme changes in liver cells during development of amoebic liver abscess and enzyme inhibition is explored in detail as rehabilitative action of nitroimidazole to regenerate liver cells. Characteristics of enzyme regulatory behavior highlight the metabolic integrity loss, energy depletion, oxidative phosphorylation, fatty deposition, phagocytosis and slow cell death as main culprits in development of liver abscess and hypoxia. Nitroimidazole inhibits several rate limiting enzymes to control oxygen (hypoxia) levels and keeps balance of NADPH, ATP, fatty acids, amino acids and proteins. In hepatocytes, enzyme inhibition and regulatory behavior of apoptosis (caspases, cysteine protease); carbohydrate metabolizing (glucokinase, pyruvate kinase, phosphofructokinase); energy metabolism (NADPH cytochrome P450 Reductase, Phosphodiesterase); DNA synthesis (Thymidylate Synthetase) describe the process of liver regeneration. In Kupffer cells, enzyme inhibition of drug metabolizing enzymes (oxidoreductases, proteases, esterases, phosphatases) predicts the liver regeneration and recovery from liver abscess. In last decade, several studies reported the use of enzyme inhibition mechanisms in enhancing liver regeneration and recovery as tool of tissue engineering and liver transplantation. In conclusion, enzyme inhibition mechanisms in liver damage and regeneration explain the better information of liver transplantation to maintain complex liver cell environment in balance. Enzyme inhibition mechanisms throw a light on characterization of enzyme regulation in engineered liver cells, amoebic liver abscess development and desired design of targeted nitroimidazole analogues to treat liver abscess.

14. Appendix 1: A protocol of hepatocellular dysfunction

Patients: In 10 control subjects, stool, ELISA, serum and liver biopsies were collected[1]. Only proved 10 subjects free from any hepatic disease were examined for enzyme estimations in isolated liver cells from biopsy specimens. Other ten subjects were treated with nitroimidazole (Tiniba and Zil from Hindustan Lever Ltd, Bombay) therapy 2 x 5 gm one-time dose thrice at 2 days interval. At the end of dose, liver biopsy and serum collection was done.

- All the 10 control subjects had no clinical findings of any hepatic disease and showed stool -ve, ELISA -ve. All 10 showed normal liver and no abscess confirmed by ultrasound and liver scan. The subjects were selected of 35 ± 15 years (mean age ± sd), average monthly income 1750 ± 100 INR, no hepatomegaly, no diarrhea, fever and pain. The subjects after nitroimidazole treatment showed induced hepatomegaly insignificant less than 2 cm by liver scan. The subjects with amoebiasis and amoebic liver abscess are not included and not shown here.

[1]The patients were studied for ongoing other research study on amoebic hepatitis vs amoebic liver abscess by Hepatocellular Dysfunction Criteria at Liver Unit, AIIMS. The results of hepatic damage are shown in Figure 3.

The ELISA was done by method of Prakash et al.[44]. Liver biopsy was taken by Manghini needle [45].

Biochemical assays: In biopsy sonipreps and serum samples the hepatocellular enzymes were estimated as described elsewhere [Sharma, 2009]. All substrates of enzymes were obtained from Sigma Chemical Company, St Louis, USA. The estimations were done on UV spectrophotometer Cecil Inc. England [Sharma, 2009].

Electron microscopy: The biopsy specimens at stored in glutaraldehyde at -20°C. For fixing samples were fixed in dental wax and emersed in pool of 0.1 M phosphate buffer containing 0.1 M Ca^{++}. The samples were cut 1 mm cube by Gillette blade. The cubes were fixed for 2 hours in 0.2 M phosphate buffer in 4 changes at 30 minutes interval in capped vials. The vials were washed for 2 hours in 0.2 M phosphate buffer 4 times at 30 minutes interval in capped vials. The vials were post fixed in buffered 1% osmium tetroxide for 1-2 hours at 4°C, dehydrated in cold 10% ethanol for about 5 minutes followed by dehydrations in cold 50%, 70%, 80%, 95% ethanol for about 5 minutes each and tissues were kept at room temperature. Further tissues were dehydrated at 100% ethanol for 15 minutes. Electron Microscope PA model was used for screening hepatic cell damage by epoxy resin blocks and osmium tetra oxide staining [46].

Art of liver cell isolation and enzyme characterization

The procedures of liver biopsy excision and preparation of liver cells were followed as per ethical committee of institute as described in following section [Sharma, 2009].

Reagents Used

The collagenase enzyme was purchased from Sigma-Aldrich, St Louis, MO and pronase enzyme was purchased from Boerhinger Meinheim GMbH.

Isolation and preparation of Kupffer cells: The liver biopsy samples (6-10 grams) were digested with collagenase enzyme (Sigma-Aldrich, St Louis, MO) 15 mg/ml for 10 minutes and bottom pallet was used for hepatocyte experiments. Other remained supernatant part of digested liver was further digested with 2 mg/ml pronase enzyme (Boerhinger Meinheim GMbH) in 0.2 % Kreb's Hansleit Buffer pH 7.4 containing 5 mm glucose and 3 mM calcium chloride after separation of hepatocytes. The said suspension was incubated for 45 minutes at 37˚C stirring 250 revolutions per minute. After incubation, the cell suspension was filtered through nylon sieve 79 × 79 microns and filtrate was centrifuged and washed twice with KHB containing 5 mM glucose and 3 mM calcium chloride pH 7.4. After centrifugation and final washing the filtrate was centrifuged at 350 × g in ice cold centrifuge. The settled pallet at bottom was washed with TC 199 medium. The number of Kupffer cells was counted on Naubourgh counting chamber and visibility was determined by trypan blue.

Kupffer cell fractionation: The isolated Kupffer cells were divided in two parts. One part was used for Kupffer cellular enzymes and other part was used for in vitro experiments. For enzyme estimations Kupffer cells from freshly harvested preparations were sonicated at zero degree temperature in said KHB buffer in soniprep. Later these were fractionated for different cellular fractions as described following by methods [Alabraba et al.2007; Heuff et al. 1994; Kirn et al.1982].

1. In refrigerated centrifuge above soniprep preparations were centrifuged at 1000 × g to take out cell nuclear fraction and again centrifuged at 9000 × g to take out mitochondrial fraction as pallet at bottom.
2. Above supernatant was centrifuged at 23000 × g to isolate microsomal bodies fraction along with fraction rich in peroxisomes.
3. The light turbid supernatant was centrifuged at 100000 × g to isolate lysosomal fraction as clear white pallet at bottom. Sometimes microsomes were contaminated with lysosomes. The lysosomal purity evaluation was used to exclude microsomes free from lysosomes [Wisse et al.1996].

Kupffer cells in cultures: The Kupffer cell cultures were maintained for 48 hours after isolation from pronase perfused liver biopsy samples [Brouwer et al. 1988]. The in vitro cultured cells were screened for trypan blue exclusion [Froh et al.2003]. The Kupffer cells were used for their in vitro phagocytic action upon foreign particles which were sheep erythrocytes, latex beads and zymosan suspended in 10 mM phosphate buffer saline pH 7.4 at 4°C. After harvesting Kupffer cells were added in phosphate buffered saline pH 7.4 containing 4.6 x 10^6 adherent cells and three ml PBS-TC 199 medium in culture flasks containing Kupffer cells in mediukm with particle cell ratio 1:5 for erythrocytes, 1:40 for latex beads and 1:4 for trophozoits. The ratio for zymosan and cell was 20:1. They were kept up to 48 hours or more in carbogen atmosphere (95 % O_2 and 5% CO_2) in desiccator with twice changes of medium after every 24 hours.

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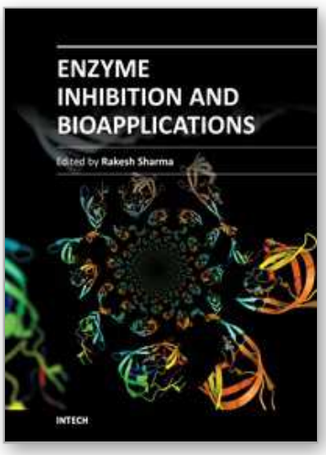
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Enzyme Inhibition and Bioapplications is a concise book on applied methods of enzymes used in drug testing. The present volume will serve the purpose of applied drug evaluation methods in research projects, as well as relatively experienced enzyme scientists who might wish to develop their experiments further. Chapters are arranged in the order of basic concepts of enzyme inhibition and physiological basis of cytochromes followed by new concepts of applied drug therapy; reliability analysis; and new enzyme applications from mechanistic point of view.

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