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ROLE OF OXIDATIVE STRESS IN ANTITUBERCULOUS DRUGS (INDIVIDUALS AND COMBINED) CYTOTOXICITY IN HEPG2 CELLS

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ABSTRACT

Hepatotoxicity is a common side and toxic effect of Antituberculous (Anti-TB) drugs with reported higher incidence with anti-TB combinations. Oxidative stress was shown to have a role. This study examined oxidative stress effects of the first line Anti-TB drugs; Rifampicin (RIF), isoniazid (INH) and pyrazinamide PZA (individually and combined) on HepG2 cells. MTT assay (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) was used to study the cytotoxic effect of the tested Anti-TB drugs. The effect of anti-TB drugs on total glutathione HepG2 cells and the production of reactive oxygen species (ROSs) were studied (individually and in combinations). Furthermore, the protective effect of the antioxidant reduced glutathione was assayed. The data revealed that the tested anti-TB were cytotoxic to HepG2 cells. RIF was the most potent. The tested drugs in their estimated IC_{50}s, to different extents, enhanced significantly (P<0.0001) ROSs production and decreased total glutathione (P <0.0001). Furthermore, 48 hours pre-treatment with INH (3mM) significantly increased ROS production and decreased glutathione with RIF (0.1mM) (P <0.01 and P <0.05 respectively) and PZA (10 mM) (P <0.01 and P <0.05 respectively). Combined RIF (0.1mM) and INH (3mM) significantly decreased total glutathione (P<0.05 for each) and increased ROSs production (P<0.05) in HepG2 cells (P<0.05 for each). Interestingly, reduced glutathione (GSH) significantly decreased the cytotoxicity of RIF and INH (P=0.005 and 0.015, respectively). These data showed that oxidative stress play a crucial role in anti-TB induced hepatotoxicity, which can be alleviated by inclusion of antioxidant in therapy, though there is need of clinical trials. Moreover, combined anti-TB therapy should be considered as a risk factor with any other oxidative liver injuries.

Key words: Anti-Tb drugs hepatotoxicity, oxidative stress, rifampicin, isoniazid, pyrazinamide

INTRODUCTION

Tuberculosis (TB) is one of the most serious infectious diseases affecting approximately one third of the world population specially in developing world (Brewer & Heymann, 2004). In 2012, there were an estimated 8.6 million incident cases of TB and 1.3 million people died from the disease (WHO report, 2013). Recently, anti-TB drugs are available with high cure rates. However, their hepatotoxic effect is the major problem suffered by patients going under the scheduled prolonged therapeutic courses of the anti-TB drugs. Generally, the incidence of anti-TB drugs induced hepatotoxicity was estimated to be 5%-28% of patients treated with anti-TB drugs (Ostapowicz *et al.,* 2002; Navarro & Senior, 2006). Shakya *et al* (2006) reported that anti-TB induced hepatotoxicity can cause different forms of pathological liver insults which may lead to permanent injury and death, if not predicted in the early stages.

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Drugs induced hepatotoxicities were reported in patients receiving the first line anti-TB drugs; Isoniazid (INH), Rifampicin (RIF) and Pyrazinamide (PZA) (Singla *et al.,* 2010). INH was reported to be the main offending agent for the occurrence of anti-TB- drugs hepatotoxicity (Singh *et al.,* 2011). A lot of risk factors were suggested for anti-TB drugs induced hepatotoxicity mainly young aged populations (Shakya *et al.,* 2006), female gender (Makhlouf *et al.,* 2008) and malnutrition (Mahmood *et al.,* 2007). Oxidative stress mediates the onset and propagation of many pathological insults and drug induced toxicities (Pereira *et al.,* 2012). Investigating the potential mechanism of oxidative stress in hepatotoxicity would require in-depth understanding of how ROS are generated, disruptions that occur in oxidant homeostasis, mitochondrial dysfunction and how clinically approved drugs induced side effects (Ott *et al.,* 2007; Daevall *et al.,* 2012). This knowledge is critical in preventing drugs induced liver injury (DILI) or hepatotoxicity.

Anti-TB drugs induced oxidative stress was suggested to play a crucial role in their induced hepatotoxicity (Attri *et al.,* 2000; Funde *et al.,* 2013). However, the oxidative effect of each individual agent is difficult to be evaluated in patients, as they must undergo the combination therapeutic courses containing more than single anti-TB agents. Rifampicin is also reported to infrequently cause hepatocellular injury and potentiate hepatotoxicities of other anti-TB medications (Menzies *et al.,* 2004). Other studies incriminate rifampicin to activate hepatocyte pregnane X receptors, leading to induction of cytochromes and induction of uridine diphosphate-glucuronosyl-transferases and P-glycoprotein transport implicated in the metabolism of other drugs (Burk *et al.,* 2004; Rae *et al.,* 2001). Rifampicin interacts with numerous drugs metabolized by these and other hepatic enzymes (Niem *et al*., 2003). Therefore, prescribing combination of these three Anti-Tb drugs in the management of tuberculosis might interfere with other metabolic pathways to promote observed upregulation of ROSs and downregulation of total glutathione to exacerbate oxidative stress and drug induced hepatotoxicity. INH metabolite, mono-acetyl hydrazine produced by acetylation and dehydrazination (Timmins & Deretic, 2006), was reported to be responsible for its hepatotoxic effects through interaction with cytochrome P_{450} system, modulating increased oxidative stress and altering mitochondrial permeability alterations by peroxidation of their membranes lipid contents (Sardao *et al.,* 2008; Chowdhury *et al.,* 2006). Pyrazinamide alters nicotinamide acetyl dehydro-genase levels in rat liver (Shibata *et al.,* 2001), which might result in generation of free radical species inducing oxidative stress.

There may be shared mechanisms of injury for isoniazid and pyrazinamide, due to molecular structural activity relationship (SAR). Therefore, the justification for the present work is to enhance better prediction of mechanisms of the role of oxidative stress in anti-TB drugs induced cytotoxic effect on Hep G2 cells via highlight the biochemical alterations, namely quantification of reduced glutathione (GSH) and increased reactive oxygen species (ROSs) production levels underlying the tested drugs induced cytotoxic effect on HepG2 cells. The effect of the drugs was assayed individually and in combinations to simulate their clinical therapeutic courses. In addition, protective effect of exogenous reduced glutathione (GSH) was investigated.

MATERIALS AND METHODS:

Chemicals

Chemicals and media components, used in this study, were purchased from Sigma Chemicals, USA unless other source is mentioned.

Cell culture

Human hepatocellular carcinoma cells (HepG2 cells) were grown in serum-free PC-1 medium (Cambrex, Verviers, Belgium) supplemented with 2 mM L-glutamine. Cells were incubated at 37ºC in a humidified atmosphere with 5% CO_2.

MTT (3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) assay

The MTT assay, an indicator for cell viability, relies on changes in redox potential of the cells in response to different agents. Following manufacturer protocol, HepG2 cells were seeded ($1x10^4$ cells per well) in 96-well plates. After being confluent, cells were exposed to antituberculous drugs RIF (in concentrations 1, 10, 100 µM and 1mM), INH and PZA in concentrations (1, 10, 100 µM and 1 mM) for a 4, 24 and 48 hrs in the presence of the drug or DMSO. The MTT absorbance values were expressed as a percent of the vehicle control (defined as 100%). Each experiment was performed in triplicate with at least 3 wells of each drug concentration in each experiment.

Measurement of total glutathione

Reduced glutathione (GSH) was measured according to Senft *et al* (2000). Cells were exposed to IC_{50}s of Anti-TB drugs and lower concentrations (30 µM, 10mM and 3 mM of RIF, PZA and INH, respectively) for 24 hr in 6 well-plates (10^6 cells per well). Lower concentrations were tested individually

or in combinations among the different Anti-TB drugs. Then cells were scraped into ice-cold phosphate buffered saline and centrifuged at 700 g for 2 mins. The pellets were resuspended in ice-cold lysis buffer and incubated on ice for 10 mins. After that, they were centrifuged at 15000 g for 5 mins to generate lysates and protein pellets. The GSH level of the lysate was quantified using the fluorescent substrate o-phthaladehyde (OPT) with an excitation/ emission wavelengths of 350/420 nm.

Reactive oxygen species (ROS) detection

3, 7-dichlorodihydrofluorescein diacetate (DCFDA) assay was used to detect ROS. Cells were cultured in 96-well plates ($3x10^3$/well) and treated for 24 h with anti-TB at either its estimated IC_{50}s for viability or with lower concentrations (100 μM, 10mM and 3 mM of RIF, PZA and INH, respectively) for 24 hr in 6 well-plates (10^6 cells per well). Lower concentrations were tested individually or in combinations among the different Anti-TB drugs used individually or in combinations. The assay was done following the protocol published in (Elmorsy *et al*., 2014). DMSO was used as a vehicle control and wells with non-stained cells were used as blank. Each experiment was performed in triplicate, where n represents the number of experiments (triplicates) performed.

Effect of Reduced glutathione (GSH)

The MTT was done as shown in sections 2.4 in the presence of reduced GSH (10 μM).

Statistical analysis

The statistical analyses were conducted using PRISM 5 (GraphPad Software Inc., San Diego, CA). For IC_{50} estimation, a non-linear curve fitting log (inhibitors)- variable slope equation was used. For comparisons, one-way ANOVA test with Dunnett's multiple comparisons post-test were used. Statistical significance is defined as $P<0.05$. Significance is indicated in the figures as *** for $p<0.001$, ** for $p<0.01$ and * for $p<0.05$.

RESULTS

Anti-TB drugs decreased the viability of Hep-G2 cells

The MTT assay showed that the tested Anti-TB drugs decreased the redox-potential of HepG2 cells in a concentration and exposure times dependent manners (Figure 1). RIF was the most cytotoxic with the lowest IC_{50} (0.6 mM; 24hours post-exposure), while PZA was the least cytotoxic with IC_{50} about 80 mM (24hours after exposure) (Table 1). PZA showed a non-significant (P=0.189) cytotoxic effect even with 1mM concentration in 4 hours post-treatment course, while RIF (100 and 1000 μM) and INH (100 and 1000mM) significantly decreased the viability of HepG2 cells within 4 hours after exposure (P=0.005 and 0.002, respectively).

The effect of anti-TB on GSH

As shown in Figure 2, all the tested anti-TB showed a significant reduction (P***$\leq$0.0001) of the intracellular GSH content in comparison with the controls in their IC_{50}s. Total glutathione was reduced

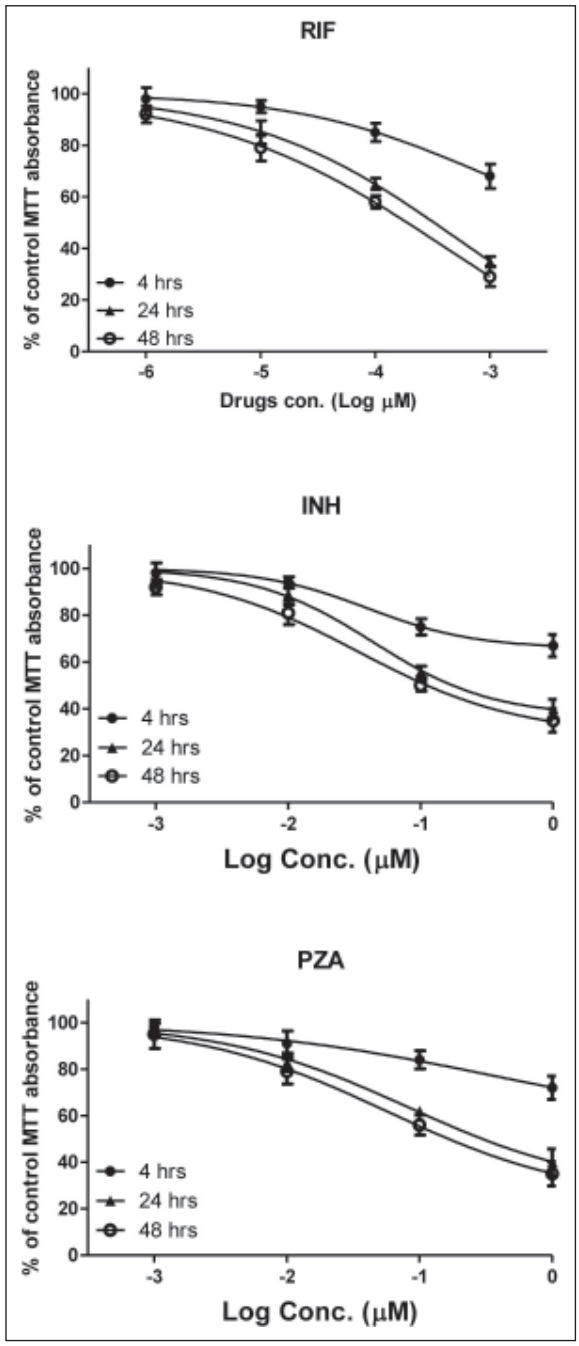

Fig. 1. MTT assay of the cytotoxicity of anti-TB drugs on HepG2 cells.

HepG2 cells were treated with the anti-TB drugs RIF, INH, & PZA in a wide range of concentrations for 4 hours, 24 hours and 48 hours.

Table 1. Estimated IC_{50}s (means and 95% confidence intervals) of the tested anti-TB drugs in mM concentrations

		RIF	INH	PZA
4 hrs	Mean	12	132	124
	95% confidence interval	0.3–48	34–596	91–186
24 hrs	Mean	0.6	76	80
	95% confidence interval	0.05–29	51–113	45–141
48 hrs	Mean	0.02	70	77
	95% confidence interval	0.007–0.88	37–130	34–178

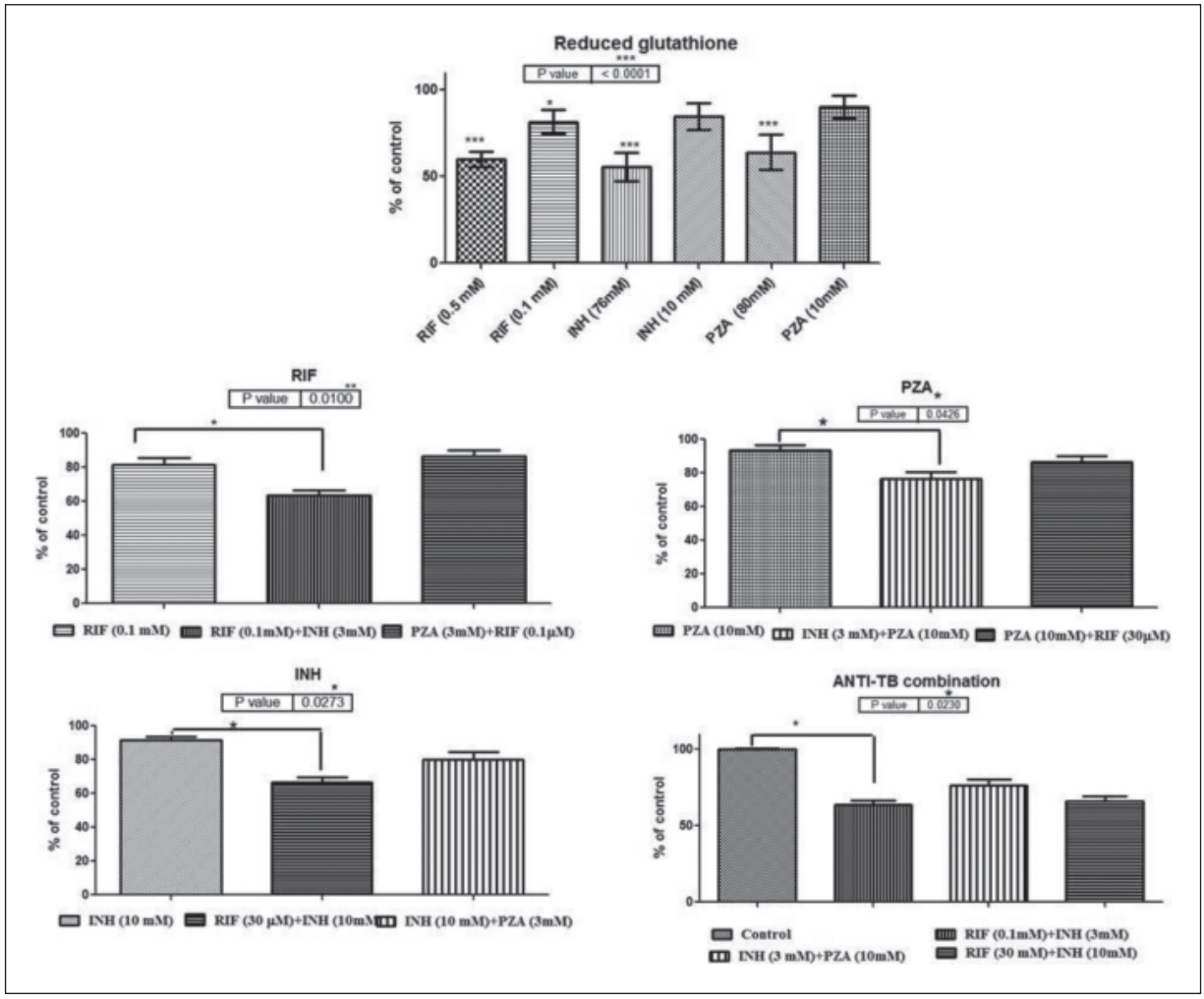

Fig. 2. Effect of anti-TB drugs on the intracellular content of reduced glutathione in HepG2.
HepG2 cells were exposed to the anti-TB drugs (individually and in combinations) in different concentrations for 24 hours. Then the intracellular contents of glutathione were assessed.

by about 40%, 45% and 35% with RIF, INH and PZA respectively. Dunn's post-test showed that only RIF in the lower concentration (0.1 mM) showed significant (P<0.05) reduction in HepG2 total GSH (~20% blow the control levels). In 10 mM concentrations, both INH and PZA showed no significant decrease in HepG2 cells glutathione stores by ~ 15% and 10% of the vehicle control cells levels, respectively. In addition, INH in 3mM was shown to increase significantly the effect of low concentrations of RIF (0.1 mM) and PZA (10mM) on HepG2 cells total GSH (P*=0.01and 0.042,

respectively) with further more reduction of HepG2 cells glutathione by ~18% and 16% respectively in comparison to the individual drugs treated cells. Interestingly, combination of RIF (0.1 mM) and INH (3 mM) significantly decreased HepG2 cells total GSH in comparison with the vehicle control treated cells (P*=0.023).

Anti-TB drugs increased Hep-G2 cells ROS production

ROS experiments revealed that the tested anti-TB increased significantly ROS production in HepG2 cells (P***≤0.0001) in comparison with the vehicle controls in their MTT estimated IC_{50}s (P***≤0.0001 for each anti-TB) (Figure 3). ROSs production was increased to 138%, 144% and 136% with RIF, INH and PZA respectively in comparison to the vehicle control treated cells. In the lower concentrations, [RIF (0.1mM), INH (10mM) and PZA (10mM)], all the tested drugs did not show a significant effect on HepG2 cells ROSs production. In addition, pretreatment with INH (3mM) for 48 hours significantly increased the effect of RIF (0.1mM) on HepG2 cells production of ROS (P =0.0098). On the other hand, pretreatment with RIF (30μM) for 48 hours significantly increased effect of INH (10mM) on HepG2 cells production of ROS (P =0.0066). Combination of RIF (0.1 mM) and INH (3mM) significantly increased cellular ROS production in comparison with control cells (P =0.021*).

Reduced glutathione decreases the cytotoxic effect of anti-TB drugs on Hep-G2 cells

Reduced GSH was shown to significantly counteracted the cytotoxic effect of RIF and INH (P=0.005 and 0.015, respectively) in their MTT estimated IC_{50}s while it showed no significant (P=0.057) effect on the cytotoxic effect of PZA in its IC_{50}. Reduced glutathione increased the viability

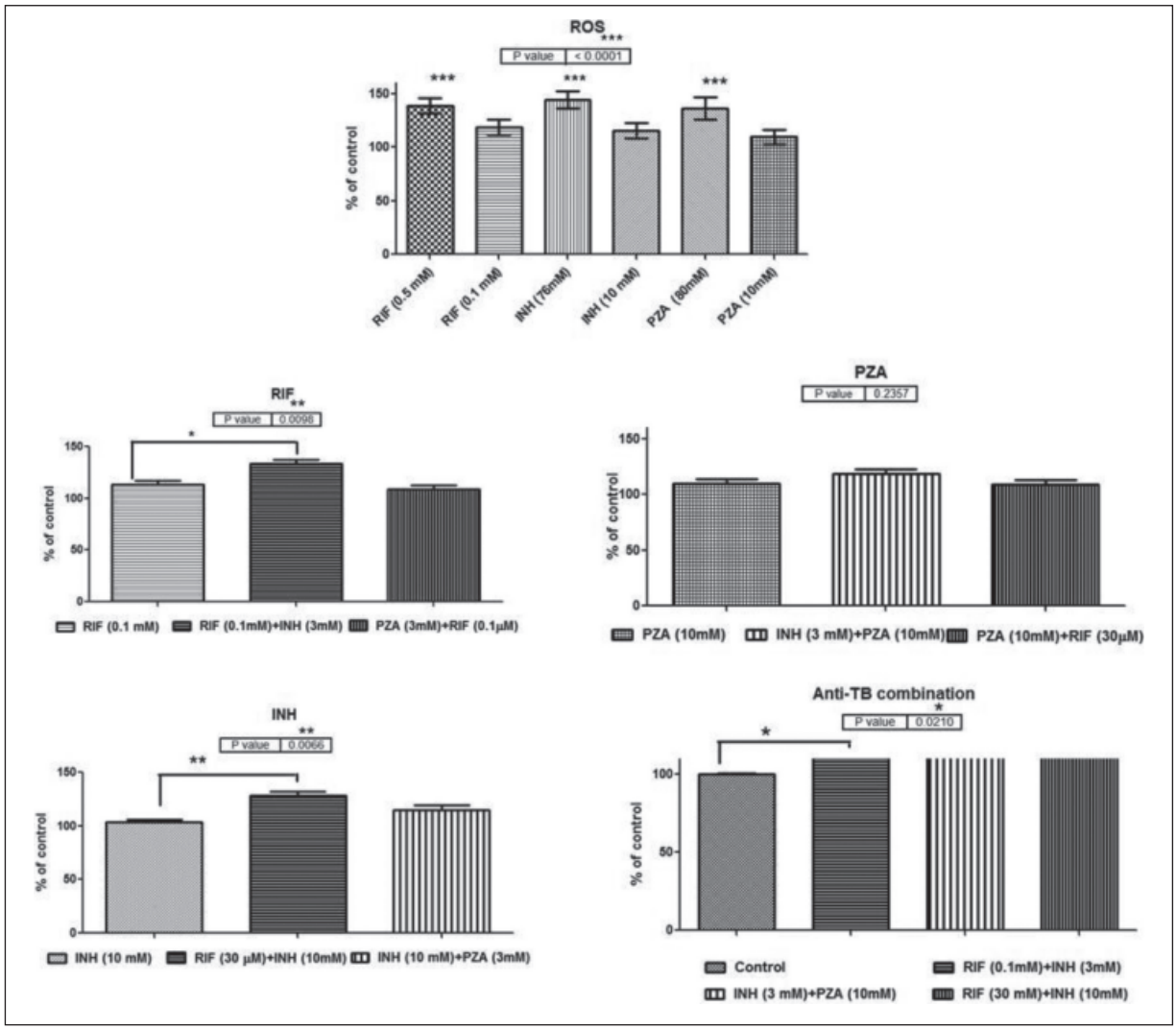

Fig. 3. Effect of anti-TB drugs on HepG2 reactive oxygen species (ROSs) production.
HepG2 cells were treated with different concentrations of the anti-TB drugs (individually and in combinations) for 24 hours. Then the intracellular content of ROSs production was studied.

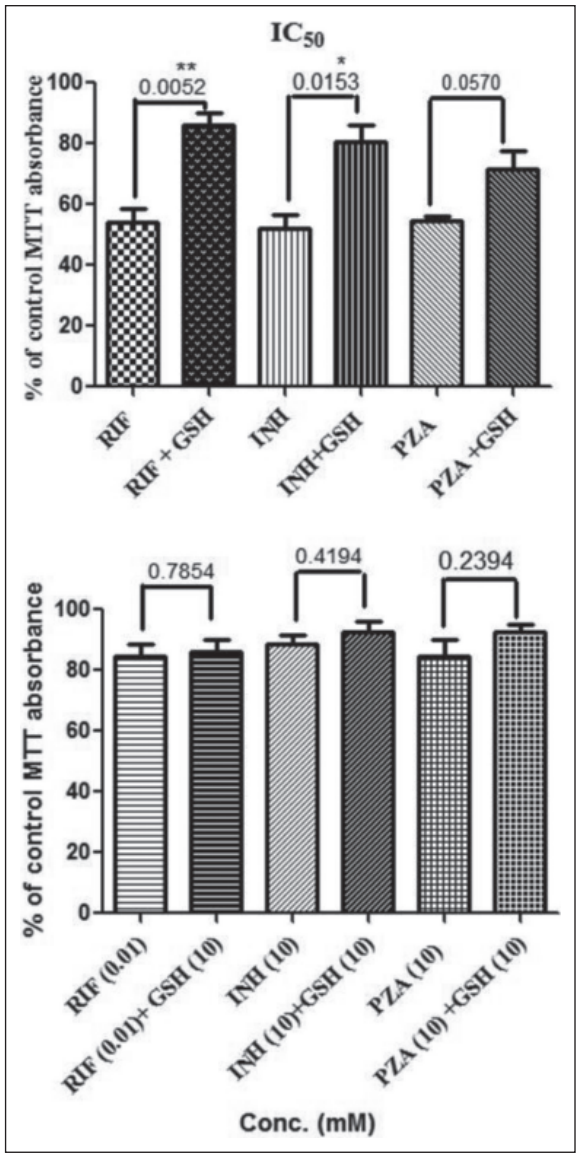

Fig. 4. The protective effect of reduced glutathione (10mM) on the anti-TB drugs induced cytotoxic effect on HepG2 cell.
HepG2 cells were exposed to RIF, INH, & PZA in absence and present of reduced glutathione (10mM) forv 24 hours. Then the cytotoxicity was studied by MTT assay.

of anti-TB drugs treated cells by ~34%, 28%, 18% of the control for RIF, INH and PZA, in their IC_{50}s, treated cells respectively. However, in the lower concentration, reduced GSH showed no significant effect with the all tested anti-TB drugs (Figure 4).

DISCUSSION

Tuberculosis induces oxidative stress as an underline mechanism for their hepatotoxic effect. HepG2 cells were used as a cell line model as they are widely accepted model for liver studies. Anti-TB drugs were assayed in a wide range of concentrations covering their therapeutic, supra-therapeutic, toxic and lethal levels (Winek *et al.,* 2001; Kayhan & Akgunes, 2011). Higher concentrations were used to induce the desired cytotoxic effect within the limited period of our experiments to simulate the clinical state with prolonged clinical therapeutic courses of the anti-TB drugs (for at least 6 months in the shortest protocols).

The present data revealed that the tested drugs were cytotoxic to HepG2 cells. RIF was shown to be the most potent. The tested anti-TB drugs, in their estimated IC_{50}s, were found to significantly increased ROSs production and decreased intracellular total glutathione. These results are in agreement with the previous studies which showed that anti-TB induce oxidative stress in rats and human studies. In rats, the free radical scavenger glutathione-related thiols, and antioxidant glutathione peroxidase and catalase activities, are diminished by isoniazid, although glutathione reductase activity is increased (Sodhi *et al.,* 1996; Attri *et al.,* 2001).

Regarding combinations in lower concentrations, pretreatment with INH (3 mM) was shown to increase the effect of both RIF (0.1mM) and PZA (10mM) on the ROSs production and HepG2 intracellular glutathione content. In addition, RIF (30 μM) pre-treatment for 48 hours was found to significantly increase the effect of both INH (10mM) on the both fore-mentioned parameters. This is supporting the previous data showed that combination of anti-TB drugs increased the risk of hepatotoxicity. Taneja & Kaur (1990) reported that the incidence of INH associated hepatitis was 6%, this incidence was increased five folds when INH was combined to RIF.

Regarding the combining of INH and RIF in therapy, it is reported that the rate of symptomatic hepatitis is 2.55% (Steele *et al.,* 1991) while combination of RIF and PZA has either less or equal hepatotoxicity to isoniazid. The presence of rifampicin in a multidrug treatment regimen increased the incidence of significant hepatotoxicity for adults from 1.6 to 2.55% and in children from 1.0 to 6.9% (Steele *et al.,* 1991). The influence of pyrazinamide on TB DILI is ambiguous. Some studies indicate little to no increased rate of hepatotoxicity (Parthasarathy *et al.,* 1986), whereas others point to it as a contributor to increased incidence or severity of hepatotoxicity (Teleman *et al.,* 2002; Yee *et al.,* 2003), although dosing variations and patient selection biases may have contributed to these reported results.

In addition, the present study revealed that introduction of exogenous glutathione diminished RIF an INH induced cytotoxic effect in their estimated IC_{50}s. This in concordance with other study in which antioxidant N-acetyl-cysteine, a

substrate for glutathione synthesis, inhibits isoniazid-induced liver injury in pretreated rats (Attri *et al.,* 2001). In addition, the active components of silymarin was shown to have a protective effect against hepatotoxic actions of the first line anti-TB drugs used in the chemotherapy of tuberculosis in male's albino wistar rats model (Eminzade *et al.,* 2008).

The data of this study is clinically important due to some concerns. Firstly, the present results are highlighting the significance of oxidative stress in anti-TB drugs induced hepatotoxicity. Secondly, the study is appreciating the intake of exogenous antioxidant as adjuvant therapy with the anti-TB drugs to alleviate their hepatotoxic effects and improve their tolerability. Thirdly, the data is supporting more oxidative stress studies in the newer anti-TB drugs development assays for better outcomes and less incidence of hepatic injuries. Fourthly, anti-TB drugs should be considered as risk factors for severity of toxic agents, which are well known to induce their toxic effect via oxidative stress as antipsychotics and paracetamol toxicities.

In summary, we reported the significant oxidative stress effects observed with Anti-TB drugs on HepG2 cells. RIF is the most offending drug. Oxidative stress plays a crucial role in their induced cytotoxicities via production of excess amounts of ROSs and depletion of glutathione stores. The addition of exogenous antioxidant, GSH, significantly decreased the cytotoxic effects of INH and RIF, though this needs further expanded clinical trials.

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