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Original Article

Co-Administration Exposure Toxicity of Isoniazid and Rifampicin in Chang Liver Cell Lines

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Abstract

The study is designed to investigate the exposure toxicity of isoniazid (INH) and rifampicin (RIF) in combination on Chang liver cell lines. Each 50, 100, and 200 ng/ml of INH and RIF were co-administered to the Chang cell lines for a period of 24 hours; the untreated cell lines were served as a control. Alanine aminotransferase (ALT), Aspartate aminotransferase (AST) and alkaline phosphatase (ALP) activity in the culture medium were assayed to determine the integrity of the cell lines. β-Galactosidase and cathepsin activities were also assayed in the lysosomal fraction. A comet assay was performed to determine DNA damage in the cell lines. The mRNA expressions of CYP2E1, BAX, BCL2, P53, and caspase-3 were studied. Cells treated with INH and RIF showed abnormal levels of ALP, AST, and ALT activities in the culture medium. However, no such abnormalities were inferred in the medium of the unexposed control cells. Altered enzymatic activities were seen in the lysosomal fractions of the treated cells, whereas no such abnormalities were detected in the untreated control cell lines. 71% of DNA damage was recorded in the intoxicated cells, whereas the DNA of the control cell lines remained intact. A significant increase in the CYP2E1, P53, and caspase-3, along with alterations in BAX and BCL2 mRNA expressions, was recorded in the intoxicated cells. In contrast, the untreated control cells showed optimal mRNA expression. This study demonstrated that exposure to INH and RIF in Chang liver cell lines compromised cellular integrity, altered the mRNA expression, and induced significant DNA damage.

Keywords: *Apoptosis; CYP2E1; Isoniazid, Oxidative Stress; Rifampicin*

Introduction

Isoniazid and Rifampicin combination are the commonly used drug regimens for the management of latent tuberculosis (Batool *et al*., 2026). However, it is accompanied by various adverse effects such as hepatotoxicity, neurological, gastric, and haematological complications (Shivaji & Prince, 2025). Approximately 5–28% of people have been reported for hepatic dysfunction on anti-tuberculosis drug ingestion, and the majority of them were found to be reported for the elevation of serum transaminases with a traceable amount of liver injury (Tostmann *et al.,* 2008). The hepatic discomfort is mainly due to the disproportionate rise of reactive oxygen species from the reactive metabolites of INH and RIF. In the liver, INH is metabolized as acetyl hydrazine, hydrazine, and acetyl isoniazid. Studies revealed that acetylhydrazine and hydrazine could cause hepatic necrosis (Wang *et al*., 2016). Moreover, co-administration of RIF aggravates hepatotoxicity by contributing more acetylhydrazine and hydrazine from the INH through CYP2E1 activation (Bishnu *et al*., 2025). Hydrazine consumes the reserve glutathione in the hepatocytes by reacting with their sulfhydryl group and resulting in cell death. Besides that, RIF shortens the half-life of the acetyl hydrazine; as a result, it is quickly converted to an active metabolite, which leads to hepatic necrosis (Naji *et al.,* 2021). Studies have shown that the pathogenicity of INH and RIF also lies in apoptosis induction in the hepatocytes (Lin *et al.,* 2025). However, the mechanism of INH and RIF induced toxicity remains ambiguous. Several studies have highlighted the possibilities of lipid peroxidation, oxidative stress, genotyping and accumulation of toxic metabolites as the causes of hepatotoxicity (He *et al*., 2020).

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Therefore, the study was proposed to uncover the pathogenicity of INH and RIF toxicity in Chang cell lines by assessing the biochemical indices and mRNA expressions of CYP2E1, P53, BAX, BCL2, and caspase 3 on its exposure. The Chang liver cell lines used in this study were among the preferred cell lines for evaluating drug-induced liver toxicity (Ekwall & Acosta, 1982).

Methodology

Chang Cell Lines Maintenances

Chang liver cells are a type of human hepatoma cell line, procured from the National Centre for Cell Science, Pune, India. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin. The cells were cultured in 75 cm² culture flasks and incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

Passaging of Cells

The culture medium in the T-75 culture flask was aspirated, and the cells were subjected to trypsinization by adding 2 mL of trypsin-EDTA solution. The flask was incubated at room temperature for 1 minute. The trypsin-EDTA solution was aspirated, and the cells were incubated in a CO_2 incubator for 1–3 minutes. After incubation, the Chang liver cells were scarped and mixed with 1 mL of FBS-containing medium and transferred to a 1.5 mL centrifuge tube. The cell suspension was centrifuged at 12,000 rpm for 10 minutes. The resulting supernatant was discarded, and the cell pellet was resuspended in the fresh growth medium. The cell suspension was then transferred to sterile T-75 culture flasks, and the final volume was made up to 10 mL with growth medium.

Stock Solution

For the preparation of the stock solutions, 100 µg each of isoniazid and rifampicin was dissolved separately in 100 mL of sterile double-distilled water. The solutions were transferred to separate 100 mL standard volumetric flasks and stored. The resulting stock concentration was 1 µg/mL (1000 ng/mL).

In vitro Assay Protocol

Chang liver cells were seeded at a density of 1×10^6 cells/well in 6-well plates and allowed to grow for 24 hours before treatment. The cells were divided into four groups. The dosage of the isoniazid (INH) and rifampicin (RIF) combination was selected based on the study by Sankar *et al*. (2015).

Group I: Untreated control (normal liver cells).

Group II: Liver cells treated with 50 ng/mL each of INH and RIF (low dose).

Group III: Liver cells treated with 100 ng/mL each of INH and RIF (moderate dose).

Group IV: Liver cells treated with 200 ng/mL each of INH and RIF (high dose).

At the end of the exposure period, the culture media were collected for biochemical estimations, and the cells were trypsinized and harvested for analysis of mRNA expression of CYP2E1, BAX, BCL-2, CASPASE-3, AND P53, and for assessment of comet assay.

Biochemical Indices Determination

The AST, ALT and ALP levels in the supernatant of the cell lines were assayed in accordance with González *et al.* (2017).

Lyosomal Fraction Preparation

The lysosomal fraction of the treated and untreated Chang cell lines was prepared in accordance with Plummer (1987). The confluent cells were homogenized with the presence of surcorse (0.25 M) and 1 mm of TCA. The homogenate was centrifuged at 2,000 rpm for 10 minutes, and the resulting supernatant was collected. The pellet was resuspended in a sucrose buffer solution and subjected to

a second centrifugation at 2,000 rpm for 10 minutes. The initial lysosomal pellets were prepared by pooling the supernatants from each tube and centrifuged at 7,400 rpm for 10 min. Fresh lysosomal pellets were prepared by centrifuging the supernatants again at 11,400 rpm for 10 min. The lysosomal pellets obtained were resuspended in a sucrose solution and centrifuged once more to re-pellet them. The finally generated lysosomal pellets were resuspended in the 2 ml of sucrose solution and stored in the ice for further analysis.

Assay for Lysosomal –Galactosidase

The β-galactosidase activity in the lysosomal fraction was determined based on Conchie and Levvy (1957). In brief, the reaction mixture was prepared by adding 0.1 ml of freshly obtained lysosomal extract, 0.2 ml of sodium acetate buffer, and 0.1 ml of 0.66 M p-nitrophenyl β-galactopyranoside, followed by incubation at 37°C for 1 hour. After that, reactions were arrested by adding 0.2 ml of 5% TCA and incubating the tubes for 15 min at 37°C. After the incubation, 3 ml of 0.1 N NaOH was added to the tubes and centrifuged for 10 min at 1000 rpm. The optical density was measured at 400 nm, and the activity of -Galactosidase was expressed as µ mol p-nitrophenol liberated/mg of protein. The lysosomal extract of untreated cellines were set as control and treated similarly as per the test.

Lysosomal Cathepsin D assay

Lysosomal Cathepsin D activity was assayed as per Anson (1938). A dissolved hemoglobin solution was prepared by mixing 1% hemoglobin with 0.2 M acetate buffer (pH 3.8) and 0.8 ml of 0.05 M acetate buffer (pH 3.8). Then, 0.2 ml of the lysosomal fraction was added to the hemoglobin-containing tube and incubated at 37°C for 1 hour. Subsequently, 2 ml of 10% TCA was added to stop the reaction, and the mixture was centrifuged at 1000 × g for 15 minutes. To the 2 ml of supernatant, 3 ml of 0.5 N NaOH and 1.5 ml of 1:2 Folins phenol reagent were added and measured at 620 nm. The untreated lysosomal fraction was set as a control treated similarly as per the test. The activity of cathepsin D was expressed as the micromoles of tyrosine liberated per minute/g protein.

m RNA Expression Analysis

Chang cells were scraped from the medium and homogenized. Trizol reagent was added to the homogenized cells and incubated at room temperature for 5 minutes, followed by centrifugation at 1000 rpm for 5 minutes. Supernatant was collected and 200 ml of chloroform was kept for incubation at room temperature for 5 mts. After incubation, tubes were treated with 500 ml of isopropyl alcohol and incubated at room temperature for 10 min. Then, centrifuge the tubes at 12000 rpm for 15 minutes to pellet out the RNA precipitate. The RNA precipitate was washed thrice with 75% ethanol and centrifuged again at 12000 rpm for 15 minutes. After that, the pellets were suspended with 20 ml of RNase-free water and stored at -80 °C for further processing.

Semiquantitative RT-PCR Analysis

RT-PCR was performed to synthesize cDNA, and it was used as a template for PCR amplification of CYP2E1, P53, CASPASE-3, BCL-2, and BAX. GAPDH was used as an internal control for normalization. The primer sequences used in the study are presented in Table 1.

A PCR reaction mixture was prepared with a total volume of 25 µL was prepared containing 12.5 µL of 2× PCR Master Mix, 2.5 µL each of forward and reverse primers, 1.5 µL of cDNA (RT sample), and 3.5 µL of sterile distilled water. The reaction mixture was mixed gently and subjected to PCR amplification. The PCR conditions consisted of an initial denaturation at 94°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, gene-specific annealing for 1 min, and extension at 72°C for 1 min. The annealing temperatures were 56°C for GAPDH, 60°C for CYP2E1, 50°C for caspase-3, 58.8°C for Bax, 56.7°C for Bcl-2, and 58°C for p53. A final extension was performed at 72°C for 5 min.

The amplified PCR products were separated by agarose gel electrophoresis and visualized using a fluorescent imaging system (Bio-Rad, USA). The expression levels of the target genes were normalized to GAPDH. The intensity of each DNA band was quantified by densitometric analysis using Bio-Rad software.

Table 1: DNA Primers for Expression

Primer	Forward Primer	Reverse Primer
GAPDH	5’-CGACAGTCAGCCGCATCTT-3’	5’-CCAATACGACCAAATCCGTTG-3’
BAX	5'-TTTTGCTTCAGGGTTTCATC-3'	5'-GACACTCGCTCAGCTTCTTG-3'
BCL2	5'-ATGTGTGTGGAGAGCGTCAACC-3'	5'-TGAGCAGAGTCTTCAGAGACAGCC-3'
Caspase 3	5′-AATTCAAGGGACGGGTCATG-3′	5′-GCTTGTGCGCGTACAGTTTC-3′
P53	5'-GGATGCCCGTGCTGCCGAGGAG-3'	5'-AGTGAAGGGACTAGCATTGTC-3'
CYP	5′-GTGATGCCCTGGCTGCAG-3′	5′-AATCGAGCTGGATCAAAGTTC-3′

Comet Assay

The Comet assay was performed according to the method of Singh *et al*. (1988). Treated (200 ng/ml INH and RIF) and untreated Chang liver cells were trypsinized and centrifuged at 800 rpm for 10 min. Cell suspensions were prepared by adding 0.8% low-melting-point agarose to the trypsinized cells. One hundred microliters of the cell mixture was transferred onto pre-coated agarose slides, which were then immersed in lysis buffer (2.5% SDS) for 15 min. After that, electrophoresis was performed, and the DNA migration pattern of 100 cells was evaluated in the fluorescence microscope at the wavelength of 420-490 nm. The length of the comet tail was calculated using an image analysis system attached to a fluorescence microscope equipped with appropriate filters. The microscope images were analyzed in the CAPS software to interpret the amount of DNA damage in %, based on the tail length in between the treated groups.

Data Analysis

Data were expressed as mean ± SEM (n = 3). The data were analyzed using one-way analysis of variance (ANOVA), followed by Tukey’s multiple-comparison test to determine differences between the treated groups and the normal control group. Statistical analysis was performed using GraphPad Prism software. A value of $P \leq 0.05$ was considered statistically significant for comparisons between the normal control and treated groups.

Results

The Impact of Isoniazid and Rifampicin on Biochemical Indices in Chang Liver Cells

The biochemical indices (AST, ALT, and ALP) in the medium of the Chang liver cell line were analyzed. Figure 1 depicts the levels of the biochemical indices in the medium. The cell treated with 50 ng/ml (low dose) of INH and RIF displayed significant increases ($p<0.05$) of AST, ALT, and ALP levels in the medium when compared to the unexposed control cell line. However, AST, ALT, and ALP levels were found to be higher ($p<0.01$) in the medium of cell lines exposed to 100 and 200 ng/ml INH and RIF. It was also quite apparent that the outcome of the biochemical indices from the cell lines was directly proportional to the dosage of INH and RIF. The higher the dosage, the higher the outcome of biochemical indices from the cells.

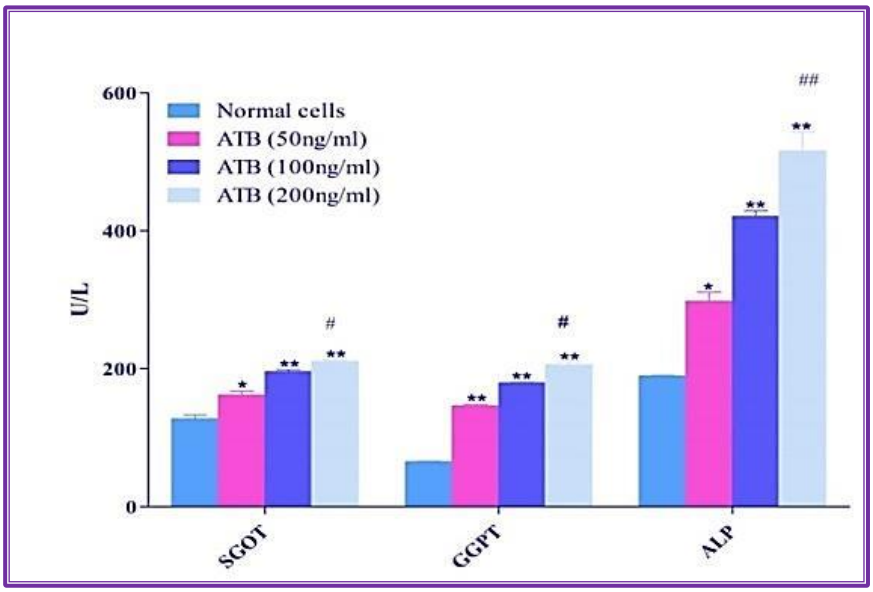


Figure 1: *Impact of Isoniazid and Rifampicin on Transaminase and Alkaline Phosphatases Activity in Chang Liver Cell Lines*

Biochemical Indices of SGOT, SGPT, and ALP in Treated Liver Cell Lines

Values are expressed as mean ± sem, where N=3* and **indicates p<0.05 and p<0.01 difference between control vs intoxicated. # indicate p<0.05 low dose vs high dose.

The Impact of Isoniazid and Rifampicin on Beta Galactosidase and Cathpesin D

The impact of INH and RIF on the lysosomal beta galactosidase and the cathpesin was studied; the data obtained is depicted in figures 2 and 3. The cell lines exposed to INH and RIF showed the dose-dependent elevated activity of beta galactosidase and cathepsin D in their lysosomal fractions when compared to untreated cell lines. This indicated that lysosomal integrity was damaged on exposure to INH and RIF.

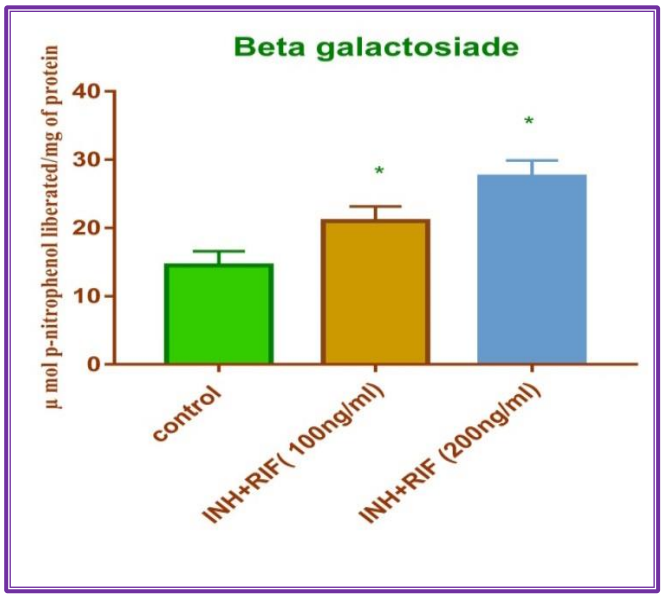


Figure 2: The *Impact of Isoniazid and Rifampicin on Beta Galactosadiase Activity*

β-Galactosidase Activity in the Lysosomal Fraction of Cell Lines Treated with INH and RIF

Values are Mean ±SEM, where n= 3. *Indicates p<0.05 significant between control vs treated cell lines.

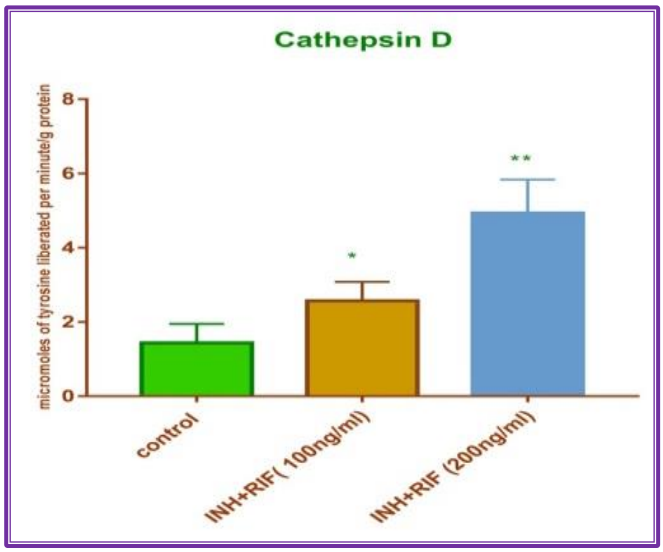


Figure 3: *The Impact of Isoniazid and Rifampicin on Cathaepsin D Activity*

Cathepsin D Activity in the Lysosomal Fraction of Cell Lines Exposed to INH and RIF

Values are Mean ± SEM, where n= 3. * Indicates $p<0.05$ significant between control vs treated cell lines

DNA damage

The comet assay was performed to detect DNA damage in the cell lines following exposure to INH and RIF. The data obtained are presented in Figure 4. The length of the comet tail was considered an indicator of the extent of DNA damage in the cell lines. A minimal amount of DNA damage, with no significant comet tail formation, was observed in the untreated control cells. However, the cell lines treated (Figure 5) with 200 ng/mL each of INH and RIF exhibited a distinct comet tail pattern, with the percentage of DNA damage reaching 71%.

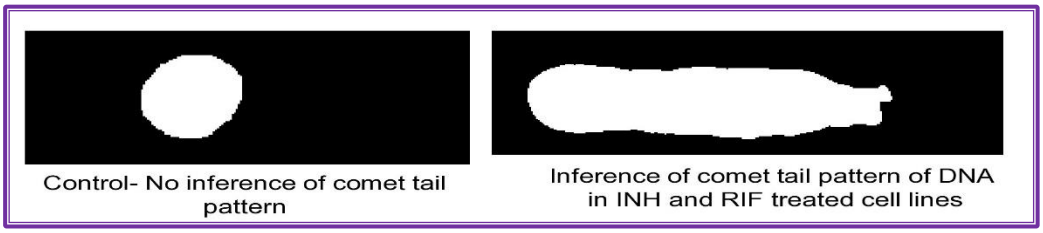


Figure 4: *Intact DNA in control Cell lines and Comet Tail Formation in INH- and RIF-Treated Cells*

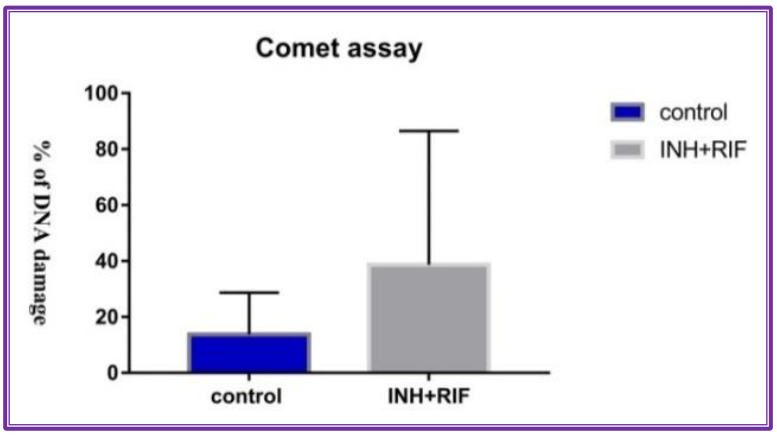


Figure 5: *Amount of DNA Damage in Chang Liver Cell Lines*

The Impact of Isoniazid and Rifampicin on M RNA Expression in Chang Cell Lines

The mRNA expression and the semi-quantitative analysis of Bcl2, Bax, P53, caspases 3, and CYP2E1 were studied in the Chang liver cell lines, and the results are represented in fig-6. Fig. 7 exhibits semi-quantitative analysis of mRNA expression in the liver cell lines exposed to 50, 100, and 200 ng/ml of INH and RIF. A significant increase in the BAX expression was observed in cell lines treated with INH and RIF, when compared to control liver cell lines. Similarly, the mRNA expression of P53, caspases 3, and CYP2E1 was found to be significantly ($p<0.01$) raised in the liver cell lines exposed to an irrespective concentration of INH and RIF. Whereas a significant decrease in the expression of Bcl2 was observed in the cellines exposed to INH and RIF.

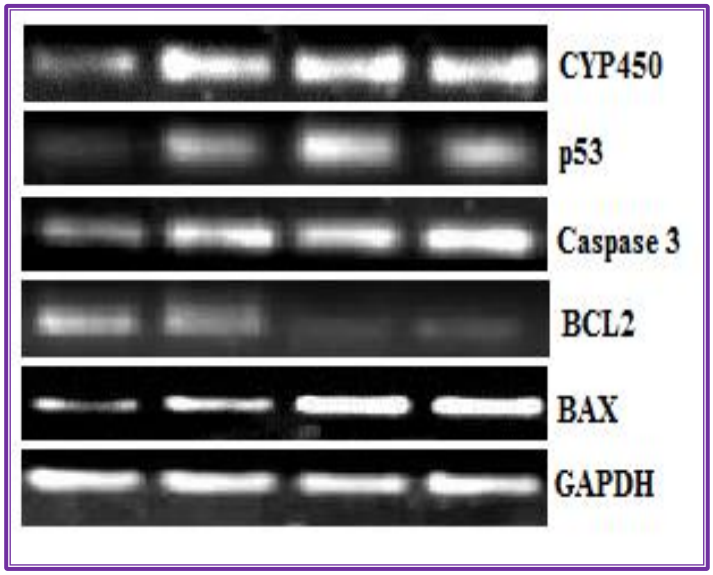


Figure 6: *Chang Liver Cell Lines M RNA Expression on Isoniazid and Rifampicin Exposure*

Lane 1: Normal chang liver cells; lane 2: ATB (50ng/ml); lane 3: ATB (100ng/ml) andlane 4: ATB (200ng/ml)

m RNA Expression of CYP2E1, p53, Caspase-3, Bcl-2, and Bax in Liver Cell Lines Treated with INH and RIF

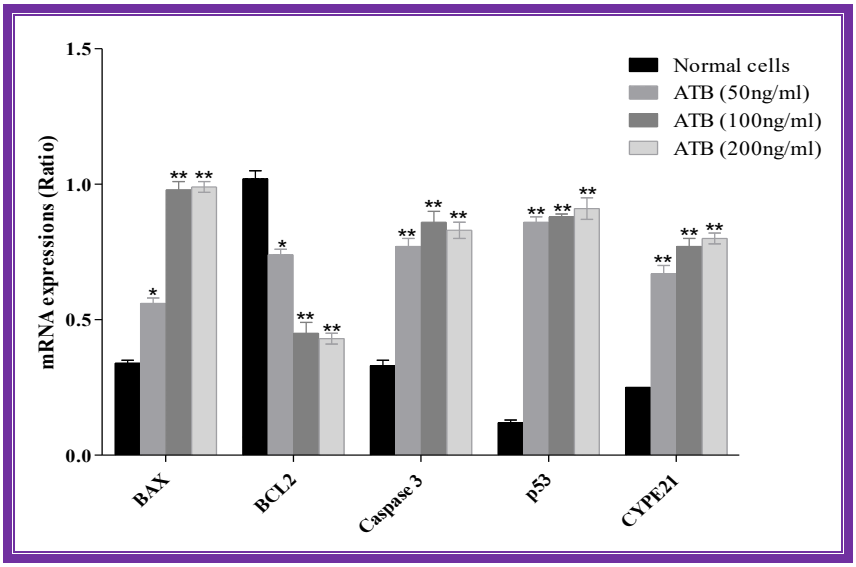


Figure 7: *Differential m RNA expression of BAX, BCL2, CASPASES 3, P53 and CYPE21 in Liver Cell Line on Isoniazid and Rifampicin Exposure*

Semiquantitative Analysis of mRNA Expression in Liver Cell Lines

Values were expressed in mean±SEM; n=3 Group; *, **indicates p<0.05 and 0.01, respectively control vs test.

Discussion

An attempt has been made to establish the INH and RIF-induced pathogenicity in Chang liver cell lines. For a period of 24 hours, 50, 100, and 200 ng/ml of INH and RIF exposure were given to the Chang cell lines. The integrity of cell lines was tested by tracing the AST, ALT, and ALP levels in the medium. Cells treated with INH and RIF showed exponential activity of AST and ALT. But unexposed cell lines have not revealed any such anomalies. Many studies have documented that INH and RIF exposure may damage the hepatocyte integrity. Therefore, cells exposed to INH and RIF have a sustained injury in their membrane, resulting in cytoplasmic transaminases (Pal *et al.,* 2008). Thus the exponential activity of AST and ALT levels in the medium could result from cellular injury.

ALP is one of the additional diagnostic biochemical markers used to detect hepatocyte dysfunction and cell damage. A high level of ALP activity is seen only if there is any hepatocyte leakage (Chopra & Griffin, 1985). A traceable quantity of ALP levels was manifested in the cell lines treated with INH and RIF. This cumulative quantity of ALP could be the outcome of cell damage.

The lysosome is one of the key organelles present in the cells that contain a variety of hydrolytic enzymes. The stability of the lysosomes is extremely important for the survival of the cell, and it also determines the fate of cell death. Any rupture in the lysosomal membrane allows for lysosomal-mediated cell death. Especially, when the cell is under stress may result lysosomal rupture and causes undesirable elevation of enzymes in the intra and extracellular spaces, induces apoptosis (Wang *et al.,* 2018). In the current study, the lysosomal fraction of intoxicated cell lines showed elevated activity of galactosidase and cathepsin D, whereas untreated cell lines showed no undesiderial enzymatic activity. The elevated activity of beta galactosidase and cathepsin D in the lyosomal fractions could be possible only if the integrity of the lysosome was compromised. Several studies have demonstrated that INH and RIF exposure may compromise the cell integrity (Zhuang *et al*., 2022).

The imbalance of cellular oxidation sinks the reserve antioxidant and makes the cells vulnerable to the modification of protein and DNA. Since the liver is the site for the metabolism of INH and RIF (Zentner *et al.,* 2020). Several studies have documented that INH and RIF may damage the DNA by inhibiting the DNA-repairing process (Shayakhmetova *et al.,* 2012). Comet assay is one of the prominent tools to analyze DNA damage by chemical and physical agents (Møller *et al*., 2025). In the present study, DNA from the liver cell lines exposed to INH and RIF exhibited a distinct comet tail pattern, whereas the untreated control cells showed predominantly intact DNA in the comet assay. The appearance of comet tails indicates DNA damage in the liver cell lines following exposure to INH and RIF.

CYP2E1 is one of the contributing factors to INH- and RIF-induced toxicity. In the current approach, cell lines treated with INH and RIF showed an up-regulated mRNA expression of CYP2E1; however, untreated cell lines did not reveal any such anomalies. CYP2E1 is a form of CYP directly linked to INH and RIF toxicity by contributing more free radicals from the reactive metabolites. Moreover, free radicals generated from the reactive metabolites directly consume the reserve endogenous antioxidant resources and make the cell less defensive against the oxidative stress. RIF further increases the toxicity by producing more reactive species from the INH by inducing CYP2E1 (Liu *et al.,* 2017). In addition to that, CYP2E1 expression is directly linked to cellular damage (Killingsworth *et al*., 2024).

In the current approach, an exponential increase in CYP2E1 and a cumulative amount of transaminases and ALP were manifested in the cell lines exposed to INH and RIF. This articulable

expression of CYP2E1, along with a perceivable amount of biochemical indices from the treated cell lines, indicates the establishment of cell injury.

P53 is one of the major transcription factors determining the cell's fate. P53 involves the maintenance of cellular homeostasis by regulating the hundreds of genes involved in the cell cycle, antioxidant system, DNA replication, repair, apoptosis, and autophagy (Beyfuss & Hood, 2018). P53 has a complex role in oxidative stress, particularly if the cell is suffering from mild oxidative stress. Then P53 acts as an antioxidant by inducing glutathione reductase. p53 also induces the BAX expression, which is crucial for the release of cytochrome c from the mitochondria to execute intrinsic apoptosis (Liu & Xu, 2011).

In the present study, liver cell lines exposed to INH and RIF exhibited upregulated mRNA expression of p53. In contrast, the untreated control cells showed approximately half the level of p53 expression observed in the INH- and RIF-treated cells. The increased expression of p53 in the treated cells suggests that the cells were experiencing cellular stress and may indicate the activation of intrinsic apoptotic pathways.

Bcl2 and Bax are the contrast proteins that regulate apoptosis. Moreover, the cell survival rate is purely dependent on the balanced ratio of Bcl2 and Bax (Putri & Nurfatihah, 2025). In particular, Bcl2 prevents apoptosis by inhibiting the release of cytochrome c from the mitochondria, whereas Bax, in contrast, promotes apoptosis by inducing the secretion of cytochrome c from the mitochondria (Gopisetty *et al.,* 2006). In the present study, cells treated with INH and RIF showed significantly upregulated mRNA expression of Bax and downregulated expression of Bcl-2. The increased expression of Bax, together with the altered Bax/Bcl-2 expression ratio, indicates that INH and RIF treatment may induce cellular stress and promote apoptotic processes in the liver cell lines.

Caspase-3 is one of the major executors of the apoptosis process (Dou *et al*., 2024). In the current approach, INH and RIF treated cell lines showed the up-regulated mRNA expression of caspases. But untreated cell lines showed the conventional expression. The up-regulated expression of caspase-3 in the intoxicated cell lines is an outcome of unbalanced BAX and BCL2 expression. Moreover, the unconventional expression of caspase 3 may shift the cells towards apoptosis.

Limitations

The study covered the toxic effects of the combined administration of isoniazid (INH) and rifampicin (RIF) in Chang liver cell lines, with particular emphasis on cellular integrity, DNA damage, and alterations in associated mRNA expression. However, the observed toxicity was dose-dependent and cannot be accounted for as individual effects of INH and RIF on the cell lines. Furthermore, the alterations in mRNA expression reported in the study were not validated at the protein-expression level. Therefore, further studies are warranted to establish the molecular mechanisms underlying INH- and RIF-induced toxicity.

Future Scope

The study demonstrated lysosomal enzyme leakage; the potential effects on other cellular organelles can be explored in future investigations. Future research needs to be warranted to examine organelle-specific alterations and their contribution to the overall cellular toxicity induced by INH and RIF.

Conclusion

The study was designed to explore the co-administration toxicity effects of INH and RIF on Chang liver cell lines. Biochemical indices, such as AST, ALT, and ALP activities, were manifested in the culture medium of the intoxicated cell lines. The traces of biochemical markers confirm the cell membrane integrity damage in the cell lines. The activities of β-galactosidase and cathepsin D in the culture medium indicate the lysosomal rupture in the intoxicated cell lines. The appearance of a comet-tail pattern of DNA on the comet assay reveals the DNA damage in the treated cell lines, whereas its absence in the unexposed control cell lines indicates the intact DNA. Cell lines exposed

to INH and RIF displayed a differential pattern of mRNA expression of CYP2E1, p53, BAX, BCL2, and caspase-3, whereas the control cell lines exhibited optimal mRNA expression. The reported toxicity in the cell lines was dose-dependent; these findings demonstrated the toxicity associated with the co-administration of INH and RIF and should not be equated with the individual toxic effects of each drug.

Conflict of Interest

The authors declare that they have no competing interests.

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