Comparative study of protective effect of silymarin and n-acetyl cysteine on isoniazid induced hepatotoxicity in mice

Sarwat Jahan1*, Lubna Danish2, Manzoor Khan3, Marva Sandhu2, Naiila Abrar4, Riffat Siddiq5, Abid Hussain6

1 Khyber Medical College, Khyber Medical University, Peshawar, Pakistan.
2 Islamic International Medical College (IIMC), Riphah International University, Rawalpindi, Pakistan.
3 Khyber teaching Hospital, Khyber Medical University, Peshawar, Pakistan. KMU Peshawar, RMC Peshawar
4 HITEC Institute of Medical Sciences, National University of Medical Sciences, Rawalpindi, Pakistan.
5 Rehman College of Dentistry, Khyber Medical University, Peshawar, Pakistan.
6 North West School of Medicine, Khyber Medical University, Peshawar, Pakistan.

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ABSTRACT
Drug induced liver injury (DILI) leads to acute hepatitis in 10%, liver failure, and death in 30%. One of the major drugs causing DILI is Anti-tuberculous drugs and since tuberculosis is affecting 1/3rd of the total world population, their use is quite common. Use of Isoniazid (INH) is limited owing to hepatotoxicity following the stress produced by oxidative species. Silymarin has hepatoprotective potential because of anti-oxidant property. N-Acetylcysteine (NAC) on the other hand triggers the cellular protective mechanisms by replenishing glutathione in the cells. The main aim of this study was to compare the hepatoprotective activity of Silymarin and NAC against INH induced toxicity. Total 50 BALB/C mice were sorted into a total of 5 groups, with 10 mice in each group for 2 weeks by random sampling. Control group was administered distilled water through I/P route daily. The INH group was administered I/P 100 mg/kg INH daily. The INH/Silymarin group was administered 150 mg/kg INH and 50 mg/kg Silymarin I/P daily. The INH/NAC group was administered 150mg/kg INH and 300mg/kg of NAC I/P daily. The INH/NAC/silymarin group was administered 150mg/kg INH, 300mg/kg of NAC and 50mg/kg of silymarin I/P daily. On day 14, the dissection of all the mice was done. Liver function tests were performed and histopathology was done. Both NAC and Silymarin demonstrated hepatoprotection that was statistically similar. However, the mice of silymarin group looked weak and less active and six dies within 5-8 days after the end of the experimental doses of the drugs.

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INTRODUCTION
DILI includes varying degrees of responses that occur as a result of exposure to synthetic or natural chemical substances. DILI is a serious rising issue in the fields of medicine, science, and public health. The incidence is approximately around 10 to 15 /100,000 of individuals [1]. About 10 % of all the acute hepatitis cases worldwide are due to DILI. Medications are withdrawn from the market most commonly due to DILI [2]. It is the major cause of acute liver failure among patients. Death rate among patients suffering from DILI is about 30 percent [2].

One of the major groups of drugs that lead to the drug induced liver injury is the Anti-Tuberculous drugs. It is one of the top 10 causes of death in the world [3]. About 1 in 3 individuals have infection of TB worldwide. In 2010, WHO...
statistics showed about that around the world there are about
8.8 million of cases.

As the incidence of tuberculosis is still quite high world-
wide as is the use of ATT and hence, the incidence of DILI
caused by ATT. One of the notorious ATT drugs producing
hepatotoxicity is Isoniazid (INH). Isoniazid is known to
cause severe and sometimes fatal hepatitis. Within the first 2
months of therapy, isoniazid-induced liver toxicity occur in
about 50% of patients [4]. Although the main mechanism of
hepatotoxicity of INH is still to be understood but according
to animal models the hepatotoxicity by INH is due to oxida-
tive stress, lipid peroxidation, reduced levels of glutathione
[5] or activation of CYP2E1 or a combination of either all or
some of these mechanisms.

The hepatotoxicity leads to induction of apoptosis of
hepatocytes, breaks in the DNA strands and mitochondrial
membrane disruption. The reactive metabolites produced by
INH metabolism, including N-acetylsalicylazid (AcINH) and
Hydrazine (Hz) damage the hepatocytes [6].

Silymarin (milk thistle) is extracted from the seeds of Si-
lybum marianum has documented hepatoprotective proper-
ties. Their mechanisms of hepatoprotection are not fully un-
derstood. However, there are four proposed mechanisms: It
has antioxidant property and regulates intracellular glutathione;
prevents the entry of toxins in the hepatocytes by alter-
ning the membrane permeability; promotes liver regeneration
by enhancing RNA synthesis; is an inhibitor the transfor-
mination of stellite hepatocytes into myofibroblasts [13].

N-Acetylcysteine (NAC) is a prodrg that forms L-
cysteine primarily used as a mucolytic agent. It triggers the
cellular protective mechanisms by replenishing glutathione
in the cells. During oxidative burst, NAC reduces the release
of reactive oxygen species from the neutrophils. It replenish-
es the hepatic stores of glutathione by providing the sub-
strate cysteine. It has the capability of arresting the hepatic
stellate cells in the G1 phase thus halting the process of fi-
brogenesis, NAC decreases the peroxidation of membrane
lipids and produces anti-toxin effect as it can increase the
hepatic blood flow, replenishes glutathione and scavenges
free toxic radicals, NAC causes the down regulation of
the enzyme glutathione S-transferase, responsible for the metab-
olism of glutathione and NAC inhibits the activation of NF-
κB, an important factor in fibrogenesis [8].

This study is designed to understand and compare the
protective effect of Silymarin and NAC on the hepatotoxic-
ity induced by INH.

MATERIALS AND METHODS

Study Design

This study was a laboratory based randomized controlled
trial.

Study Settings

The study was carried out in the Veterinary research in-
stitute, Peshawar in collaboration with the Pharmacology
Department of, Khyber girls’ medical-college and Khyber
medical university, Peshawar. Male mice, 25-30 grams of
weight were kept in the animal house for one week prior to
the study in Veterinary research institute Peshawar under
standard conditions of with temperature of 20-25°C. Mice
were given proper diet and water throughout the duration of
the study.

Sample Collection Technique

BALB/C mice were initially selected through random
sampling method.

Sample Size

A total of 50 mice of BALB/C type were randomly sor-
ted into a total of 5 groups; each of the groups including the
one used as control had 10 animals.

Inclusion Criteria

• Male BALB/C mice
• Mice aged 5 - 7 weeks
• Mice weighing 25 – 30 g

Exclusion Criteria

• Inactive mice
• Mice having a prominent deformity

Data Collection Procedure

All the animals were sacrificed on day 14. Terminal car-
diac blood samples were taken for the assessment of the liver
enzymes, bilirubin, enzyme alkaline phosphatase, enzyme
aspartate aminotransferase and enzyme alanine aminotrans-
ferase. Liver was dissected out, preserved in 5% formalin
and sent for histopathology.

Duration of Study

The study was conducted for a period of 2 weeks from 1st
July 2018 to 14th July 2018.

Generation of Isoniazid Induced Hepatotoxic Model

A pilot project was carried out to confirm the hepatotoxic
dose of INH and hepatoprotective doses of silymarin and
NAC, in male mice of BALB/C type. INH was given 150mg
per kg, 50mg per Kg Silymarin & 300mg per Kg NAC keeping
the time span of 24 hours between injections for 2 weeks
was given.

At the end of 2 weeks terminal sampling and dissection
was selected on the basis of previous researches.

Experimental Outline

The selected mice were sorted randomly into a total of 5
groups containing 10 mice in each group for 2 weeks. Strict
aseptic measures were observed during solution (drug) pre-
paration and administration. Fresh solutions of drugs were
was prepared before each dose.

This paper is available online at: http://ijpt.iiums.ac.ir
Group 1 (n=10) served as a control group and received intra-peritoneal doses of distilled water daily. In Group 2 (INH group), the liver toxicity was produced in total 10 mice by giving injection of INH 150mg per kg intra-peritoneally for a time interval of total 2 weeks. In Group 3 (INH/Silymarin group), 10 mice were given intraperitoneal injection of INH 150mg/kg and Silymarin 50mg per Kg for a time interval of total 2 weeks. In Group 4 (INH/NAC group), 10 mice were given intraperitoneal injection of INH 150mg/kg and NAC 300mg per Kg given one hour before INH. Each dose of the drugs in every group was given at an interval of 24 hours.

<table>
<thead>
<tr>
<th>Grouping</th>
<th>Isoniazid</th>
<th>Silymarin</th>
<th>NAC</th>
<th>Dissection</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Day 14</td>
</tr>
<tr>
<td>Mice 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 2</td>
<td>150mg/kg [33, 34] of INH in water daily.</td>
<td>-</td>
<td>-</td>
<td>Day 14</td>
</tr>
<tr>
<td>Mice 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 3</td>
<td>150mg/kg of INH in water daily.</td>
<td>50mg/kg [35, 36] of silymarin in water daily.</td>
<td>-</td>
<td>Day 14</td>
</tr>
<tr>
<td>Mice 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 4</td>
<td>150mg/kg of INH in water daily.</td>
<td>-</td>
<td>300mg/kg [37] of NAC in water daily.</td>
<td>Day 14</td>
</tr>
<tr>
<td>Mice 10</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group 5</td>
<td>150mg/kg of INH in water daily.</td>
<td>50mg/kg of silymarin in water daily.</td>
<td>300mg/kg of NAC in water daily.</td>
<td>Day 14</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Table 2. The histopathological changes were graded according to Knodell Modified scoring system</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Peri-portal or peri-septal interface hepatitis (piecemeal necrosis)</td>
</tr>
<tr>
<td>Observation:</td>
</tr>
<tr>
<td>Absent</td>
</tr>
<tr>
<td>Mild (focal, few portal areas)</td>
</tr>
<tr>
<td>Mild/moderate (focal, most portal areas)</td>
</tr>
<tr>
<td>Moderate (continuous around, &lt;50% of tracts or septa)</td>
</tr>
<tr>
<td>Severe (continuous around, &gt;50% of tracts or septa)</td>
</tr>
<tr>
<td>B. Confluent necrosis</td>
</tr>
<tr>
<td>Observation:</td>
</tr>
<tr>
<td>Absent</td>
</tr>
<tr>
<td>Focal confluent necrosis</td>
</tr>
<tr>
<td>Zone 3 (centri-lobular) necrosis in some areas</td>
</tr>
<tr>
<td>Zone 3 necrosis in most areas</td>
</tr>
<tr>
<td>Zone 3 necrosis 1 occasional portal-central (P-C) Bridging</td>
</tr>
<tr>
<td>Zone 3 necrosis 1 multiple P-C bridging</td>
</tr>
<tr>
<td>Panacinar or multi-acinar necrosis</td>
</tr>
<tr>
<td>C. Focal (spotty) lytic necrosis, apoptosis and focal inflammation</td>
</tr>
<tr>
<td>Observation:</td>
</tr>
<tr>
<td>Absent</td>
</tr>
<tr>
<td>One focus or less per 10 X objective</td>
</tr>
<tr>
<td>Two to four foci per 10 X objective</td>
</tr>
<tr>
<td>Five to ten foci per 10 X objective</td>
</tr>
<tr>
<td>More than ten foci per 10 X objective</td>
</tr>
<tr>
<td>D. Portal Inflammation</td>
</tr>
<tr>
<td>Observation:</td>
</tr>
<tr>
<td>None</td>
</tr>
<tr>
<td>Mild, some or all portal areas</td>
</tr>
<tr>
<td>Moderate, some or all portal areas</td>
</tr>
<tr>
<td>Moderate/marked, all portal areas</td>
</tr>
<tr>
<td>Marked, all portal areas</td>
</tr>
<tr>
<td>Maximum possible score for grading = 18</td>
</tr>
<tr>
<td>Minimal inflammation = 1-3</td>
</tr>
<tr>
<td>Mild inflammation = 4-8</td>
</tr>
<tr>
<td>Moderate inflammation = 9-12</td>
</tr>
<tr>
<td>Severe inflammation = 13-18</td>
</tr>
</tbody>
</table>
**Design of the Experiment**

**Blood Sampling Procedure**

Terminal blood sampling was performed through cardiac puncture for all the groups at the end of 2 weeks, 24 hours after the last dose of the drugs. Animals were dissected and 0.5-1.5 ml of blood was gradually withdrawn with 1 ml syringe. Samples were taken to Clinical Pathology Laboratory where they were centrifuged for ten minutes to separate the serum for the assessment of ALT, ALP, AST and Bilirubin.

**Liver Histopathology**

The histopathological changes were graded according to Knodell Modified scoring system. As duration of the study was short, fibrosis was not looked for (Table 1 and Table 2).

**Statistical Analysis**

The expression of the obtained results was done in the form of mean value and standard deviation. The statistical analysis was done on statistical package for social sciences SPSS 23. Comparison of biochemical markers at initial and final hours in the same group was subjected to the one-way ANOVA test. The Post- Hoc test of Tukey was then applied to the data. The range of the significant value was taken to be, $p < 0.05$.

**Validity of animal species or model selection**

For this research study we chose BALB/C mice because of the fact that the characteristics that these mice possess make them an ideal model for hepatotoxicity studies. This includes induction of injury to the liver in a big percentage of these animals, low cost of these mice as well as the ease of their management[9].

**RESULTS**

**Group 1**

The group 1 received I/P normal saline daily for 2 weeks. LFTs remained within normal limits. Histopathology of group 1 coincided with the biochemical parameters, graded as normal according to ISHAK’s criteria (Fig. 1).

**Group 2**

INH receiving mice were less active and worn-down as compared to mice of control Group 1 during the experimental period. Liver enzymes were significantly raised and light microscopy of slides classed slides as moderate damage (Fig. 2). All the mice in this group died within 2 to 3 days after receiving the last experimental dose of INH.

**Group 3**

In Group 3 which received isoniazid 150mg/kg and Silymarin 50mg/kg daily intraperitoneally for 2 weeks, the liver function test parameters remained within normal limits. In this group, after light microscopy of H & E stained slides were classed to have minimal histopathological changes (Fig. 3). On observation however, the mice of this group were weak and less active. Six out of the ten mice in the group died within 5-8 days after the last doses of the experimental drugs.

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**Figure 1.** Biochemical and histological parameters of group 1 samples. a) Normal liver parameters with mean serum ALT level was $35.89 \pm 5.24$ U/L, ALP exhibited a mean value of $32.20 \pm 9.51$ U/L, AST a mean value of $42.43 \pm 2.63$ U/L, b) Normal serum bilirubin showing a mean value of $0.12 \pm 0.05$ mg/dl, c) The histology of the liver slice fixed in 5% formalin and stained with H & E showed normal hepatocytes.
Group 4
In INH/NAC group the mice looked healthy and active. The liver function tests demonstrated normal parameters. The light microscopy of H & E stained slides was classed to
have normal histological structure (Fig. 4).

**Group 5**

In Group 5 which received isoniazid 150mg/kg, Silymarin 50mg/kg and N-Acetylcysteine 300mg/kg daily intraperitoneally for 2 weeks, the liver function test parameters remained within normal limits. In this group, after light microscopy of H & E stained slides were classed to have normal histological structure (Fig. 5).

**Comparison between Groups**

Comparison of mean serum liver function parameters between all the groups showed a significant difference in ALT levels of Group 2 with p less than 0.05 in range (Table 3).

**DISCUSSION**

The issue of hepatic injury induced by drugs still remains an unresolved. Its impact on the healthcare system is even greater than the total number of cases that are reported every year. It was ranked at the top among the adverse effects pro-

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**Figure 4.** Biochemical and histological parameters of group 4 samples. a) Mean serum ALT of 37.4 ± 5.40 U/L; ALP registered a mean value of 26.53 ± 4.94 U/L, AST of 41.57 ± 2.16 U/L, b) Normal serum Bilirubin of 0.14 ± 0.07 mg/dl, c) Light microscopy of H & E stained slides was classed to have normal histological structure.

**Figure 5.** Biochemical and histological parameters of group 5 samples. a) Normal Lfts with mean serum ALT of 34.25 ± 5.56 U/L; ALP registered a mean value of 27.96 ± 5.64 U/L, AST of 42.48 ± 2.16 U/L, b) Normal Bilirubin of 0.13 ± 0.07 mg/dl, c) Light microscopy of H & E stained slides were classed to have normal histological structure.
Protection of alloxan monohydrate-induced testicular toxicity by Gundelia tournefortii

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Table 3. Comparison between Groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>ALT</th>
<th>ALP</th>
<th>AST</th>
<th>Bilirubin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
</tr>
<tr>
<td>Group 2</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
</tr>
<tr>
<td>Group 3</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
</tr>
<tr>
<td>Group 4</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
</tr>
<tr>
<td>Group 5</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

*The mean difference is significant at the 0.05 level

Figure 6. Comparison of Liver function Tests (LFTs) in different groups. Comparison between all the groups demonstrated a significant difference in ALT levels of Group 2 with p less than 0.05 in range. No statistical difference was found between group 3 and 4 although clinically NAC group showed better response

duced by drugs in 2006 [10], DILI has emerged as the most common reason for the termination of drug development programs and post-marketing drug regulatory decisions. Out of 1000 drugs known to cause hepatic damage, more than 50 approved ones have been withdrawn from markets worldwide owing to their hepatotoxic potential [11]. Serious DILI is induced by the anti tuberculosis drugs that are used in a very high number owing to the prevalence of tuberculosis infection all over the world. Ever since 1993, TB has been labeled as a public health emergency by the WHO [12].

Tuberculosis is a curable disease with very efficacious drugs available for the treatment. Isoniazid is among the commonly used anti tuberculous drugs and is very efficacious in treating the infection. However, the hepatotoxicity induced by these drugs is fatal and a big limiting factor in the treatment continuation. The therapy has to be withdrawn,
withheld until the LFTs are back to normal or switched and the second line agents are not only expensive but also more toxic [13]. Upon restarting the first line drug after the LFTs return to normal about 11-24 percent of the patients suffer from the drug induced hepatotoxicity again [14, 15]. Decision in these cases is very controversial and difficult as there is risk of toxicity but at the same time there is requirement of threatening the infection that can be life threatening, stopping the disease progress and transmission. So, the best option in this scenario is the development of methods to prevent and reduce the hepatotoxicity of the first line anti tuberculosis drugs. There is very less evidence available for the exact pathophysiological mechanism of these drugs that has the toxic potential and in addition it is hard to predict whether the toxicity is self limiting and reversible or it is going to cause cirrhosis and liver failure.

Isoniazid being very efficacious is used commonly in the treatment of the tuberculosis infection. INH, however has a very serious risk of producing toxicity in the liver [16]. Within the first 2 months of therapy, isoniazid-induced liver toxicity occur in about 50% of patients [17]. The DILI caused by INH is due to an idiosyncratic mechanism [18]. Although the main mechanism of hepatotoxicity of INH is still to be understood but according to animal models the hepatotoxicity by INH is due to the stress produced by toxic metabolites [19], peroxidation of the lipid membrane [20], depletion of glutathione in the cells [21] or activation of CYP2E1 [22] or a combination of either all or some of these mechanisms. INH is metabolized into products that act as reactive oxygen species and increase the oxidative stress. The oxidative stress ultimately leads to the disruption of the hepatocyte membranes and cell damage. Under the oxidative stress the liver produces glutathione that possesses antioxidant properties in its reduced form. However, INH is known to deplete the stores of glutathione hence leading to hepatocyte damage. If either the production of the oxidating radicals by INH is reduced or glutathione stores are replenished the liver damage may be prevented.

The pattern in which hepatic enzymes are raised in case of INH use is mostly hepatocellular. Isoniazid induces the apoptosis of liver cells. The membrane of mitochondria is disrupted and there is breakage of the strands of DNA. The INH metabolites can bind to and produce destruction of the macromolecules of the hepatocytes [29]. Hepatocellular injury induced by INH show the pattern of hepatic necrosis. Zonal necrosis with coagulation is observed varying in intensity, either involving a fraction of a single acinar zone to extension of necrosis to the whole of acinus.

During the initial stages, the regions with necrosis contain the ghost cells of hepatocytes. When the injury progresses further there is migration of, neutrophils & macrophages to the zone of necrosis, starting from the edges. Eventually within 12 to 24 hours of the injury, the process of necrosis progresses and only pigmented macrophages are observed that have replaced the hepatocytes. The apoptosis of the hepatocytes is observed at the necrotic corners. Significant amount of inflammation, portal traditis, lymphocytic and neutrophil infiltration is present.

Realizing the increasing necessity of use of INH but high incidence of morbidity with the use of this drug, we decided to investigate the extent of INH induced liver injury and its prevention by the use of Silymarin and N-Acetylcysteine, both of which have the hepatoprotective potential and can help reduced the INH induced morbidity. For our research study we chose BALB/C mice because of the fact that the characteristics that these mice possess make them an ideal model for hepatotoxicity studies. This includes induction of injury to the liver in a big percentage of these animals, low cost of these mice as well as the ease of their management. According to Murugananandan & Sinal [23], in cases of toxicity including the metabolism by the cytochromal enzymes, mice can be used as an efficient model. Our choice of mice was also supported by Metushi [24], who showed that reactive metabolites bind more with mice and human hepatic

Figure 7. Comparison of Knodell Scoring. The group 2 score was showing moderate histopathological damage.
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23. Muruganandan S, Sinal C. Mice as Clinically Relevant Models for the Study of Cytochrome P450-dependent Metabolism. Doi.org/10.1038/cpt.2008.50


