The Hepatocellular Hypoxia Criteria: 2'Nitroimidazole Effect on Hepatocyte Carbohydrate Metabolizing Enzymes and Kupffer Cell Lysosomal Enzymes: Hypoxia Screening.

Rakesh Sharma[1]
[1]Gastroenterology Department,
All India Institute of medical Sciences, New Delhi-110029 India

Abstract

Aim: to understand the 2'-nitroimidazole induced hypoxia and liver cell interaction, we proposed a "Hapatocellular Hypoxia Criteria". *Hypothesis:* The nitroimidazole induced metabolic energy loss and oxygen depletion (hypoxia) in liver cell mitochondria causes the phagocytosis. Based on it, ten control subjects with 2'-nitroimidazole therapy were studied for their carbohydrate metabolizing enzymes in serum and hepatocellular enzymes in liver biopsy tissues. *Materials and Methods:* Proven ten control subjects were studied for hypoxia by enzyme assays. The 2'nitroimidazole treated paired ten subjects were studied for hypoxia using enzyme assays and hepatocellular cytomorphology by electron microscopy. *Results and Discussion:* Out of ten subjects on 2'-nitroimidazole, nine showed elevated carbohydrate metabolizing and lysosomal enzyme levels in serum. The enzymes 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 enzymes β-glucuronidase, alkaline phosphatase, acid phosphatase, showed enhanced levels in the serum samples. In control ten liver biopsies, the hepatocytes and Kupffer cell preparations showed altered enzyme levels. Hepatocytes showed lowered 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 on 2'-nitronidazole clinical trial, the electron microscopy cytomorphology observations showed swollen bizarre mitochondria, proliferative endoplasmic reticulum, and anisonucleosis after 2'-Nitroimidazole effect in liver cell damage. *Conclusion:* The proposed "Hepatocellular Hypoxia Criteria" served to define origin of liver hypoxia and showed altered hepatic enzyme activities with active phagocytosis and cytotoxicity in subjects after 2'-nitroimidazole treatment. The study suggests the enzyme

[1] The manuscript is part of Ph.D "Effect of imidazole compounds on liver cells during development of amoebic liver abscess" submitted by author at Meerut University. Present address: Center of Nanobiotechnology, Florida State University, Tallahassee, USA. Email: rksz2004@yahoo.com

based evaluation of nitroimidazole induced hypoxia monitoring and treatment of hepatic tumors and infected liver.

Key Words: *liver, enzymes, electron microscopy, carbohydrate metabolizing enzymes, hepatocellular dysfunction criteria*

Introduction

2'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 [1, 2]. Several 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 [3-15]. Still nitroimidazole structure-function relationship of hypoxia, radiosensitization and cytotoxicity remain less understood perhaps due to its low sensitivity to clinical investigations and available enzyme alterations at the very late necrotic stage [16-24]. However, nitroimidazole cytotoxicity was reported continuously in last three decades indicating altered enzymes, nitric oxide production, cytokines, oxygen depletion (hypoxia) and immunoactive substance release from both hepatic infections and cancer tumor tissues [25-30]. Still, studies do not establish the acceptability of growing popularity of nitroimidazole in tumor monitoring and treatment as risk free if nitroimidazole can induce tissue regeneration after hepatic infections or oxygen starved tumors [32]. The present report puts evidence of hepatic metabolic integrity loss (in mitochondria and cytoplasm) and lysosomal stimulation associated with hypoxia. The enzyme based routine evaluation of nitroimidazole or its derivatives is proposed to measure induced oxygen depletion and energy loss. The approach is applicable possibly in oxygen starved tumor cells or infected liver lesions and abscesses.

Nitroimidazole is considered clearly hepatotoxic, renal toxic with reports of diffused damage of parenchymal and nonparenchymal liver cells as initial stage of hepatic damage using electron microscopy and biochemical markers in serum with emphasis of pathophysiology [31]. Based on evidences of the previous reports on nitroimidazole action of cytotoxicity, oxygen depletion (hypoxia), radiosensitization, the present study proposed a 'hepatocellular hypoxia criteria' assuming 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. Our previous observation on nitroimidazole effect on hepatic amoebiasis and amoebic abscess development indicated the possibility of nitroimidazole concentration dependence with oxygen depletion, nitric oxide production, cytokine synergy and

liver cell enzyme alterations as inter-related consequences of liver regeneration [32]. The major players were the energy metabolizing and lysosomal enzymes as initial liver damage. Using this information, the liver cell enzyme profile was designed to define the hypoxia sensitivity of nitroimidazole by 'Hepatocellular Hypoxia Criteria'. The novelty of hepatocellular hypoxia criteria was the detailed cytomorphic-biochemical information using altered enzyme activities during initial cytomorphic changes in hypoxic hepatic cells 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. However, the possibility of various hepatocytoxicity mechanisms was reported as initial loss of metabolic integrity in experimental animal models exposed to nitroimidazole derivatives. Several new nitroimidazole derivatives are emerging as potential cancer chemosensitizers, hypoxia markers and hypoxia imaging contrast agents [32-36]. Such ongoing developments need the clear and complete information on action of new nitroimidazole derivatives in tumor selective cytotoxicity, oxygen depletion and hepatocellular DNA and enzyme alterations before they can be used in hypoxia monitoring and therapy. Further we believe that initially glucose and calcium hemostasis are the primary targets of hepatic hypoxia followed by induced metabolic integrity loss leading to apoptosis and regulatory failure in glycolytic, TCA cycle, gluconeogenesis and Ca^{++} mediated cAMP related biodegradation of molecules [31].

Hepatocellular Hypoxia Criteria: The criterion was a step by step sequence of 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 keep 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 cytotoxicity 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.

In the present study, the hepatic hypoxia criterion focus was on 3 major 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 due to energy loss; 2. Changes in peripheral biomarkers such as cytokine immunity, altered glutathione reductase, nitric oxide production, super oxide dismutase enzymes (data not shown); 3. Severe energy loss and oxygen depletion

results with changes in liver cellular morphology and tissue shrinkage. The study highlights the sensitivity of different enzymes as response of nitroimidazole induced cytotoxicity. We proposed a simple scheme of "Hepatocellular Hypoxia Criteria" as shown in Table 1.

Origin of hypoxia: 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 source of energy molecule synthesis and gluconeogenesis it serves mainly as available central ATP control tool of glucose homeostasis in liver cytototoxicity or regenerated liver cell [37, 38]. The citrate synthase, malate dehydrogenase, isocitrate dehydrogenase and succinate dehydrogenase enzymes serve as TCA cycle regulatory enzymes. These enzymes in hepatocytes likely define the nature of hepatocyte tumor hypoxia and hepatocyte response after nitroimidazole therapy [37, 38, 39]. At the cost of ATP from TCA cycle and oxygen from cell, oxidative phosphorylation maintains 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 indicator of oxidative phosphorylation status and oxygen insufficiency (hypoxia) in liver. Other glutathione reductase and superoxide dismutase enzymes are indicators of hypoxic state of liver cells [2, 26, 40, 41]. Any energy imbalance and oxygen insufficiency (hypoxia) in liver cell are indicated by these enzymes.

Table 1: 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 (shown highlighted in yellow).

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)		**glycogenolysis**
lysosome (L)		**lysosomal enzymes**
	⇩	

nuclear changes (N) **oxygen flux related**

Hepatocellular enlargement (Apoptosis)

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 membrane damage increased lipid synthesis

⇩

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 negative** **-ve Hypoxia biomarkers**
OR if unchanged or poor recovery abnormal ELISA,enzymes

⇩

Surgical intervention

Origin of hypoxia: 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 source of energy molecule synthesis and gluconeogenesis it serves mainly as available central ATP control tool of glucose homeostasis in liver cytototoxicity or regenerated liver cell [37, 38]. The citrate synthase, malate dehydrogenase, isocitrate dehydrogenase and succinate dehydrogenase enzymes serve as TCA cycle regulatory enzymes. These enzymes in hepatocytes likely define the nature of hepatocyte tumor hypoxia and hepatocyte response after nitroimidazole therapy [37, 38, 39]. At the cost of

ATP from TCA cycle and oxygen from cell, oxidative phosphorylation maintains 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 indicator of oxidative phosphorylation status and oxygen insufficiency (hypoxia) in liver. Other glutathione reductase and superoxide dismutase enzymes are indicators of hypoxic state of liver cells [2, 26, 40, 41]. Any energy imbalance and oxygen insufficiency (hypoxia) in liver cell are indicated by these enzymes.

In present study, emphasis has been concentrated only upon 2'-nitroimidazole induced carbohydrate metabolizing enzymes and description of nitroimidazole induced oxygen deprivation by 'hepatocellular hypoxia criteria'. Based on this approach nitroimidazole was evaluated for its safe antiparasitic or anticancer drug value and liver cells' oxygen depletion in liver cells (nitroimidazole tumor therapy). Earlier studies also reported nitroimidazole evaluation by its oxygen deprivation action, cytotoxicity action and antiparasitic action [1-30]. The paucity of information on nitroimidazole-liver tissue interaction can be predicted partly by data of initial sequential biochemical changes in liver cells which further lead to detectable hypoxia [43]. The novelty of representative serum enzyme measurement was to establish 'hepatocellular hypoxia criteria' as hypoxia biomarker of nitroimidazole therapeutic evaluation and hepatic regeneration if any. The hypoxia is a sequence of initial energy loss and oxygen insufficiency leading to apoptosis and organelle changes. The major finding was that carbohydrate metabolizing enzymes showed metabolic integrity loss in hepatocyte cells and oxygen depletion after nitroimidazole treatment. The serum enzyme profile serves as quick hypoxia assessment.

Methods and Materials

Patients: In 10 control subjects, stool, ELISA, serum and liver biopsies were collected[2]. 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. 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 [46]. All substrates of enzymes were obtained from Sigma

[2] 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.

Chemical Company, St Louis, USA. The estimations were done on UV spectrophotometer Cecil Inc. England [46].

Electron microscopy: The biopsy specimens at stored in glutaraldehyde at -20°C. For fixing samples were fixed in dental wax and immersed 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].

Results

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 $\pm$ 15 years (mean age $\pm$ sd), average monthly income 1750 $\pm$ 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.

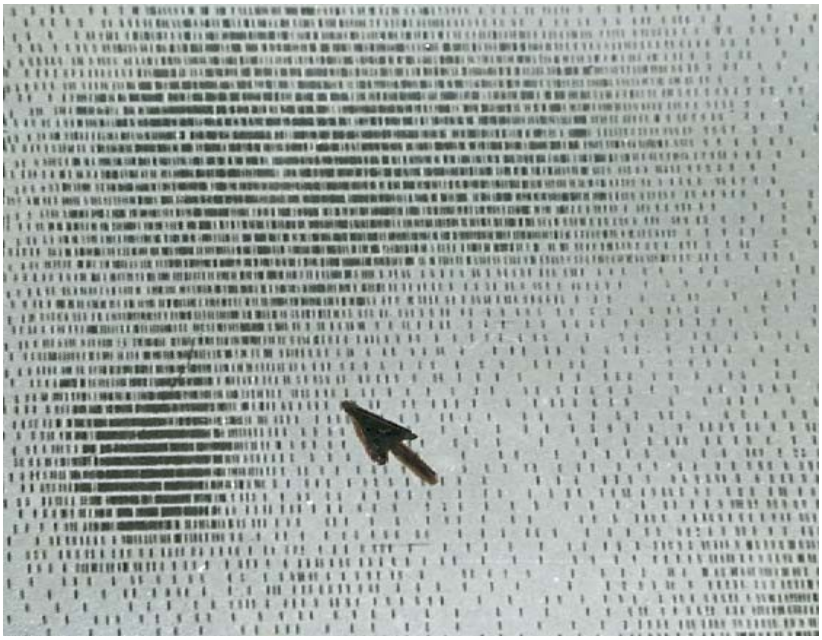

Figure 2: The liver scan (on left panel) is shown for assessing the position of hepatic hypoxia and associated hepatomegaly and for biopsy collection site as shown with arrow.

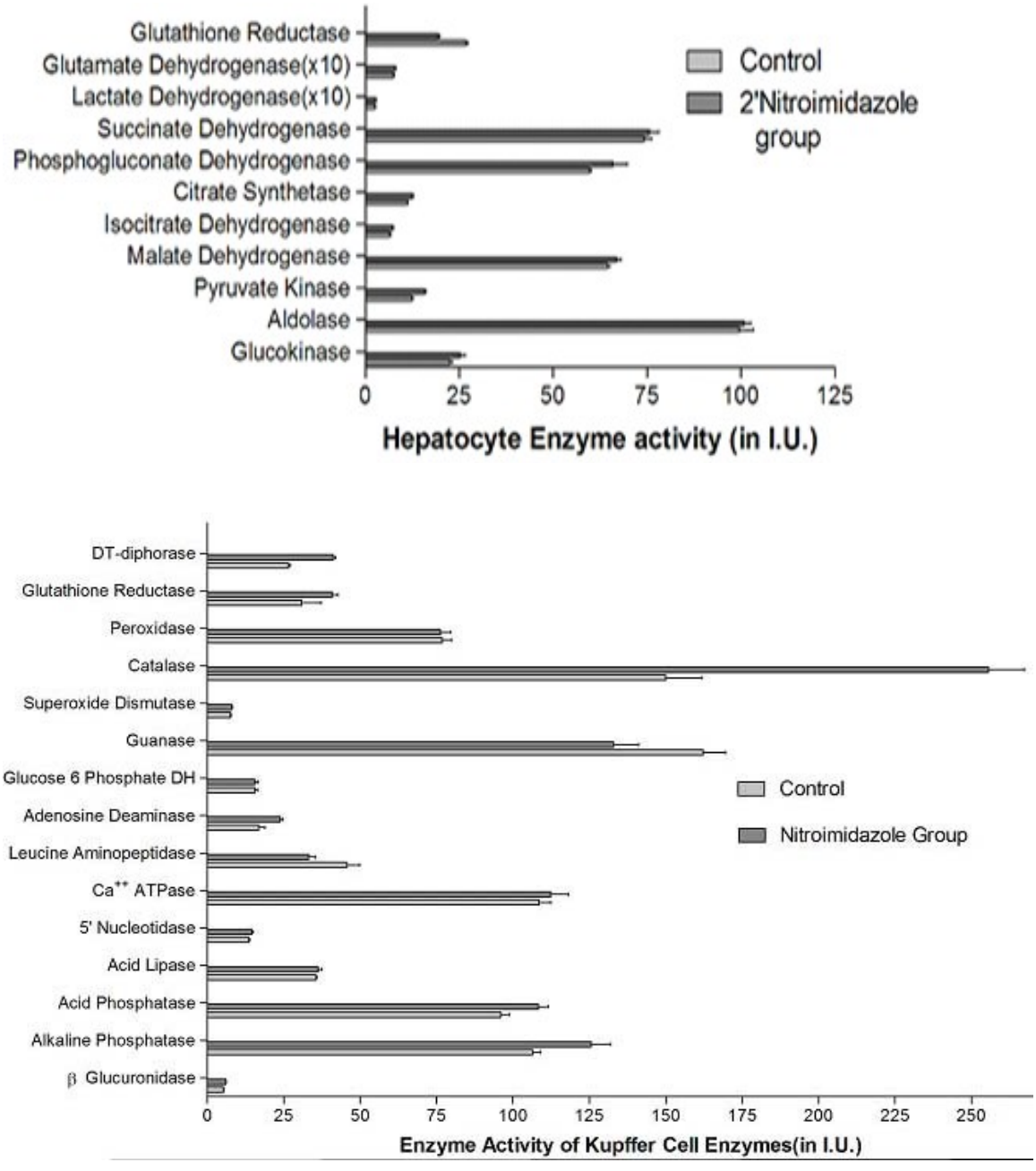

Figure 3: The histogram bars show the effect of nitroimidazole on biomarker enzymes and comparison of different enzymes in hepatocytes and Kupffer cells in control vs nitroimidazole treated subjects (panel on top and bottom)

Figure 3(continued)

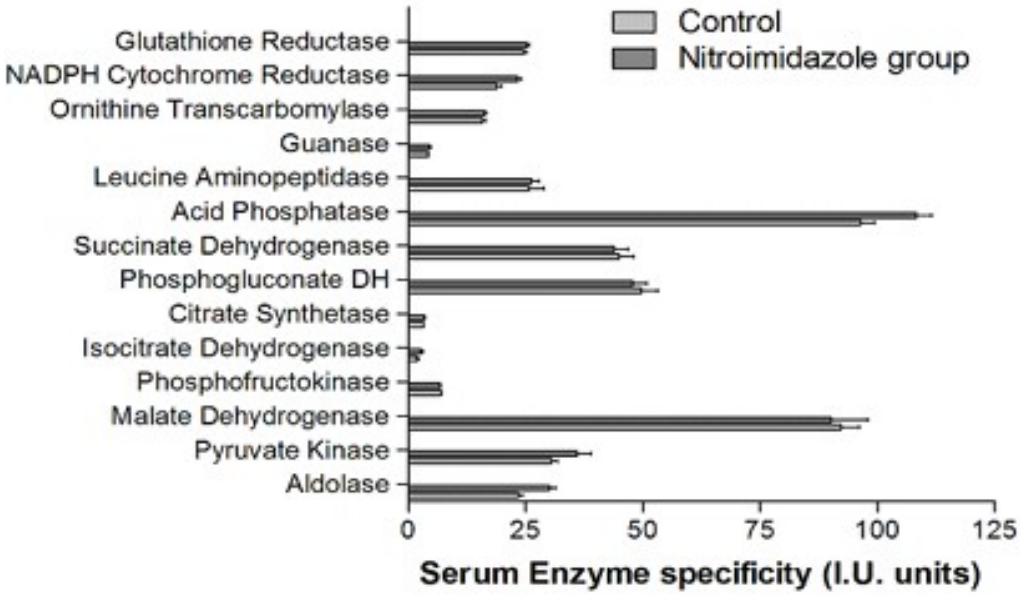

Figure 3: The histogram bars show the effect of nitroimidazole on biomarker enzymes and comparison of different enzymes in serum biomarker enzymes.

Initial loss of metabolic integrity of glycolysis and ATP:

In serum and liver cells of these subjects, exhibited more or less specific characteristic changes in enzymes. In nitroimidazole treated subjects the glucokinase levels were more or less normal in both serum and biopsy (100%). In nitroimidazole treated subjects the phosphofructokinase levels were nonspecific. In nitroimidazole treated subjects the levels were normal (80%). In nitroimidazole treated subjects the lactate dehydrogenase levels were reversed to normal. In nitroimidazole treated subjects the levels were normal (100%). In nitroimidazole subjects, the levels were normal (80 %). In nitroimidazole treated subjects, the isocitrate dehydrogenase enzyme levels were normal (80 %) in serum. In nitroimidazole treated subjects, the citrate synthase levels were normal. In nitroimidazole treated subjects, the phosphogluconate

dehydrogenase levels were normal (80 %) in both. In nitroimidazole treated subjects, the succinate dehydrogenase levels were normal (60 %) in both. The enzymes are shown in Figure 3 and Tables 2-4.

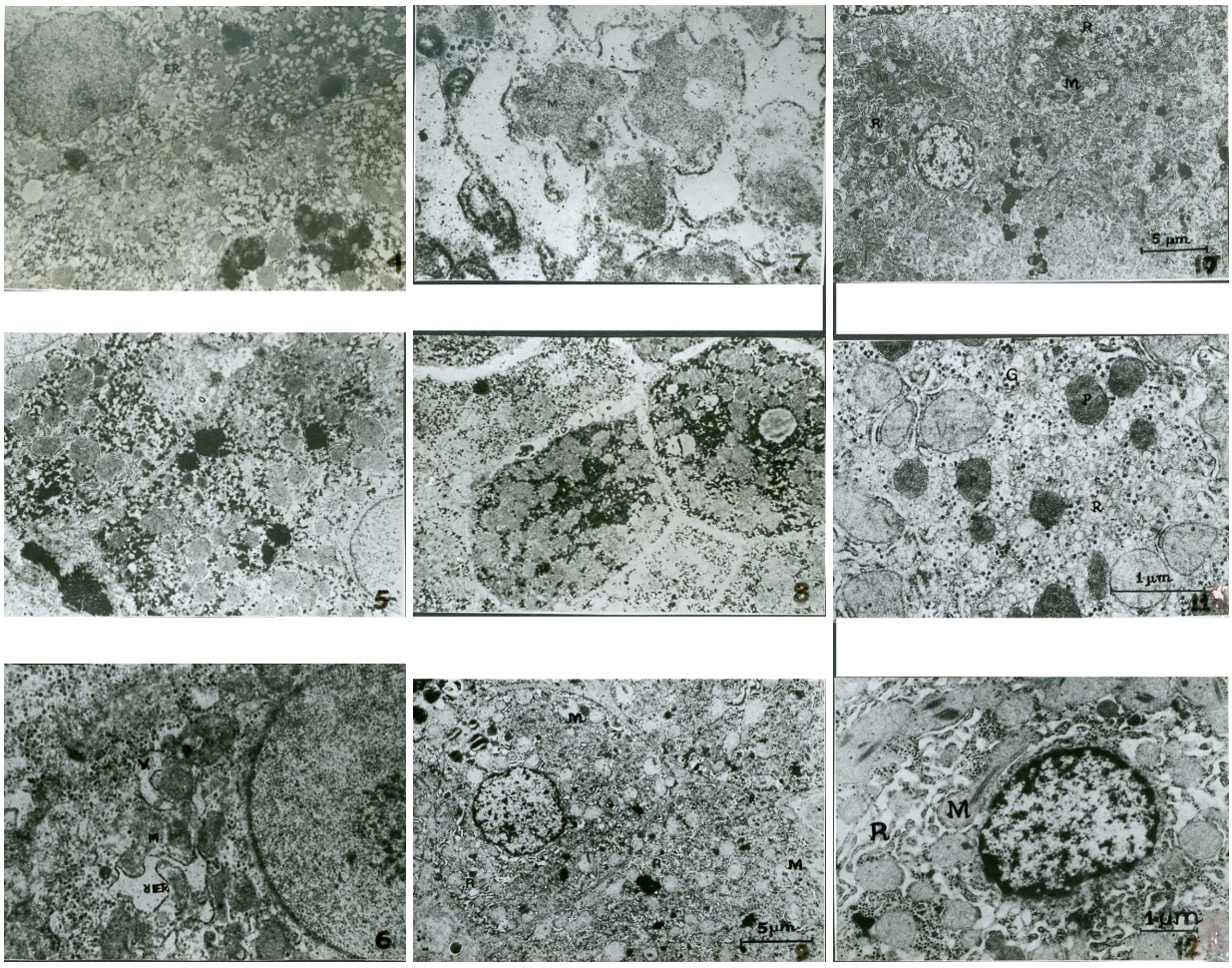

Figure 4: Ultrastructural changes are shown in hepatocyte organelles during hypoxia: exfoliation of endoplasmic reticulum (top on left); anisonucleosis (mid and bottom on left); intercellular junction gaps (top and mid panels in center); nuclear inclusions (bottom on center); swollen and bizarre mitochondria (top on right); inclusions in peroxisome (mid on right); mitochondrial atrophy with lipid vesicles (bottom on right).

Hypoxia marker enzymes and Kupffer cell lysosomal enzymes:

In nitroimidazole treated subjects, the β-glucuronidase levels were normal (50 %) in Kupffer cells and also normal in serum (60%). In nitroimidazole treated subjects, the acid phosphatase levels were normal in both. In nitroimidazole treated subjects, the levels were normal in serum (80 %) and remained high in Kupffer cells (60%). In nitroimidazole treated subjects, the levels

were normal in both Kupffer cells and serum (80%). The enzymes are shown in Figure 2 and Table 3.

The ultrastructure of liver cells showed characteristic changes in nitroimidazole exposed liver. Hepatocyte mitochondria became swollen, bizarre with dense matrix and showed destroyed cristae, endoplasmic reticulum dilated vesicles, giant nuclei with diffuse proliferation of endoplasmic reticulum and clear anisonucleosis features. Kupffer cell hyperplasia was observed with swollen lysosomal contents as shown in Figure 4.

In above, 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.

Discussion

The liver is made of parenchymal hepatocytes and nonparenchymal Kupffer cells as sole targets that exhibit their intracellular biochemical changes. The enzyme biomarkers could be analyzed in serum and hepatocytes as clinically significant indicator of hepatic damage. In hepatic cytotoxicity, initially metabolic integrity loss leads to oversecretion of liver cell enzymes including lysosomal enzymes. Soon after, the ultrastructural changes in liver cells by electron microscopically are suggestive of acute organelle degeneration.

It was evident that ultrastructural parenchymal cytotoxicity was associated with nitroimidazole overdosage (normal dosage is 2 x 3 gm one time in amoebic hepatitis and thrice in amoebic abscess). The regenerative change 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 as unequivocal diffuse parenchymal injury exhibiting diffused sinusoidal and portal infiltration events [47, 48, 49]. Still such changes may be misleading as these can be expected to occur in any other inflammatory disease of liver associated with negative symptoms. So, in present study, biomarker enzymes with electron microscopy evidence of cytotoxicity in liver biopsy samples after nitroimidazole treatment are evidenced by possible hepatic hypoxia criteria.

Degenerative changes of hepatocytes were consistently observed suggestive of necrosis. At maximum, these conditions were excluded among the present control group of subjects.

Evidence of hepatic regenerative reaction and nitroimidazole cytotoxicity (hypoxia induced changes) was characterized by cytokine synergy, unusual degree of anisonucleosis, nitric oxide production and the presence of giant nucleus in hepatocytes after liver regenerative therapy [50]. Endoplasmic reticulum showed a diffuse and intense proliferative activity in these liver cells with normal appearance of mitochondria as earlier reported [47, 48, 49]. However, intramitochondrial inclusion bodies were absent but they have been reported as very prominent features [47]. The cause of the ultrastructural changes after nitroimidazole cytotoxicity as shown in this study can be supported by initial enzyme alterations reflecting loss of metabolic integrity probably induced by free radical formation from nitroimidazole [30]. 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 as reported earlier [30].

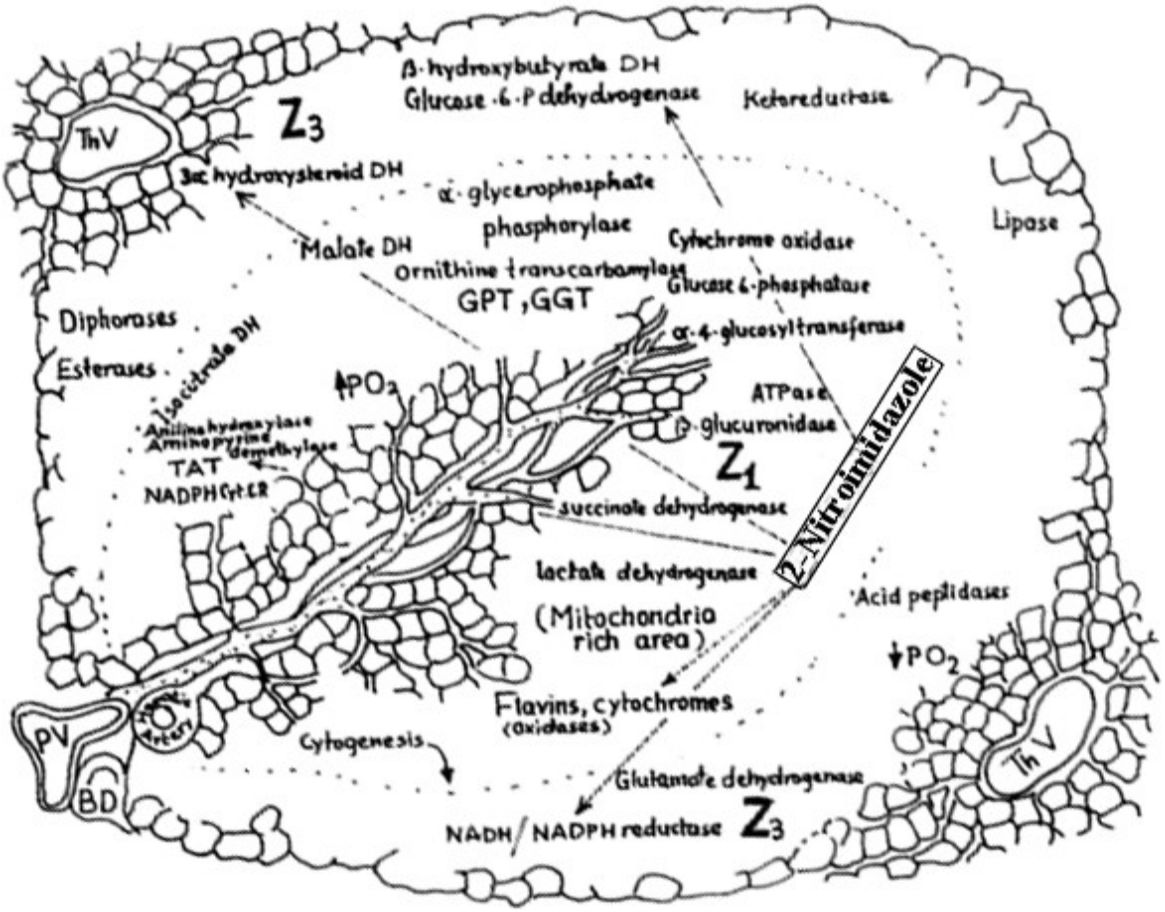

Figure 5: The sketch of nitroimidazole induced intracellular enzymes changes indicating the points of metabolism and metabolic control during hypoxia and subsequent liver recovery after nitroimidazole treatment . The enzymes distribution in organelle and enzyme location in different hepatic sites explains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

The 'Hepatocellular hypoxia criteria' was proposed as a sequence of events of 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 have been cited as main initial determinants of hypoxia [51]. Present study addressed the question of glucose metabolizing pathways viz. glycolysis, TCA cycle and gluconeogenesis. In hepatic cells, metabolic alterations of these pathways in hepatitis and hepatic tumors are best correlated with respective enzymes and ultrastructural changes in cells. The following description is broad explanation of different enzymes secreted from hepatocytes as a result of nitroimidazole induced cytotoxicity and oxygen depletion. Form biochemical stand point, different regulatory enzymes are discussed as biochemical events of hypoxia development as shown in Figures 3 and 4.

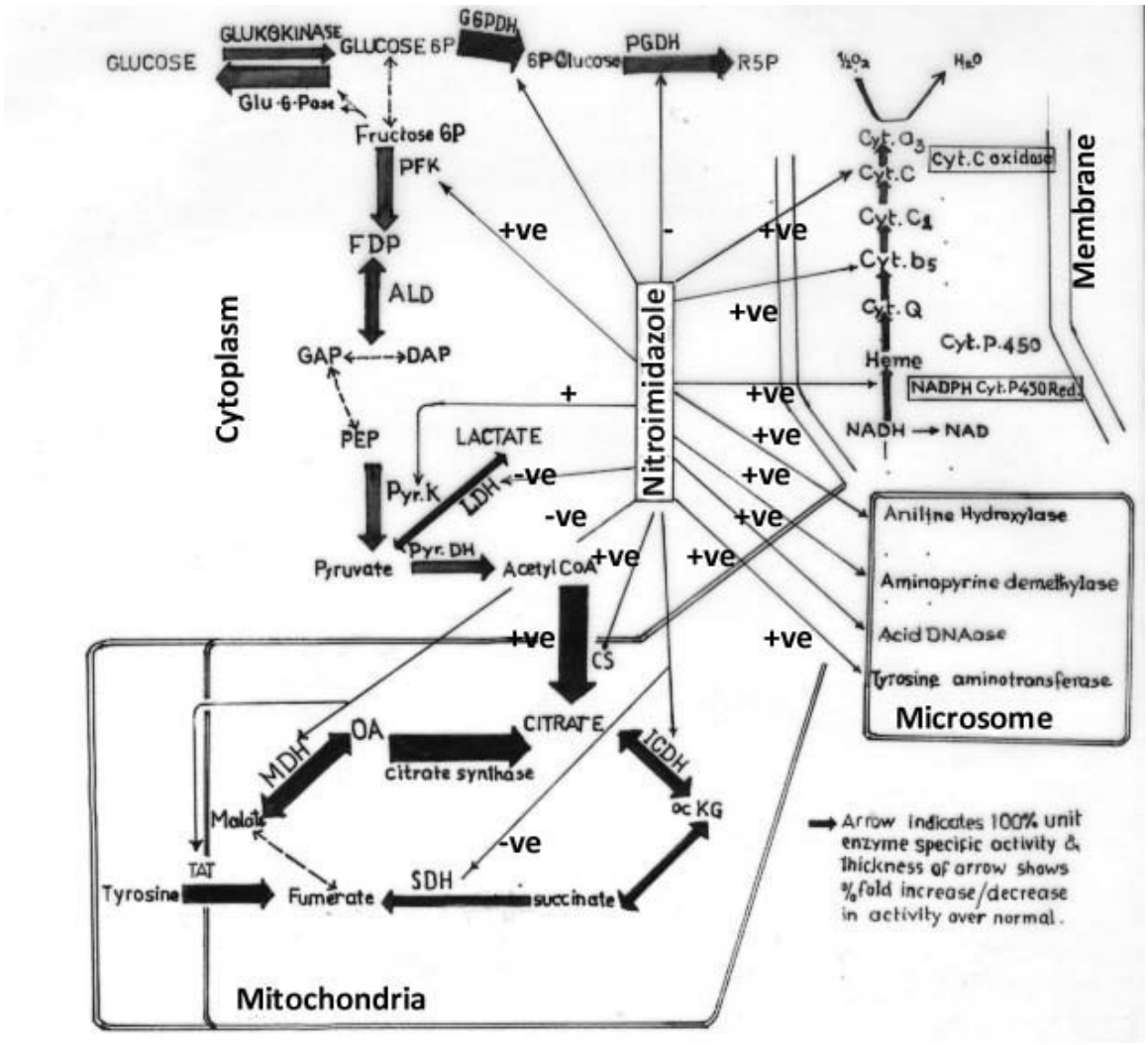

Figure 4: The sketch of relative biomarker enzyme changes (as arrow thickness) in liver due to nitroimidazole effect**.** The +ve sign denotes the relative increase in enzyme activity and –ve sign denotes the decrease in enzyme activity in liver cells in different organelles. The figure sketch also represents a sequence of metabolic steps of hepatocellular hypoxia criteria [Reproduced from Sharma R. Ph.D dissertation].

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 [1]. In serum, the enzymes exhibit their significance but aldolase, pyruvate kinase and LDH, MDH, ICDH observed as distinguishing the diffused injury or abscess formation [52]. 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.

Hypoxia is represented as a state of oxygen depletion in cell. Initially liver cell gets 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 make high energy metabolites in the cell (metabolic integrity). Infected liver cells and tumor cells already have less available oxygen (oxygen starved). Nitroimidazole cytotoxicity was established two decades ago as potential oxygen quenching chemical in most of the infected and tumor cells. Here the fact “Nitroimidazole oxygen quenching actions makes oxygen starved cells further worse to die and leaving normal cells functional” can be observed as potential tumor hypoxia therapy and antiparasitic treatment by nitroimidazole.

During metabolic integrity loss and resultant hypoxia state, liver cell signals the alarm to produce nitric oxide, release of cytokines (IFN-γ with IL-1β or TNF-α). These changes trigger the sequence of Kupffer cell stimulation and lysosomal action. As a result, lysosomal enzymes, growth factor, plasminogen stimulating factor were consistently observed suggestive of Kupffer cell suicidal phagocytic action (hepatic necrosis) by nitroimidazole analogs and reviewed widely [32, 50]. In drug induced hepatitis, liver lysosomal enzymes have been reported elevated or stimulation for any cellular defense apart from respiratory burst and chemotaxis by liver cells [53]. The present study showed the associated electron microscopic observations that

Kupffer cells accumulate around and exhibited hyperplasia condition showing degenerated nucleus, enlarged lysosomal vesicles. The enlarged lysosomal vesicles were further correlated by higher lysosomal enzyme levels in serum. In liver biopsies these lysosomal enzymes were significantly enhanced as shown in Table 2-3. 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 [54]. No data is available on liver cell enzymes estimated in liver cells from liver biopsy of nitroimidazole treated livers. Liver cell enzymes may expand 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, cyclooxygenase2, lactate dehydrogenase enzymes have been reported significant in hepatic hypoxia or hepatic damage evaluation with addition of new members of enzymes [54-56].

There appear two main reasons of enzyme level recovery by nitroimidazole. First, these enzyme changes were recovered by nitroimidazole therapy as drug changes the energy status in heaptocytes and Kupffer cell macrophagal activity diminishes the hepatic enzyme secretion and results in decreased enzyme levels towards normal. Second, regenerating hepatocytes may also regulate the normal cell recovery process and initializes the signaling Kupffer cells to keep stored enough lysosomal enzymes.

Challenges, Limitations and Futuristic approaches

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. In future, chip technology or silica or polymer coated enzyme estimation techniques may be more reliable in small sample collections with high degree of accurate enzyme estimation.

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, enzyme levels in 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.

Acknowledgements

The author acknowledges the assistance provided by Professor Rakesh K Tandon to provide assistant research officer job during this work with grant assistance from Indian Council of medical Research (ICMR) and Dr V.S.Singh for guidance supported with valuable Council of Scientific and Industrial Research fellowship and grant from ICMR, New Delhi.

References

1. Thiim M, Friedman LS. Hepatotoxicity of antibiotics and antifungals. Clin Liver Dis. 2003 May;7(2):381-99.

2. Brezden CB, Horn L, McClelland RA, Rauth AM. Oxidative stress and 1-methyl-2-nitroimidazole cytotoxicity. Biochem Pharmacol. 1998 Aug 1; 56(3): 335-44.

3. Ersoz G, Karasu Z, Akarca US, Gunsar F, Yuce G, Batur Y. Nitroimidazole-induced chronic hepatitis. Eur J Gastroenterol Hepatol. 2001 Aug;13(8):963-6.

4. Brezden CB, McClelland RA, Rauth AM. Apoptosis and 1-methyl-2-nitroimidazole toxicity in CHO cells. Br J Cancer. 1997;76(2):180-8.

5. Noss MB, Panicucci R, McClelland RA, Rauth AM. Preparation, toxicity and mutagenicity of 1-methyl-2-nitrosoimidazole. A toxic 2-nitroimidazole reduction product. Biochem Pharmacol. 1988 Jul 1;37(13):2585-93.

6. Sugiyama M, Tsuzuki K, Haramaki N. DNA single-strand breaks and cytotoxicity induced by sodium chromate(VI) in hydrogen peroxide-resistant cell lines. Mutat Res. 1993 Apr;299(2):95-102.

7. Berube LR, Farah S, McClelland RA, Rauth AM. Depletion of intracellular glutathione by 1-methyl-2-nitrosoimidazole. Int J Radiat Oncol Biol Phys. 1992;22(4):817-20.

8. Sapora O, Paone A, Maggi A, Jenner TJ, O'Neill P.Induction of mutations in V79-4 mammalian cells under hypoxic and aerobic conditions by the cytotoxic 2-nitroimidazole-aziridines, RSU-1069 and RSU-1131. The influence of cellular glutathione. Biochem Pharmacol. 1992 Oct 6;44(7):1341-7.

9. Moselen JW, Hay MP, Denny WA, Wilson WR. N-[2-(2-methyl-5-nitroimidazolyl)ethyl]-4-(2-nitroimidazolyl)butanamide (NSC 639862), a bisnitroimidazole with enhanced selectivity as a bioreductive drug. Cancer Res. 1995 Feb 1;55(3):574-80.

10. Stratford IJ, Williamson C, Hardy C. Cytotoxic properties of a 4-nitroimidazole (NSC 38087): a radiosensitizer of hypoxic cells in vitro. Br J Cancer. 1981 Jul;44(1):109-16.

11. Adams GE, Ahmed I, Clarke ED, O'Neill P, Parrick J, Stratford IJ, Wallace RG, Wardman P, Watts ME. Structure-activity relationships in the development of hypoxic cell radiosensitizers. III. Effects of basic substituents in nitroimidazole sidechains. Int J Radiat Biol Relat Stud Phys Chem Med. 1980 Dec;38(6):613-26.

12. Noss MB, Panicucci R, McClelland RA, Rauth AM. 1-Methyl-2-nitrosoimidazole: cytotoxic and glutathione depleting capabilities. Int J Radiat Oncol Biol Phys. 1989 Apr;16(4):1015-9.

13. Mulcahy RT, Gipp JJ, Carminati A, Barascut JL, Imbach JL. Chemosensitization at reduced nitroimidazole concentrations by mixed-function compounds combining 2-nitroimidazole and chloroethylnitrosourea. Eur J Cancer Clin Oncol. 1989 Jul;25(7):1099-104.

14. Widel M, Watras J, Suwinski J, Salwinska E. Radiosensitizing ability and cytotoxicity of some 5(4)-substituted 2-methyl-4(5)-nitroimidazoles. Neoplasma. 1982;29(4):407-15.

15. Rauth AM, Mohindra JK. Selective toxicity of 5-(3,3-dimethyl-1-triazeno)imidazole-4-carboxamide toward hypoxic mammalian cells. Cancer Res. 1981 Dec;41(12 Pt 1):4900-5.

16. Chao CF, Subjeck JR, Johnson RJ. Nitroimidazole inhibition of lactate production in CHO cells. Int J Radiat Oncol Biol Phys. 1982 Mar-Apr;8(3-4):729-32.

17. Whitmore GF, Varghese AJ, Gulyas S. Reaction of 2-nitroimidazole metabolites with guanine and possible biological consequences. IARC Sci Publ. 1986;(70):185-96.

18. Cowan DS, Matejovic JF, McClelland RA, Rauth AM. DNA-targeted 2-nitroimidazoles: in vitro and in vivo studies. Br J Cancer. 1994 Dec;70(6):1067-74.

19. Melo T, Duncan J, Ballinger JR, Rauth AM. BRU59-21, a second-generation 99mTc-labeled 2-nitroimidazole for imaging hypoxia in tumors. J Nucl Med. 2000 Jan;41(1):169-76.

20. Moller P, Wallin H, Vogel U, Autrup H, Risom L, Hald MT, Daneshvar B, Dragsted LO, Poulsen HE, Loft S Mutagenicity of 2-amino-3-methylimidazo[4,5-f]quinoline in colon and liver of Big Blue rats: role of DNA adducts, strand breaks, DNA repair and oxidative stress. Carcinogenesis. 2002 Aug;23(8):1379-85.

21. Ballinger JR, Kee JW, Rauth AM. In vitro and in vivo evaluation of a technetium-99m-labeled 2-nitroimidazole (BMS181321) as a marker of tumor hypoxia. J Nucl Med. 1996 Jun;37(6):1023-31.

22. Edwards DI. Mechanisms of cytotoxicity of nitroimidazole drugs. Prog Med Chem. 1981;18:87-116.

23. Edwards DI, Knox RJ, Knight RC. Structure-cytotoxicity relationships of nitroimidazoles in an in vitro system. Int J Radiat Oncol Biol Phys. 1982 Mar-Apr;8(3-4):791-3.

24. Ballinger JR. Imaging hypoxia in tumors. Semin Nucl Med. 2001 Oct;31(4):321-9.

25. Riche F, d'Hardemare AD, Sepe S, Riou L, Fagret D, Vidal M. Nitroimidazoles and hypoxia imaging: synthesis of three technetium-99m complexes bearing a

nitroimidazole group: biological results. Bioorg Med Chem Lett. 2001 Jan 8;11(1):71-4.

26. Melo T, Ballinger JR, Rauth AM. Role of NADPH:cytochrome P450 reductase in the hypoxic accumulation and metabolism of BRU59-21, a technetium-99m-nitroimidazole for imaging tumor hypoxia. Biochem Pharmacol. 2000 Sep 1;60(5):625-34.

27. Su ZF, Zhang X, Ballinger JR, Rauth AM, Pollak A, Thornback JR. Synthesis and evaluation of two technetium-99m-labeled peptidic 2-nitroimidazoles for imaging hypoxia. Bioconjug Chem. 1999 Sep-Oct;10(5):897-904.

28. Rumsey WL, Patel B, Kuczynski B, Narra RK, Chan YW, Linder KE, Cyr J, Raju N, Ramalingam K, Nunn AD.Potential of nitroimidazoles as markers of hypoxia in heart. Adv Exp Med Biol. 1994;345:263-70.

29. Koch CJ, Evans SM. Non-invasive PET and SPECT imaging of tissue hypoxia using isotopically labeled 2-nitroimidazoles. Adv Exp Med Biol. 2003;510:285-92.

30. Hodgkiss RJ, Webster L, Wilson GD. Measurement of hypoxia in vivo using a 2-nitroimidazole (NITP). Adv Exp Med Biol. 1997;428:61-7.

31. Sharma R. The Hepatocellular Dysfunction Criteria: Hepatocyte Carbohydrate Metabolizing Enzymes and Kupffer Cell Lysosomal Enzymes in 2'Nitroimidazole Effect on Amoebic Liver Abscess (Electron Microscopic – Enzyme Approach). Parasitology Research(Nova). 2009; 1(2)—(in press)

32. Sharma R, Kwon S. Role of immunoregulators as possibility of tumor hypoxia-induced apoptosis. J Apoptosis Research. 2009;1(2):--(in press)

33. Mahy, P, De Bast M, Leveque PH, Gillart J, Labar D, Marchand J, Gregoire V. Preclinical validation of the hypoxia tracer 2-(2-nitroimidazol-1-yl)- N-(3,3,3-[(18)F]trifluoropropyl)acetamide, [(18)F]EF3. Eur J Nucl Med Mol Imaging. 2004;31(9):1263-72.

34. Chu T, Li R, Hu S, Liu X, Wang X. Preparation and biodistribution of technetium-99m-labeled 1-(2-nitroimidazole-1-yl)-propanhydroxyiminoamide (N2IPA) as a tumor hypoxia marker. Nucl Med Biol. 2004;31(2):199-203.

35. Grönroos T, Bentzen L, Marjamäki P, Murata R, Horsman MR, Keiding S, Eskola O, Haaparanta M, Minn H, Solin O. Comparison of the biodistribution of two hypoxia markers [18F]FETNIM and [18F]FMISO in an experimental mammary carcinoma. Eur J Nucl Med Mol Imaging. 2004 Apr;31(4):513-20.

36. Ziemer LS, Evans SM, Kachur AV, Shuman AL, Cardi CA, Jenkins WT, Karp JS, Alavi A, Dolbier WR Jr, Koch CJ.Noninvasive imaging of tumor hypoxia in rats using the 2-nitroimidazole 18F-EF5. Eur J Nucl Med Mol Imaging. 2003 Feb;30(2):259-66.

37. Jankovic B, Aquino-Parsons C, Raleigh JA, Stanbridge EJ, Durand RE, Banath JP, MacPhail SH, Olive PL. Comparison between pimonidazole binding, oxygen electrode measurements, and expression of endogenous hypoxia markers in cancer of the uterine cervix. Cytometry B Clin Cytom. 2006 Mar;70(2):45-55.

38. Papadopoulou MV, Ji M, Rao MK, Bloomer WD. Reductive metabolism of the nitroimidazole-based hypoxia-selective cytotoxin NLCQ-1 (NSC 709257). Oncol Res. 2003;14(1):21-9.

39. Eschmann SM, Paulsen F, Reimold M, Dittmann H, Welz S, Reischl G, Machulla HJ, Bares R. Prognostic impact of hypoxia imaging with 18F-misonidazole PET in non-small cell lung cancer and head and neck cancer before radiotherapy. J Nucl Med. 2005 Feb;46(2):253-60.

40. Heimbrook DC, Shyam K, Sartorelli AC.Novel 1-haloalkyl-2-nitroimidazole bioreductive alkylating agents. Anticancer Drug Des. 1988 Mar; 2(4): 339-50.

41. Berube LR, Farah S, McClelland RA, Rauth AM. Depletion of intracellular glutathione by 1-methyl-2-nitrosoimidazole. Int J Radiat Oncol Biol Phys. 1992;22(4):817-20.

42. Adams GE, Ahmed I, Clarke ED, O'Neill P, Parrick J, Stratford IJ, Wallace RG, Wardman P, Watts ME. Structure-activity relationships in the development of hypoxic cell radiosensitizers. III. Effects of basic substituents in nitroimidazole sidechains. Int J Radiat Biol Relat Stud Phys Chem Med. 1980 Dec;38(6):613-26.

43. Bos HJ, Van Den Eijk AA, Steeremberry PA. Application of ELISA in serodiagnosis of amoebiasis. Trans Roy Soc Trop Med Hyg. 1976;6:440.

44. Mehghini Giorgie. New Series. 1959;Vol 4(9):682-692.

45. Worthington Enzyme Manual. Worthington Biochemical Corporation, 730 Vassar Avenue, Lakewood, New Jersey 08701, Editor: Worthington, V. 1993:1-1947.

46. Tandon BN, Tandon HD, Puri BK. An electron microscopic study of liver in hepatomegaly presumably caused by amoebiasis. Exp Mol Pathol 1975; 22:118-132.

47. Horlen CK, Seifert CF, Malouf CS. Toxic metronidazole-induced MRI changes. Ann Pharmacother. 2000 Nov;34(11):1273-5.

48. Tabak F, Ozaras R, Erzin Y, Celik AF, Ozbay G, Senturk H. Ornidazole-induced liver damage: report of three cases and review of the literature. Liver Int. 2003 Oct;23(5):351-4.

49. Larrey D. Drug-induced liver diseases. J Hepatol. 2000;32(1 Suppl):77-88.

50. Michalopoulos GK, DeFrances MC. Liver regeneration. Science. 1997, 276:60-66.

51. Ramirez-Emiliano J, Flores-Villavicencio LL, Segovia J, Arias-Negrete S. Nitric oxide participation during amoebic liver abscess development. Medicina (B.Aires) 2007; 67(2): 167-176.

52. Das P, Debnath A, Munoz ML. molecular mechanisms of pathogenesis in amoebiasis. Ind J Gastroenterol 1999, 18(4):161-166.

53. Virk KJ, Ganguly NK, Dilawari JB, Mahajan RC Role of lysosomal enzymes in tissue damage during hepatic amoebiasis. An experimental study. Liver. 1989 Dec ;9 (6):338-45.

54. Virk KJ, Mahajan RC, Dilawari JB, Ganguly NK. Role of beta-glucuronidase, a lysosomal enzyme, in the pathogenesis of intestinal amoebiasis: an experimental study. Trans R Soc Trop Med Hyg. 1988 ;82 (3):422-5.

55. Ventura-Juárez J, Jarillo-Luna RA, Fuentes-Aguilar E, Pineda-Vázquez A, Muñoz-Fernández L, Madrid-Reyes JI, Campos-Rodríguez R. Human amoebic hepatic abscess: in situ interactions between trophozoites, macrophages, neutrophils and T cells. Parasite Immunol. 2003 Oct;25(10):503-11.

56. Sánchez-Ramírez BE, Ramírez-Gil M, Ramos-Martínez E, Rohana PT. Entamoeba histolytica induces cyclooxygenase-2 expression in macrophages during amebic liver abscess formation. Arch Med Res. 2000 Jul-Aug;31(4 Suppl):S122-3.