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Protective role of sildenafil citrate in isoniazid-rifampicin induced histomorphological changes in liver of albino mice

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Abstract

Objectives: The objective of the study was to reveal the reversal of histo-morphological changes in mice liver induced by combined isoniazid-rifampicin (INH-RIF) therapy with sildenafil treatment.

Methods: Twenty-one mice weighing between 25–35 g were enrolled in the study. Randomisation was carried out by simple balloting method. The selected mice were sorted into three groups with 7 mice, each group. In group C (n=7) control group, mice were administered 0.4ml of saline per kg body weight daily intra peritoneally for 21 days. In group R (n=7) INH-RIF group, rifampicin (50 mg/kg) and isoniazid (50 mg/kg), dissolved in 4 ml/kg isotonic saline, were administered intra-peritoneally (ip) daily for 21 days. In group S (n=7) sildenafil administered group, 10 mg/kg sildenafil was given orally by gastric gavage on daily basis along with the intraperitoneal injection of INH-RIF (50 mg/kg each) daily for 21 days.

Results: Histopathology revealed hepatotoxicity in group R (INH-RIF), while significant improvement was observed in group C (INH-RIF-sildenafil).

Conclusion: Sildenafil citrate possesses hepatoprotective role against INH-RIF induced hepatotoxicity.

Keywords: hepatotoxicity; histomorphology; INH-RIF; oxidative stress; sildenafil citrate

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Introduction

Tuberculosis (TB) remains one of the leading causes of illness and death worldwide including Pakistan.[1] WHO still considers isoniazid-rifampicin (INH-RIF) combination therapy as a basic pillar of anti-tuberculous therapy.[2] INH when administered in an inactive form is activated by the catalase-peroxidase enzyme in Mycobacterium tuberculosis known as KatG which results in mycolic acid synthesis inhibition. Toxic effects of INH are mediated by its metabolites hydrazine and acetyl hydrazine produced by acetyltransferase followed by oxidation by cytochrome p450 to form hepatotoxic metabolites.[3,4] RIF causes unconjugated hyperbilirubinemia. When given in combination, RIF accelerates INH metabolism producing toxic metabolites resulting in pathological changes in mice liver.[3]

As per literature antitubercular drug induced hepatotoxicity varies from 2–28%, and 11% of patients. About 20% of patients receiving combined therapy results in asymptomatic elevation of liver enzymes. Studies have shown raised levels of alanine aminotransferase (ALT) and aspartate transaminase (AST) as markers of hepatotoxicity. A rise in ALT and AST values 3–4 times of the normal range with nausea, vomiting, pain abdomen, tiredness and, jaundice is considered as evidence of acute hepatic toxicity.[5,6] Hepatic histologic alterations were revealed in a qualitative histological animal study treated by INH-RIF.[4–7] Hepatocytes were enlarged having an abundance of eosinophilic cytoplasm and signs of vacuolization. Sinusoids were dilated with a large number of erythrocytes in the lumen. Central veins showed dilatation and congestion. In another study, the mechanism of liver injury secondary to combination therapy of INH-RIF was studied in rats in which the liver showed portal triaditis and microvascular fat deposition.[8]

The literature revealed increase oxidative stress in INH-RIF therapy which was evident from a decrease in

the levels of glutathione superoxide dismutase, glutathione peroxidase, and glutathione-S-transferases. Both the altered profile of antioxidant enzymes and increased lipid peroxidation pointed towards the pathogenesis. Fulminant hepatic failure can be one of the worst complications of combination therapy.[9]

Sildenafil citrate is a phosphodiesterase inhibitor that was approved initially for erectile dysfunction and pulmonary hypertension.[10] Recently literature revealed that sildenafil inhibits oxidative stress as well as lessens inflammatory changes.[11] The hepatoprotective role of sildenafil has been observed in thioacetamide-induced liver fibrosis as well as deranged liver function tests.[12] Paracetamol is one of the known hepatotoxic drugs and was administered in Wister rats along with sildenafil it was found that phosphodiesterase 5 inhibition has a preventive role in paracetamol- induced liver injury.[13] In another related study by Ayman et al.,[14] the protective role of sildenafil in cisplatin-induced nephrotoxicity was manifested by improvement in renal function tests (RFTs) as well as reversal of histological changes in rats kidneys. There is evidence that sildenafil has an important protective role in oxidative stress-related cardiac, lung, and kidney injuries, but data is very much scarce regarding the role of sildenafil in liver injury in both biochemical as well as histological aspects, therefore this experimental study has been designed in a mouse model to find out the hepatoprotective role of sildenafil, in the face of combined INH-RIF therapy.

Materials and Methods

This study was conducted in Anatomy Department of Khyber Girls Medical College Peshawar in collaboration with Pakistan Council of Scientific and Industrial Research (PCSIR) laboratories of Peshawar. Total duration of study was six months. Thirty-one healthy male albino mice seven mice in each group of 6–8 weeks age were bought from Veterinary and Research Laboratories of Khyber Pakhtunkhwa Peshawar. Sample size was calculated by a formula which is E = total number of animals - total number of groups, where E is the degree of freedom and its value should lie between 10 and 20. So, E = 21 – 3 = 18. The surplus 10 mouse were kept as a reserve using attrition formula. Simple random sampling was performed by balloting method into three different groups. Mice of 6–8 weeks age having 25 to 30 g weight were included whereas inactive and deformed mice were excluded. The mice were purchased from Veterinary Research laboratories Peshawar, weighing 25–35 g and housed in the animal house of PCSIR. They were kept in 12 hours light / 12 hours dark cycle at temperature of (23±2°C) for one week to be acclimatized. The mice in each group were numbered. The dosage was standardized according to international protocols of study of drugs in the animals. At the commencement of the research, the general physical examination (GPE) of the mice were done and it was ensured that all the mice were healthy and without any apparent deformity. All the animals were weighed by electronic animal weighing scale before the commencement and before culling of mice. All the weights of mice were recorded.

Two groups of drugs that are INH-RIF and Sildenafil citrate dose calculation and solution preparation was done in the Anatomy Department of Khyber Girls Medical College (**Table 1**).

The groups were as follows:

Group C (n=7): The control group were administered 0.4 ml of saline per kg of body weight daily intra peritoneal for 21 days.

Group R (n=7): In group R, rifampicin (50 mg/kg) and isoniazid (50 mg/kg), dissolved in 4 ml/kg isotonic saline, was administered intraperitoneally (i.p.) daily for 21 days.

Group S (n=7): In group S 10 mg/kg sildenafil was given orally by gastric gavage on daily basis along with the intraperitoneal injection of INH-RIF (50 mg/kg each) daily for 21 days.

Table 1

Details of administered antitubercular drugs (dosage, strength and supplier).

Chemical	Physical state	Strength	Supplier	Use	Dose
Saline	Solution	500 ml	Otsuka	Group C	4 ml/kg
Sildenafil	Tablets	50 mg	Pfizer	Group S	10 mg/kg
INH	Tablets	300 mg	TB control board	Group R Group S	50 mg/kg
RIF	Tablets	300 mg	TB control board	Group R Group S	50 mg/kg

Table 2
Necrosis and inflammation scores by modified Knodellar index.

Piecemeal necrosis(A)	Score	Confluent necrosis (B)	Score	Focal lytic necrosis, apoptosis (C)	Score	Portal inflammation (D)	Score
Absent	0	Absent	0	Absent	0	None	0
Mild (focal, few portal areas)	1	Focal confluent necrosis	1	One focus or less per10x objective	1	Mild, some or all portal areas	1
Mild/moderate (focal, most portal areas)	2	Zone 3 necrosis in some areas	2	2–4 foci per 10x objective	2	Moderate, some or all portal areas	2
Moderate (cont around <50% of tracts or septa)	3	Zone 3 necrosis in most areas	3	5–10 foci per 10x objective	3	Moderate/marked, all portal areas	3
Severe (cont around >50% of tracts or septa)	4	Zone 3 necrosis+occasional portal-central bridging	4	More than ten foci per 10x objective	4	Marked, all portal areas	4
		Zone 3 necrosis + multiple portal-central bridging/panacinar necrosis		5/6			

Animals were culled on day 21. Anaesthesia was administered by chloroform-soaked cotton, placed in 50ml conical plastic tube. As per laboratory animals dissection protocols, all the animals were dissected by central incision. Livers were retrieved and weighed one by one. The livers from mice were preserved in 10% neutral buffered formalin solution. The tissue samples were transported to the histopathology section of PCSIR labs.

After fixation in 10% formalin solution, the cassettes of cut liver were placed in the tissue processor. Tissue sections were cut. Liver tissue was stained with hematoxylin eosin stain. All the slides were studied under the light microscope according to modified Knodellar criteria to observe quantitative and qualitative histological features of the mouse liver to differentiate the changes occurred in different groups of mice (**Table 2**). Microscopy was done for each group of mice with 10× and 40× lenses of microscope for the detailed observation.

Results

Before the start of research all the mice in groups C, R and S were completely active having sharp response to touch stimuli whereas at the completion of 21 days, the mice in group R were having sluggish response to touch stimuli as compare to other two groups (groups C and S).

The mean weight of mice before and after experiment was calculated and t test was applied (**Table 3**). There was no statistically significant change in between weights before treatment and after treatment in any of the three groups (Df=6, $p<0.05$; 2-tailed). When Tukey post hoc test was applied there was no significant weight change in between groups C, R and S (**Table 3**).

The mean absolute weight of liver ranged from minimum 1.5 g for group C to maximum 1.86 g for group R (**Figure 1**). No statistical significant difference between absolute liver weights was found in different groups except groups C and R (p=0.02).

Histological variables

Mean Knodellar score was calculated, ranging from minimum 5 in groups C and S to maximum score 10 in group R (**Figure 2**). One-way ANOVA evaluated the degree of variance between groups and within groups, which was significant (p=0.000). Post-hoc Tukey test showed the signifi-

Table 3
Mean weight of mice before and after treatment.

Groups	Mortality (n)	Body weight before treatment	Body weight after treatment	Paired sample test	Anova/Post-hoc Tukey
Control	0/7	28.7±2 g	28.1±2 g	0.231	C=R
INH-RIF	0/7	27.5±2 g	27.1±1 g	0.289	R=S
INH-RIF-sildenafil	0/7	28.7±2 g	28.1±3 g	0.231	C=S

Paired t test $p \geq 0.05$; Post-hoc Tukey $p \geq 0.05$.

cant difference between the mean Knodellar score in all three treated groups. Significant increase was found in the mean Knodellar score in group R as compare to group C, whereas a significant decrease was noted in group S as compare to group R. The score was equal in between groups C and S, which potentiates our alternate hypothesis.

In order to check qualitative variables (ordinal data) Kruskal Wallace test was applied to show association between histological changes in liver like piece meal necrosis, confluent necrosis, focal lytic necrosis and portal inflammation with type of administered drugs assuming significant ($p \leq 0.05$) (**Table 4**).

Moreover, the histological changes occurred in all the three groups were compared individually with each other in order to see whether the effect of sildenafil on histomorphological changes in liver is significant or not. For this reason, Mann-Whitney U test was applied. When group C was compared with group R significant histological changes (Piece meal necrosis, focal lytic necrosis and Portal inflammation) were observed in R group ($p \leq 0.05$) (**Figures 3a** and **3b**). In group S versus R there was less degree of piecemeal necrosis and portal inflammation ($p \leq 0.05$) (**Figures 4a** and **4b**).

Significant improvement was observed in overall Knodellar score in group S as compare to group R. There was also significant improvement in histological parameters like piece meal necrosis and portal inflammation, when INH-Rifampicin was administered along with Sildenafil citrate.

Discussion

The most striking finding of our research study was that INH-RIF had established hepatotoxic effects and sildenafil citrate had antioxidant properties which proved to be hepatoprotective against the toxic effects. In the era of laparoscopic and robotic surgery, one cannot precede regarding the management without a histopathological picture of the tissue. Every drug has its manifestations in the body that appear either in form of the biochemical changes at the enzymatic level or histopathological changes at the tissue level.

One of the established mechanisms of drug-induced hepatotoxicity in literature is oxidative stress. It is also a fact that diets, as well as drugs having antioxidant properties, have a hepatoprotective role in countering the toxic effects of drugs.[15,16]

Bodyweight is one of the benchmark tools to check the toxic effects of a specified drug. In our study, there was no significant difference in body weights either before or after

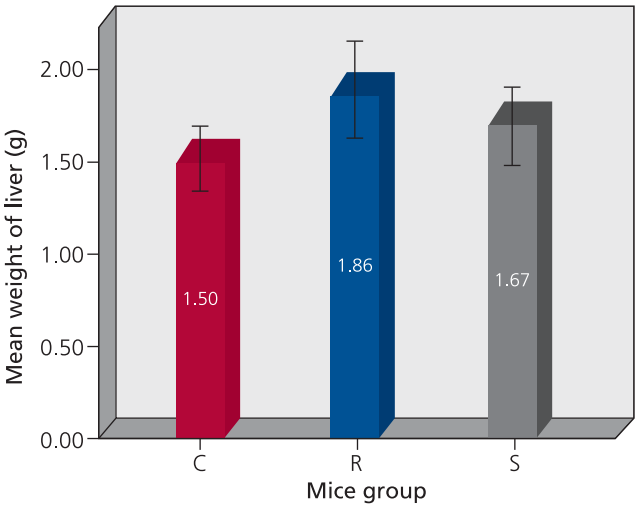

Figure 1. Comparison of mean weight of mice livers (error bars±2SD).

Table 4

Comparison of histological parameters in mice liver by applying Kruskal Wallice and Mann-Whitney U tests.

Histological parameters	Overall group comparisons (Kruskal-Wallis test)	Pair wise significant comparisons (Mann-Whitney U test)
Piecemeal necrosis	0.000	C ↔ R R ↔ S
Confluent necrosis	0.119	
Focal lytic necrosis	0.168	C ↔ R
Portal inflammation	0.000	C ↔ R R ↔ S C ↔ S

↔ shows $p \leq 0.05$

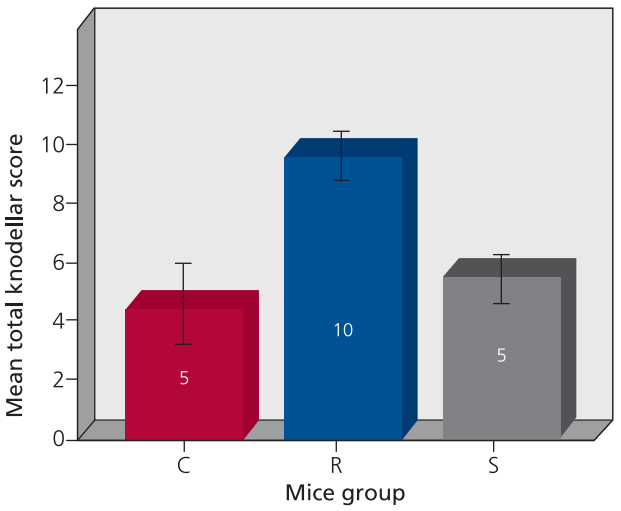

Figure 2. Mean difference of Knodellar score in between groups C, R and S (error bars 95% CI).

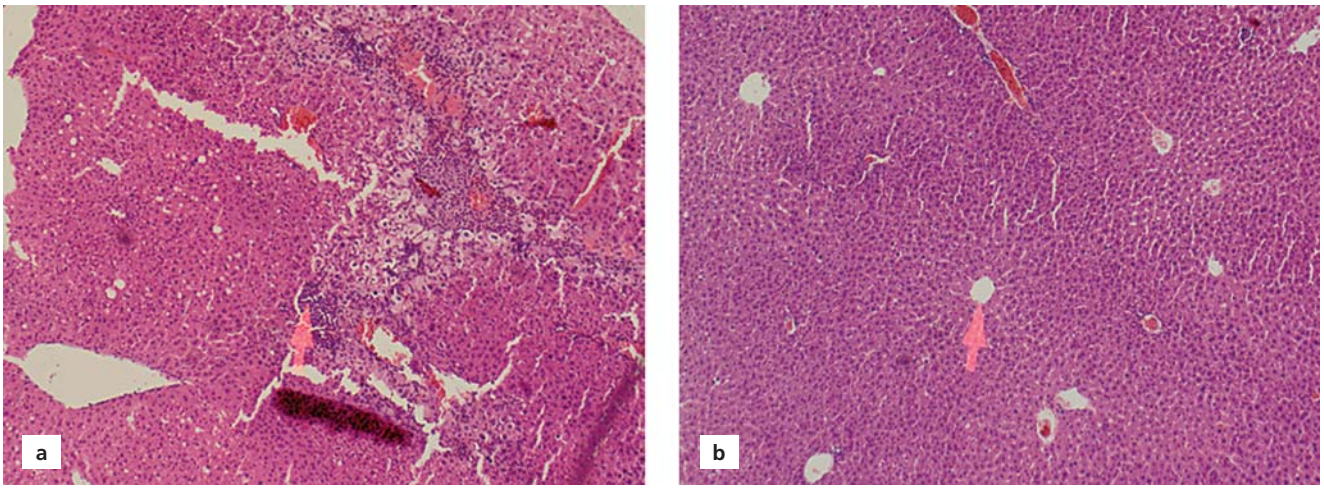

Figure 3. (**a**) Liver histology in group R showing grade 3 focal lytic necrosis and portal inflammation. (**b**) Normal liver histology in group C.

treatment. In a study on INH-RIF by Pal et al.,[17] hepatotoxic model was produced by injecting 50 mg/kg INH-Rifampicin and garlic was administered as a hepatoprotective agent showing no significant change between the three groups. In another study by Pal et al.,[18] carotenoids effects were observed in INH-RIF hepatotoxicity, there was no significant change in body weights. Overall, our results are following the literature.

In our study a significant increase in absolute liver weight was observed in-between groups C and R as well as the significant increase was found in relative liver weight in group R as compared to group C which was per the study conducted by Wang et al.,[19] where the protective effects of naranginin were studied against INH-RIF induced hepatotoxicity. In group S, the liver weight decreased but that was not significant and the same was the case in the above-mentioned study where the decrease in liver weight in naranginin 50 mg/kg administered group was not significant. On the other side in another group of mice where naranginine was administered in 100 mg/kg, the decrease in the liver weight was significant $p<0.05$. Based on these facts we can also presume that if sildenafil citrate is given in increased dosage in future research there may be a significant decrease in liver weight. In a very detailed research study conducted by Yang et al.,[20] protective effects of diallyl trisulfide (DATS) were studied against the histomorphological and biochemical effects of INH-RIF. The mice were divided into six groups and DATS (10 mg/kg, 20 mg/kg, and 40 mg/kg) were administered two hours before

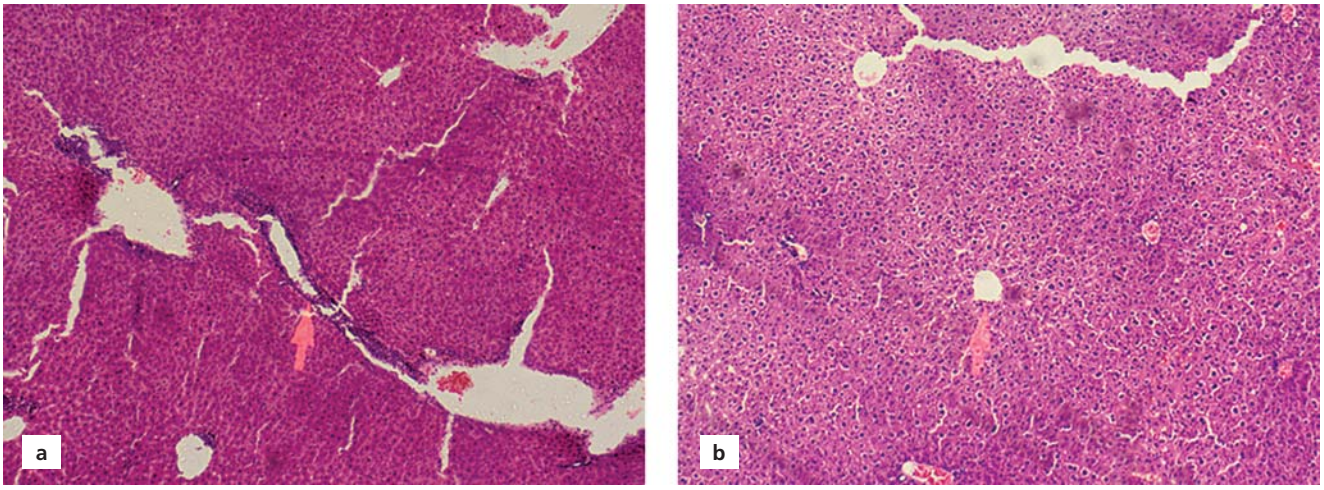

Figure 4. (**a**) Portal inflammation in group R. (**b**) Group S showing improved liver histology.

administration of INH-RIF (100 mg/kg & 100 mg/kg) respectively. The levels of AST, ALP, liver weight, and histological parameters were studied. In similarity to our study where the mean Knodellar score was the same in the control and sildenafil administered group, they observed significant improvement in the histological parameters of liver induced by INH-RIF. In our study control and sildenafil group had more or less normal lobar architecture with normal cell morphology while in the INH-RIF group there was focal lytic necrosis, piecemeal necrosis and portal inflammation which was also observed in a study by Yang et al.[20] DATS have an anti-oxidant effect like sildenafil citrate that's why significant improvement was observed in liver morphology.

Reactive oxygen and nitrogen species are one of the main causes of the initiation and progression of liver injury. Free radicals have unpaired electrons which is highly reactive. These reactive species activate lipid peroxidation, breakage of DNA strands and ultimately oxidize all molecules in cell membrane resulting in cell injury. Normally in a healthy person, there is a balance between oxidative agents and the production of antioxidants.[21]

It is a known fact that some of the non-toxic herbs are having reverse activities in the form of membrane stabilization, anti-oxidant, and having CYP2E1 inhibitory effects. Literature review suggests that decreased levels of lipid peroxide content in tissue and an increase in superoxide dismutase, catalase, glutathione, and glutathione peroxidase activities help to maintain liver cell coherence and control the derangement in the level of liver enzymes.[22]

The comprehensible mechanism of hepatotoxicity is not clear, but the proposed mechanism for INH and RIF-induced damage involve lipid peroxidation and oxidative stress resulting in lowering of phospholipids protein synthesis with modifications in cell wall layout, reduced glutathione level, and activation of CYP2E1.[21,22] PDEs play a vital role in the control of normal and pathological cellular signalling mechanisms. Mainly the PDE5 family inhibition increases cGMP levels as it hydrolyses specifically cGMP. Sildenafil, a PDE5 inhibitor, is mainly used for treating erectile dysfunctions and pulmonary hypertension. It also induces protective effects during ischemia-reperfusion injury in several organs like lungs and kidneys.[23] Based upon these facts, sildenafil was used as an anti-oxidant and observed its protective role in the liver. Our limitations in this research study were very small data and no guidelines can be changed based upon this data, however this study opens up new avenues to climb further upon the shoulders of this research and randomized trials should be done to establish the protective role of sildenafil in INH-RIF induced hepatotoxicity.

Conclusion

In the light of this study, it is concluded that INH and RIF exhibit hepatotoxic potentials as observed in this study from the derangements in hepatic histological parameters. We suggest that sildenafil has a hepatoprotective role against INH-RIF-induced hepatotoxicity if administered along with it, as evident by significant improvement in histological parameters.

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Conflict of Interest

All authors declare no potential conflicts of interest.

Author Contributions

NM: conceived the idea, designed the study and wrote initial manuscript; KF: helped in executing the plan after going through the study protocol, data collection, interpretation and revising the manuscript. NM, and KF, reviewed the draft critically, carried out corrections and supervised the whole study. All authors contributed significantly to the submitted manuscript.

Ethics Approval

Study was approved by Graduate Study Committee, and advanced study and research board, Khyber Medical University vide notification no DIR/KMU-AS and RB/TS/001126 dated 31/12/2019.

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