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ISONIAZID INDUCES OXIDATIVE STRESS, MITOCHONDRIAL DYSFUNCTION AND APOPTOSIS IN HEP G2 CELLS

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Abstract- Isoniazid (INH) continues to be a sheet anchor in treatment of tuberculosis, however its chronic administration is known to cause hepatotoxicity through a poorly defined mechanism. Ellucidation of mechanism underlying INH induced hepatotoxicity may be beneficial in devising ways to counteract toxic manifestations. In view of this concentration dependent effects INH were evaluated in hepatoma cell line (Hep-G2). INH exposure produced cytotoxic effects in Hep-G2 cells in a characteristic dose dependent manner. There was considerable cell detachment, loss of viability and alterations in cellular morphology that were indicative of toxic insult. We observed cell shrinkage at highest concentrations (88µM) suggesting an involvement of apoptosis. This finding was substantiated by the flow cytometry data and DNA fragmentation analysis which clearly indicated that INH induced cytotoxicity, was being mediated by induction of apoptosis. Furthermore there was mitochondrial dysfunction as indicated by significant inhibition of MTT Reduction as compared to control at all the concentrations and depletion of cellular glutathione (GSH) content along with increased production of Reactive oxygen species (ROS). Collectively these findings led us to conclude that INH induced apoptosis in Hep-G2 cells is mediated by generation of oxidative stress.

Key words: Apoptosis, Hepatotoxicity, Oxidative stress, Reactive Oxygen species.

INTRODUCTION

Mycobacterium tuberculosis infection is one of the leading causes of death in third world countries. This single infectious agent has a global prevalence of greater than 1.6 billion persons (10, 11). M. Tuberculosis shows important synergy with HIV (33) and because of increased incidence of tuberculosis that has resulted from the co-epidemic of HIV infection, use of isoniazid (INH) in the prevention and treatment of tuberculosis is on the rise (41). INH or isonicotinic acid was discovered in 1950's and since then it has remained a sheet anchor in the treatment of tuberculosis. It is a synthetic bactericidal agent that enters the mycobacterial cells through positive diffusion across bacterial envelop (4) and exerts its bactericidal effects by producing reactive species (31). INH continues to be highly effective in chemoprophylaxis and treatment of tuberculosis due to low cost per dose and reasonable bioavailability (15). It is given chronically to critically ill patients who are on multiple drug therapy to treat tuberculosis. However daily administration of INH has been reported to be associated with mild elevation of liver enzyme activities in plasma in upto 20 % of patients (21) and significant hepatotoxicity in approximately 1-2% of patients (5). This hepatotoxicity is potentially fatal if not detected early (28). Over 25 years after the toxicity was detected in INH treated patients, the mechanism remains unknown and therefore the hepatotoxicity remains neither preventable nor treatable (28). It has been suggested that acetylation followed by hydrolysis are the first step in the metabolic activation of INH resulting in formation of acetylhydrazine (37) which is a toxicologically active metabolite. However, no idiosyncratic metabolic pathway has been described that would explain idiosyncratic isoniazid hepatotoxicity

Research on mechanism of INH induced hepatotoxicity has been partly hampered due to the lack of the suitable animal model that closely parallels the toxicity in humans. Earlier studies have revealed that toxicity in was due to conversion of a metabolite of INH to a reactive intermediate (34). Although these initial studies were done using a rat model, but a growing body of evidence suggests that INH and its major metabolites are not hepatotoxic in rats (42). Hepatotoxic action of INH in dogs, rabbits, and guinea pigs has also been investigated. However

none of these experiments yielded an animal model that truly represented the signs and symptoms of toxicity as observed in humans (29). The present study therefore was conducted in Hep G2 cells, which are hepatoma cells of human origin. They display many genotypic and phenotypic characters of normal liver cells and thus are better test system as compared to rats. Peak plasma concentration of INH following an oral dose of 300 mg is 20-50µM. Based on this we selected four different concentrations viz 11µM, 22µM, 44µM, 88µM. Our aim was to evaluate the effects of pharmacological levels of drug on Hep G2 cells over an extended period of time.

Interstingly M. Tuberculosis has a predilection for the malnourished subjects (22). Golden and Randath (14) provided an evidence that malnutrition results in excessive load of free radicals. Many drugs such as acetaminophen, CCl_4 possibly cause toxicity as a result of excess production of reactive oxygen species (ROS) and free radicals (9, 25). INH is also metabolised to reactive species by the action of catalase-peroxidase (kat g) enzyme (41). Eventually this may result in overload of reactive species such as free radicals and ROS in an already malnourished subject. Administration of INH may further aggravate the situation by disrupting the delicate antioxidant/pro-oxidant balance of cells leading to generation of oxidative stress. It is with this idea in mind that the following study was undertaken. The aim of the present study was to evaluate the effect of INH on Hep G2 cells and to explore whether these effects were mediated by alteration of cellular redox status.

MATERIALS AND METHODS

Cell cultures

The human hepatoma cell line Hep G2 obtained from National Center for Cell Sciences, Pune were cultured in DMEM supplemented with 10 % FBS and 1% antibiotic/ antimycotic solution (Sera Laboratories International Ltd, U.K.) at 37°C and 5% CO_2 using standard cell culture methods. Cells were used at passages 3-10. At conflunce, cells were trypsinised, transferred into standard cell culture plates at a density of $3x10^4$ and were allowed to adhere for 24 hr. In order to evaluate the effect of INH, exponentially dividing cells were exposed to different concentrations of INH (11µM, 22µM, 44µM and 88µM). Hydrogen peroxide (300µm) was used as positive control.

Cellular morphology

Cells were incubated with different concentrations of INH (11-88µM) at 37 °C in a humidified atmosphere of 5% CO_2 for 24 hrs. INH induced morphological changes were visualized in Leica DM IRB Phase-Contrast Microscope (20X).

Cytotoxicity assay

Cytotoxic effects of INH in Hep G2 cells were assessed by cellular neutral red uptake and lactate dehydrogenase (LDH) leakage assay. Cellular neutral red uptake by control and INH treated cells was measured in accordance with the method described by Triglia et al., (38). Briefly, cells were washed twice with saline after treatment and incubated with 200µl of (0.25%) neutral red for 2 hours. Excess stain was removed; cells were washed twice with phosphate-buffered saline (PBS) and air-dried for approximately 1 hr at room temperature. The intracellular neutral red was extracted by shaking the cells with acetic acid/ethanol solution for 20 minutes and its amount was quantified by recording absorbance at 550 nm in Biotek (Power Wave XS) microplate spectrophotometer. Results were expressed as relative neutral red uptake. Lactate dehydrogenase activity in culture media was measured spectrophotometrically as an index of plasma membrane damage and loss of membrane integrity, following the method developed by Vassault (39). Reaction mixture contained 80 mM Tris-Cl (pH 7.4), 200mM NaCl and 0.2mM NADH. Reaction mixture was equilibrated to 37°C followed by addition 500 µl aliquot of media. Reaction was initiated by adding 500 µl of 9.6 mM sodium pyruvate and change in absorbance at 340 nm was monitored for 5 minutes. Activity of LDH was expressed as U/L

Apoptosis detection by annexin V binding

Apoptosis was detected by identifying cell membrane alterations that accompany programmed cell death through flow-cytometry as per the method recommended by manufacturer (Calbiochem, Germany). In the normal viable cells phosphatidyl serine is located in the cytosolic half of lipid bilayer. Following induction of apoptosis rapid alterations occur in the organization of membrane phospholoipids, which eventually lead to localization of phosphatidyl serine to the outer half of lipid bilayer (13, 20). This localization of phosphatidyl serine can be used as a biomarker for detection of apoptosis. In vitro detection of externalized phosphatidyl serine (PS) can be achieved through interaction with the annexin V (7,17). In presence of calcium, rapid high affinity binding occurs betweein annexin V and phosphatidyl serine and therefore cells progressing through apoptosis can be readily recognized by their annexin V positivity. Briefly control and treated cells were washed twice with PBS and thereafter harvested quickly by trypsinization. Cells were collected by centrifugation at 500xg and gently resuspended in cold binding buffer to achieve a cell density of $1x10^6$ cells /ml. Homogeneous cell suspension was incubated with 1.25µl Annexin V-FITC conjugate (200 µg/ml) for 15 minutes in dark, at room temperature. Thereafter cells were centrifuged at 100xg for 5 minutes at room temperature. Supernatant was discarded and pelleted cells were again suspended in 0.5ml of binding buffer. Finally 10 µl of Propidium iodide (30µg/ml) was added to homogeneous cell suspension and samples were incubated in dark for 15 minutes. Samples were placed on ice and analysed by flow cytometry for detection of apoptosis.

DNA fragmentation

Total DNA was isolated from brain as described by Sambrook et al. (27). Briefly, control and treated cell were harvested by trypsinization and collected at 1000xg. Detached cells present in culture media were also collected at 1000xg. Both the populations were pooled together and

used for subsequent steps. Pelleted cells were suspended in phosphate buffered saline(pH 7.5) followed by lysis using cell lysis buffer (10 Tris-Cl, 0.1M EDTA, 0.5% SDS, 20 µg/ml pH 8.0) at 37°C for1 hr. Samples were treated with proteinase K (100µg/ml) at 50°C for 3 hr. At the end of the incubation, samples were cooled to room temperature and equal volume of Phenol-Chloroform-Isoamyl alcohol (25:24:1) mixture was added. Aqueous and organic phase were mixed by gently swirling the tubes. Alternatively tubes were left on angular shaker for 5 minutes. Deproteination was done by centrifuging the samples at 10,000×g at 4°C for 15 minutes. Aqueous phase was collected in a fresh tube and extraction was repeated until the interface became clear. Finally DNA present in aqueous phase was precipitated by adding 2.5 volumes of chilled alcohol in presence of 0.1 volumes of 1M sodium acetate at -20°C for overnight. The precipitate was collected at 10,000×g for 15 minutes washed once with 70% ethanol, air dried and dissolved in minimum quantity of TE buffer (Tris-Cl 10 mM, EDTA 1mM, pH 8.0). Thereafter DNA samples were mixed with 6x gel loading buffer (0.25% xyelene cyanol, 0.25% bromophenol blue and 30% glycerol) and resolved electrophoretically through 1.5 % agarose gel (containing 10µl of 0.02% EtBr) in TBE buffer at 50 volts till the dye bromophenol blue migrated upto one cm from the bottom of the gel. After completion of the run, bands were visualized and analyzed in gel documentation system (Bio-Rad Lab, USA).

Mitochondrial integrity

Mitochondrial integrity was measured using 3-(4, 5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) following the method developed by Mossman (24). For evaluation of mitochondrial integrity cells were grown in standard 12 well cell culture plates. After exposing the cells to different concentrations of INH for 24 hours, 200 µl/well of 0.5% MTT was added. Cells were incubated with MTT for 2 hours at room temperature in dark. After 2 hours excess MTT was decanted and cells were air dried at room temperature for 1 hr. Blue colored formazon was extracted in 10% DMSO by shaking the plates gently for 4-5 minutes and its amount was quantified by recording the absorbance at 550 nm in Biotek (Power Wave XS) microplate spectrophotometer. Results were expressed as relative MTT reduction.

Reactive Oxygen Species

Amount of ROS in cells was measured at 1hr, 6 hr and 24 hr after treatment using 2', 7'-dichlrofluorescein diacetate (DCF-DA) that gets converted into highly fluorescent DCF by cellular peroxides. The assay was performed as described by Socci et al., (35). After completion of the stipulated treatment duration, medium was withdrawn and 0.1ml of ice–cold 40 mM tris –hcl buffer (ph 7.4) was added to the cells followed by addition of 40-µl of 1.25 mM DCF-DA. Fluorescence was recorded for 1 hour at 488nm excitation and 525 nm emissions using a fluorescence plate reader (Varian Cary Eclipse Spectrofluorimeter). Amount of ROS generated was expressed in terms of relative fluorescence unit (RFU).

Glutathione

Intracellular GSH content was measured according to the method described by Ellman et al., (12) and modified by Jollow et al., (16). Harvested cells were suspended in 0.25 M sucrose and incubated for 10 minutes at 37 °C for complete lysis. After lysis, 300µl of 4 % sulphosalycylic acid was added and supernatant was collected after centrifugation at 1000xg. An aliquot of 0.2 ml was mixed with 0.4 ml of 10 mM 5, 5'dithiobis-(2-nitrobenzoic acid) (DTNB) and 1 ml phosphate buffer (0.1M, ph 7.4). Amount of GSH was measured in terms of reduced DTNB by recording the absorbance at 412 nm. GSH concentration was expressed as mg GSH/ml.

Aspartate aminotransferase and Alanine aminotransferase

In order to evaluate effect of INH exposure on leakage of cytosolic transaminases, cells were incubated with respective concentrations of INH for 24 hr and culture medium was collected for estimation of aspartate aminotransferase and alanine aminotransferase activities. Aspartate aminotransferase and Alanine aminotransferase activities were measured with the help of Beckman Synchron CX5 clinical system and results were expressed as U/L. The activity of AST was assayed according to the method of Reitman and Frankel (26). The assay system contained 1.0 ml of substrate solution (consisting of 1.0 M aspartic acid and 2 mM 2-oxoglutaric acid in 0.1 M phosphate buffer, pH 7.4), 0.2 ml serum/liver/kidney homogenate (10% w/v). Reaction was carried out incubating the reaction mixture for exactly 60 minutes at 37°C in water bath. 1.0 ml of chromogen solution (20 mg 2,4-dinitrophenyl hydrazine in 1.0 N HCl and volume made up to 100 ml) was added, mixed and allowed to stand for 20 minutes at room temperature. 10 ml of NaOH (0.4 N) was then added and the optical density was read after 5 minutes against blank at 505nm using silica cell of 1cm light path. The controls were run parallel, but in these tubes the substrate being added after deproteinizaation. The activity of ALT was measured by the method of Reitman and Frankel (26). The assay system contained 1.0 ml of substrate (0.2 M DL-alanine, 2 mM 2-oxoglutarate in 0.1 M phosphate buffer, pH 7.4) and 0.2 ml of serum/liver/kidney homogenate (10% w/v) and incubated for exactly 30 minutes at 37°C in water bath. 1.0 ml of chromogen solution (20 mg 2, 4-dinitrophenyl hydrazine in 1.0 N HCl, made upto 100 ml) was added, mixed and allowed to stand for 20 minutes at room temperature. 10.0 ml of NaOH (0.4 N) was added, mixed and optical density was read at 505 nm after 5 minutes against reagent blank. Controls were run parallel, with substrate being added after deproteinization.

Statistical analysis

Data are expressed as means ± SEM. Data comparisons were carried out using one way analysis of variance followed by Newman keul's post test to compare means between the different treatment groups. Differences with p value < 0.05 were considered significant.

RESULTS

Fig 1 represents morphological features of INH induced cytotoxiciy in Hep G2 cells as visualized through phase contrast microscope. Control cells (Fig 1.A) appeared normal having a well-defined polygonal morphology with intact

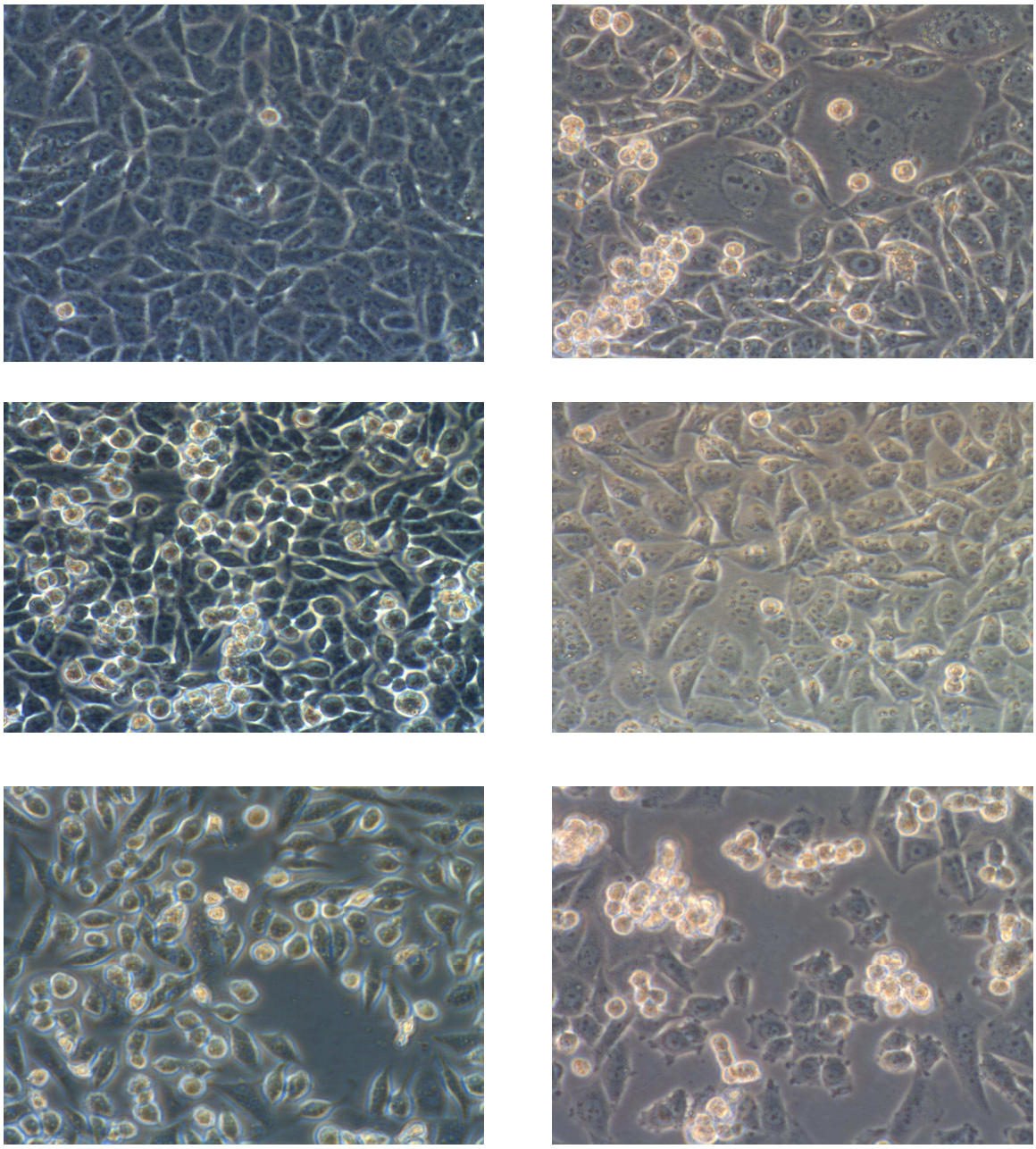
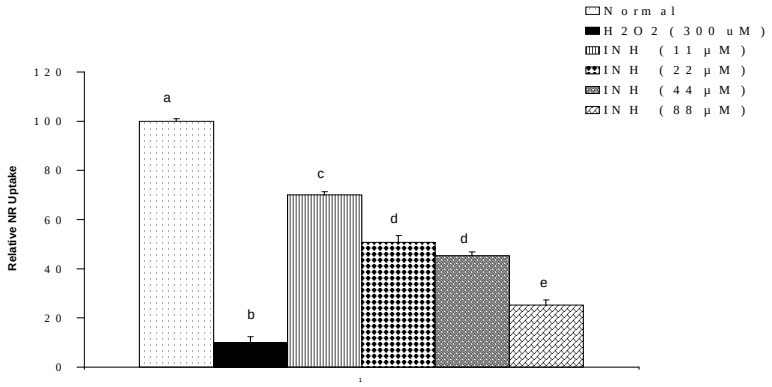


Figure 1. Morphological features of INH induced cytotoxiciy in Hep G2 cells (A-F). A: Phase contrast micrograph (20X) of normal (negative control) cells. B: H_2O_2 (300 µM, positive control) treated cells. C: INH (11µM) treated cells. D: INH (22 µM) treated cells. E: INH (44µM) treated cells. F: INH (88µM) treated cells.

membrane and well defined centrally located nucleus. Cells treated with H_2O_2 (Fig 1. B) showed significant loss of integrity. There was considerable cytotoxicity as indicated by large numbers of free-floating dead cells. Cellular detachment was accompanied by evident changes in morphological features such as cell size and structure. Cells treated with INH (11µM) although did not show such a significant loss of integrity, but there were subtle signs of toxicity in terms of loss of cell-cell contact giving an indication that there was impairment of cell-cell interaction (Fig 1.C). At the higher concentrations (22 µM, 44 µM) the toxic effects were relatively more evident. There was considerable change in cell morphology relatively larger number of cell showed detachment at this concentration. Maximum deleterious effects were observed at highest concentration (88 µM), wherein apart form other manifestations of toxic insult such as impairment of cell-cell interaction and cellular detachment, considerable degree of cell shrinkage in otherwise viable cells was also noticed (Fig 1.F).

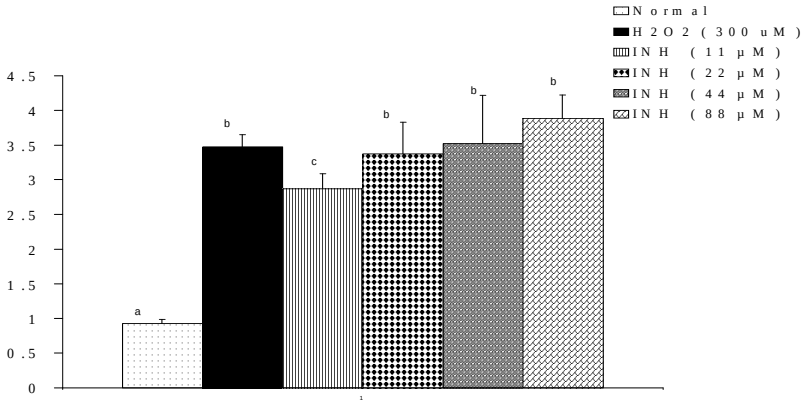
For quantitative evaluation of overall loss of cell viability caused by different concentrations of INH, LDH release assay in culture medium and cellular neutral red uptake assays were carried out. There was significant release of LDH into culture media from hydrogen peroxide and INH treated cells as compared to normal cells and this finding was concurrent with decreased neutral red uptake by INH treated cells (Fig 2 & 3). Interestingly both the parameters showed concentration dependent effect with maximum loss of viability at the concentration of 88µM.



Units and Abbreviations: NR- Neutral red uptake

Values are mean±SE; n=5. *-‡ Differences between values with matching symbol notations are not statistically significant at 5 % level of probability

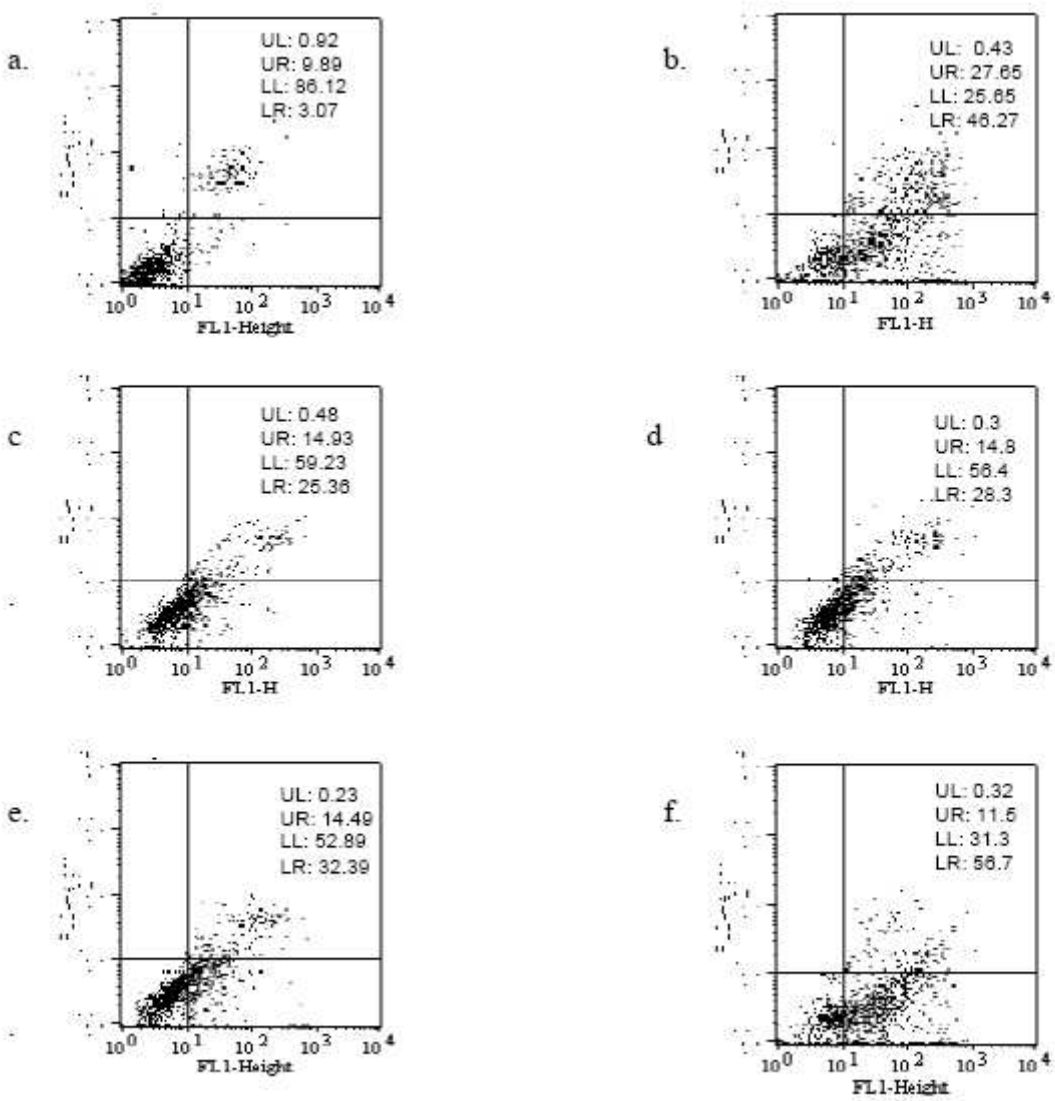
Figure 2. Effect of INH exposure on cell viability expressed as neutral red uptake by Hep G2 cells.



Units and Abbreviations: LDH-Lactate Dehydrogenase; U/L

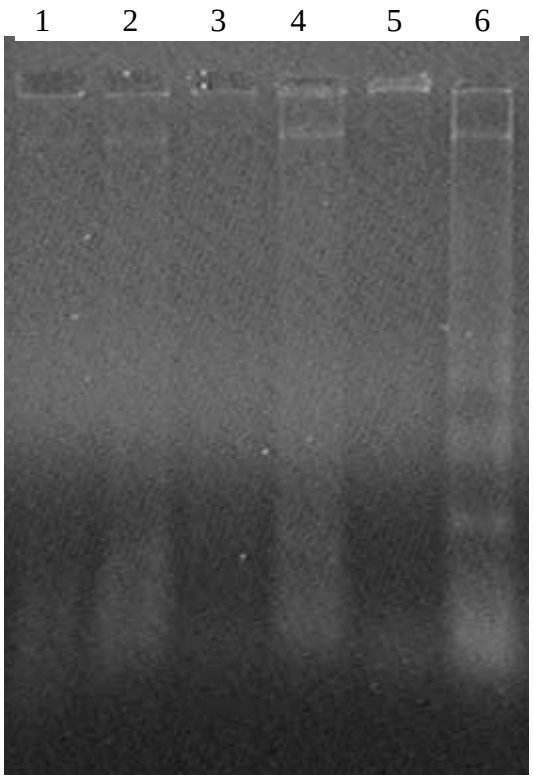
Values are mean±SE; n=5. *-‡ Differences between values with matching symbol notations are not statistically significant at 5 % level of probability

Figure 3. Effect of INH exposure on cell viability expressed as indicated by LDH release from Hep G2 cells.



A: Flow cytometric analysis of normal (negative control) cells. B: H_2O_2 (300 µM, positive control) treated cells. C: INH (11µM) treated cells. D: INH (22 µM) treated cells. E: INH (44µM) treated cells. F: INH (88µM) treated cells. Legend: UL-Upper left (unidentified cells/cell debris), UR-Upper right (PI positive non-viable cells), LL-Lower left (Normal cells), LR-Lower right (Annexin V positive apoptotic cells). All values are given in %.

Figure 4. Evaluation of INH induced apoptosis/necrosis by annexin binding and PI staining of Hep G2 cells.



Agarose gel electrophoresis of DNA extracted fron Hep-G2 cells exposed to varying concentrations of INH for 48 hrs. Lane 1: Normal; Lane 2: H2O2(300µM); Lane 3: INH (11 µM); Lane 4: INH (22 µM); Lane 5: INH (44 µM); Lane 6: INH (88 µM)

Figure 5. DNA fragmentation analysis of Hep-G2 cells treated with different concentrations of INH.

Flow cytometric analysis of control, H_2O_2 (300µM) treated, and INH exposed cell is represented in fig.4. Annexin V and propidium iodide (PI) enabled flow cytometric sorting of Hep G2 cells into live, apoptotic and necrotic cells. All PI-positive stained embryonic cells were classified as necrotic regardless of their annexin V staining. Cells with no PI staining were sorted as annexin V positive (apoptotic) and annexin V negative (normal) cells. H_2O_2 exposed cells (positive control) showed considerable cytotoxicity when compared to normal cells. INH too showed dose dependent cytotoxic effect. Interestingly there was almost a constant number of necrotic cells at all the concentrations but number of apoptotic cells increased in a dose dependent manner indicating that apoptosis was major pathway responsible for INH induced cell death. At higher concentrations, occurrence of apoptotic cell death became more prevalent and at the highest concentration (88 µM) as many as 56% cells were found positive for occurrence of apoptosis. In addition to this agarose gel electrophoresis (fig.5) of DNA samples collected after 48 hr also revealed clear signs of apoptosis at highest concentration. Chracteristic DNA lader was present in the cell exposed to the highest concentration of INH. Interestingly DNA fragmentation was not evident at lower concentrations despite presence of significant number of annexin positive cells.

Fig 6 shows effect of INH on mitochondrial integrity as indicated by MTT reduction. Succinate dehydrogenase of intact mitochondria reduces MTT to produce the blue colour formazon. Degree of MTT reduction thus gives a direct indication of the mitochondrial integrity. In our study there was significant loss of mitochondrial integrity in hydrogen peroxide treated Hep G2 cells when compared to normal cells. In addition to this exposure to INH also caused significant loss of mitochondrial function in a concentration dependent manner.

Figure 7. Represents effect of INH on ROS generation as indicated by sequential hydrolysis and oxidation of non-fluorescent DCFDA to DCF in the presence of ROS. Control cells did not show any significant increase in ROS at different time intervals (1 hr, 6hr, 24hr) while H_2O_2 (300µM) treated cells showed

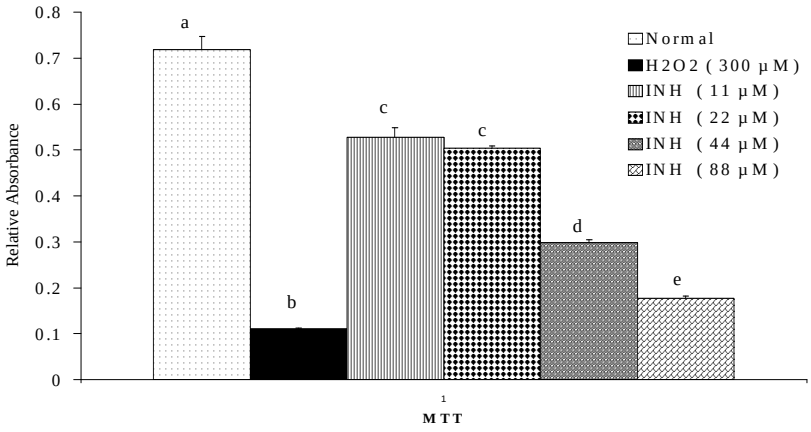
progressive increase with time. Similar changes were observed in the cells treated with different concentrations of INH (11 µM, 22µM, 44µM). However at highest concentration (88µM) there was sudden spurt in DCF fluorescence at 1 and 6 hr following which there was a steep decline at 24 hr.

Effect of INH treatment on cellular glutathione after 24 hr was evaluated and results have been represented in figure 8. INH exposure depleted cellular glutathione content drastically. However there was no concentration dependent effect. Effect of INH on leakage of cytosolic transaminases into culture media was evaluated as an index of organotypic toxicity endpoint and results have been shown in table 1. Leakage of alanine transaminase into extracellular culture media was observed in H_2O_2 treated and INH treated cells at all the four concentrations after completion of 24 hr exposure period, however the concentration dependent effect was not seen. On the other hand there was no significant increase in extracellular aspartate aminotransferase activity following INH exposure.

Table 1. Effect of INH on the extracellular leakage of cytosolic transaminases from Hep G2 cells.

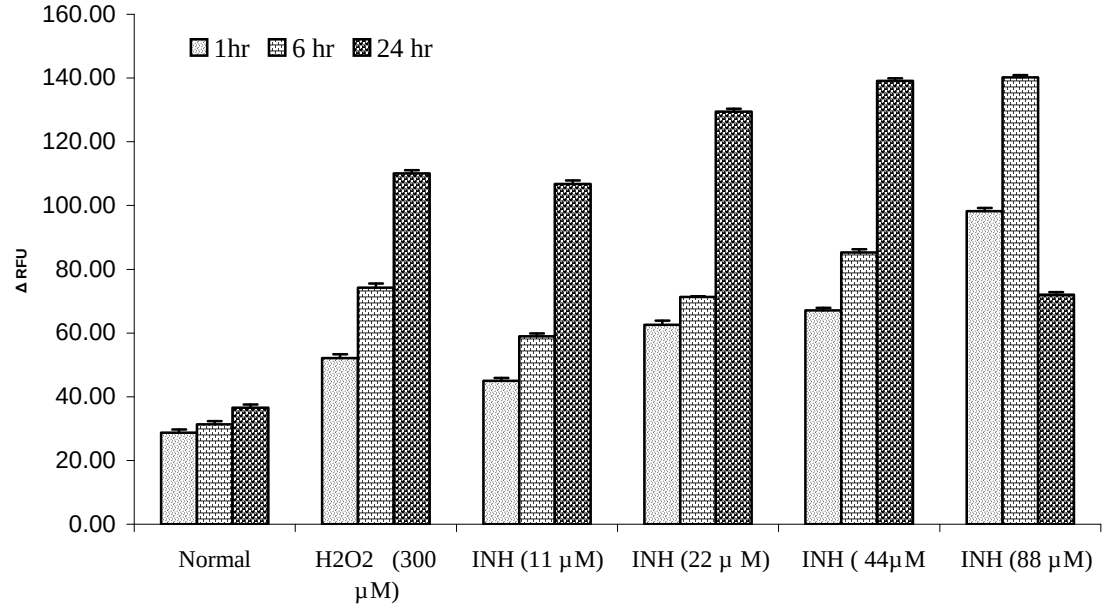
	AST	ALT
Normal	23.0±1.0^a	6.7±0.2^a
H_2O_2 (300µM)	24.3±1.9^a	11.7±1.8^b
INH (11µM)	27.0±1.9^b	9.0±0.3^c
INH (22 µM)	27.7±2.7^b	7.7±0.7^d
INH (44 µM)	27.0±3.0^b	7.0±0.0^d
INH (88 µM)	28.7±2.2^b	9.7±0.4^c

Units and Abbreviations:
AST; Aspartate aminotransferase expressed as U/ml: ALT; Alanine aminotransferase expressed as U/ml
Values are mean±SE; n=5. $^{a-c}$ Differences between values with matching symbol notations with in each column are not statistically significant at 5 % level of probability



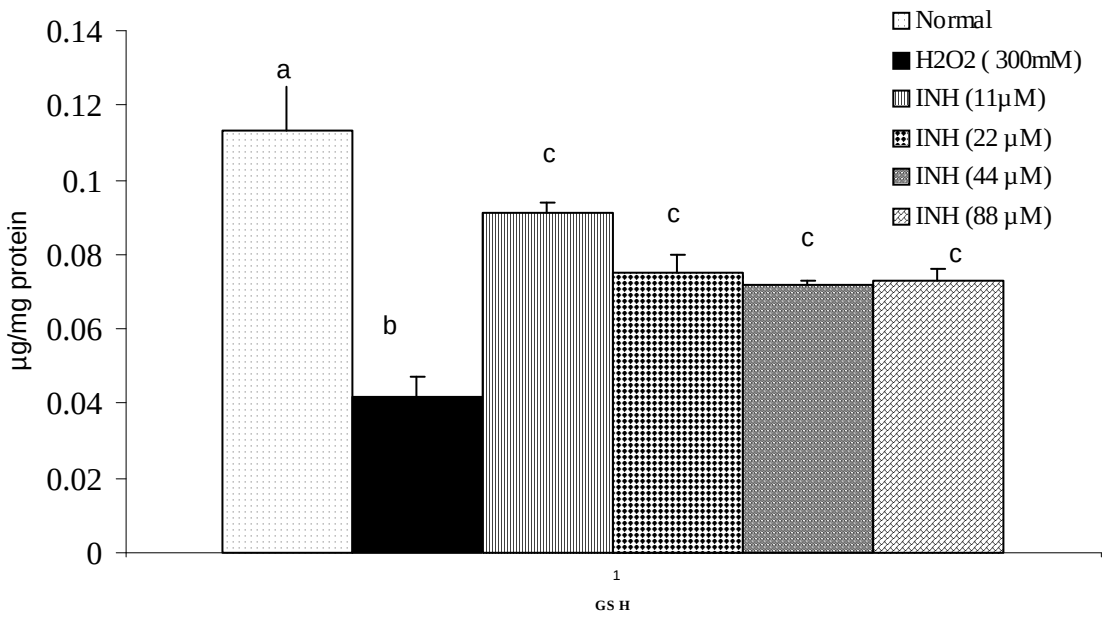
Units and Abbreviations: MTT; 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide, expressed as relative absorbance compared to blank.
Values are mean±SE; n=5. $^{a-c}$ Dffierences between values with matching symbol notations are not statistically significant at 5 % level of probability

Figure 6. Concentration dependent effect of INH on mitochondrial integrity as indicated by reduction of MTT by intact mitochondrial dehydrogenases.



Units and Abbreviations: ROS; Reactive oxygen species expressed as change in relative fluorescence unit (RFU)
Values are mean±SE; n=5. [a-g] Dffierences between values with matching symbol notations are not statistically significant at 5 % level of probability

Figure 7. Reactive oxygen species generation in Hep G2 cells by INH in a concentration and time dependent manner.



Units and Abbreviations: GSH; Glutathione expressed as ug/mg protein
Values are mean±SE; n=5. [a-c] Dffierences between values with matching symbol notations are not statistically significant at 5 % level of probability.

Figure 8. Concentration dependent effects of INH on intracellular glutathione content.

DISCUSSION

INH is being used as a first-line agent for prophylaxis and treatment of tuberculosis since 1952 (28). Although it is known to cause potentially fatal hepatotoxicity through a poorly defined mechanism but being superior drug in terms of efficacy, it cannot be excluded from the treatment regimen. Elucidation of the mechanism underlying INH induced hepatocellular toxicity can be beneficial especially in devising ways to counteract the episodes of toxicity. However efforts aimed in this direction have been greatly compromised by the difficulty surrounding the development of an animal model of INH induced hepatotoxicity (29). Therefore present study was conducted with an objective to evaluate the in-vitro effects of INH in hepatoma cell line of human origin.

In our study we observed clear signs of toxicity especially at higher concentrations of INH with clear-cut morphological alterations. There was extensive rounding of otherwise polygonal cells accompanied by cell detachment and loss of viability. There was considerable loss of cell-cell contact at two highest concentrations giving an indication that cell-cell interaction required for survival and sustenance of cell has been completely disrupted. The toxic insult or any other patho-physiological alterations that cause cellular damages are generally followed by closure of intercellular gap junctions through which various signals, ions and second messenger molecules pass among the neighboring cells (1). With exception of a few terminally differentiated cells such as skeletal muscle cells and blood cells, most of the cells are in communication with their neighboring cells via gap junctions. When a cell is damaged, its plasma membrane can become leaky. Ions present at higher concentration in the extracellular fluid, such as Ca^{2+} and Na^{+}, then move into cell and vital metabolites were leak out (1). If such cells were to remain coupled to their healthy neighbors, these too would suffer a dangerous disturbance of their internal chemistry. Thus immediate closure of the gap junction and disruption of cell-cell interaction in response to toxic insult is an attempt to isolate an otherwise abnormal cell from the surrounding normal cells. This mechanism ensures that the ionic and physiological alterations taking place in the affected cells are not propagated to the healthy and normal cell. At the lowest concentration there was considerable loss of cell-cell contact however cell were still viable as indicated by lesser LDH release and greater neutral red uptake, but as the concentration was progressively increased to 44 µM the loss of cell-cell contact became much more prominent along with significant loss of viability as indicated by large number of round, freely floating dead cells and lesser neutral red uptake. Most characteristic change was observed at highest concentration i.e. 88µM wherein apart from other features indicative of cytotoxicity considerable degree of cell shrinkage was observed. Volume decrease and consequent cell shrinkage is an important feature of apoptotic cell death (3). Apoptosis is a morphologically and biochemically defined form of cell death that occurs in response to a variety of stimulus such as toxic insult, withdrawal of growth factors, treatment with glucocorticoids, γ-irradiation, extensive DNA damage etc (18). A major hallmark of cell death is normotonic shrinkage of cells (43). The characteristic apoptotic volume decrease (AVD) starts before cell fragmentation and it is coupled to K^{+} release from cells (3, 6, 8) presumably via K^{+} channels (40, 44). There exists a possibility that volume regulation mechanisms (24) including volume regulatory Cl- and K+ channels are disordered, thereby inducing the AVD during the apoptosis process. Maeno et al., (19) too, in one of their studies have reported that normotonic cell shrinkage because of disordered volume regulation is one of the early prerequisite for apoptosis to progress. Presence of this typical normotonic cell shrinkage and volume decrease in INH exposed Hep G2 cells gives an indication that INH induced cytotoxicity is mediated by apoptosis. Presence of this typical normotonic cell shrinkage in INH exposed Hep G2 cells gives an indication that INH induced cytotoxicity is mediated by apoptosis. In order to confirm this hypothesis, Annexin V and propidium iodide (PI) enabled flow cytometric sorting of Hep G2 cells was carried out. In our study we observed that there was significant increase in number of annexin V positive (apoptotic) cells in response to INH treatment. As many as 25 % cells were undergoing apoptosis even at the lowest concentration of INH which increased further in characteristic dose dependent manner to 56% at the highest concentration (88µM). Thus the assumption about the occurrence of apoptosis that was made on the basis morphological alterations was held true and was further substantiated by the flow-cytometric analysis. In addition to this agarose gel electrophoresis of

DNA samples collected after 48 hr also revealed clear signs of apoptosis at highest concentration. Characteristic DNA ladder was observed in the cells exposed to the highest concentration of INH. Interestingly DNA fragmentation was not evident at lower concentrations despite presence of significant number of annexin V positive cells. The probable explanation for this is that externalization of phosphatidylserine is an early event, while DNA fragmentation is a terminal event. Possibly the apoptosis pathway was although initiated at lower concentrations but had still not reached terminal phase owing to lesser concentrations of the drug. On the other hand it was progressing much faster in the cell exposed to highest concentrations (88µM) resulting in marker apoptotic DNA fragmentation at the end of 48 hr.

Apart from other parameters indicative of cytotoxicity, specific organotypic endpoints such as aspartate and alanine aminotransferase also indicated that INH exposure had adverse effect on Hep G2 cells. These are cytosolic enzymes and their leakage from the damaged hepatocytes resulting in increased serum levels is indicative of hepatotoxicity (45). Apart from liver, aspartate aminotransferase is also present in cardiac renal and cerebral tissues therefore changes in serum AST levels are not definitive indicator of hepatotoxicity. Subjects with myocardial infraction also show increased serum AST activity (45). However alanine aminotrasferase is present in hepatocytes at much higher concentrations when compared to myocardial and other tissues and this has led to application of ALT determination to the study of hepatic tissue. In our study we measured the amount AST and ALT activities released from cells to the media in order to assess the cytotoxicity in terms of organotypic endpoints. There was marginal increase in extracellular AST activity while ALT, which is a more definitive marker of hepatotoxicity showed significant extracellular distribution indicating cellular damage. Collectively all these findings indicate that INH exposure accounts for cytotoxicity in Hep G2 cells by activation of apoptotic pathway. This finding is in good agreement with studies conducted by Schwab and Tuschl (30), who reported occurrence of apoptosis in hepatoma and lymphoma cells following INH exposure.

Mitochondria plays a central role in regulating, inducing and executing apoptosis pathway especially the intrinsic pathway by certain well characterized mechanisms such as Bcl2/Bax localization, cytochrome c release etc (18). Mitochondria respond to several signals such as reactive oxygen species, altered redox status and upregulation of intracellular calcium concentration. Damage to mitochondrial DNA and consequent loss of mitochondrial integrity is considered to be one of the key reasons for induction of apoptosis (18). This prompted us to explore whether mitochondrial integrity had any relationship with the INH induced apoptosis in Hep G2 cells. Mitochondrial viability and integrity was assessed using MTT viability assay that employs reduction of MTT to blue color formazon by functional mitochondrial dehydrogenases. In our study we observed marked loss of mitochondrial integrity even at the lower concentrations of the drug. The loss of mitochondrial viability in response to INH exposure showed concentration dependent effect and at highest concentration there was as much as 25% inhibition in reduction of MTT by mitochondrial dehydrogenases. One of the key factors known to be responsible for drug induced toxicity and apoptosis is alteration of cellular redox status by the drug or its metabolite. This led us to explore whether INH induced apoptosis in Hep G2 cell is due to altered cellular redox status. Glutathione is the principal intracellular non-protein thiol and plays a major role in the maintenance of the intracellular redox state. It is an important cellular antioxidant and is an integral component of first line defense system against oxidative stress. Similarly reactive oxygen species constitute an important group of pro-oxidants and they are known to be involved in the patho-physiology of various diseases and incidents of toxic insult. In our study we therefore evaluated the effect of INH exposure on cellular ROS production and glutathione content. Results indicated that there was an increased generation of ROS along with corresponding depletion of the cellular glutathione pool. Interestingly at the highest concentration (88 µM), the rate of ROS generation was much higher as compared to that at other concentration and this excessive production of ROS was concurrent with higher percentage of apoptotic cells as indicated by FACS and photomicrographs. This undesired increase in ROS and shortfall of GSH is indicative of oxidative stress in the cells following INH exposure. This finding is in accordance with the available reports that indicate involvement of oxidative stress in the INH toxicity in rats (2). Studies conducted by Sodhi et al., (36) have

indicated that enzymes involved in glutathione metabolism and disposal of reactive oxygen species reveal significant alterations following INH administration. In addition to this, they reported significant depletion of both protein and non-protein thiols in animals exposed to INH for two weeks. In vitro binding of glutathione with INH metabolites is well demonstrated by Nelson et al., (23). Thus the overall depletion of glutathione may not only be due to increased oxidation of GSH by active metabolites generated during cytochrome P 450 metabolism but also due to direct binding of the metabolites to the glutathione. Resultant depletion of thiol pool may account for the cytotoxicity due to altered redox status and resultant induction of oxidative stress.

In conclusion, growing body of evidence suggest that INH exposure causes apoptosis in Hep G2 cells possibly by induction of oxidative stress. The mitochondrion is a crucial control point in induction of apoptosis and there are typical permeability and functional changes in mitochondria during apoptosis (18). We observed that INH induced apoptosis too involved changes in mitochondrial functions, however changes that follow alterations in mitochondrial permeability such as cytochrome c release and activation of caspases are yet to be evaluated. Further studies exploring effects of INH on other components of defense system against oxidative stress (superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase etc) and elucidation of downstream apoptosis signaling cascade can provide better insight into the mechanism of action responsible for INH induced hepatotoxicity. Although involvement of mitochondria and oxidative stress generates a possibility that the intrinsic pathway is responsible for the cytotoxic effects, nevertheless extensive research for complete characterization of the pathways is required for confirming the involvement of intrinsic pathway.

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