Hepatoprotective effect of tocopherol against isoniazid and rifampicin induced hepatotoxicity in albino rabbits

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Received 3 July 2007; revised 5 September 2007

Antitubercular drug induced hepatotoxicity is a major hurdle for an effective treatment of tuberculosis. The present study was undertaken to assess the hepatoprotective potential of tocopherol (50 mg/kg and 100 mg/kg, ip) and to compare it with cimetidine (120 mg/kg, ip). Hepatotoxicity was produced by giving isoniazid (INH, 50 mg/kg, po) and rifampicin (RMP, 100 mg/kg, po) combination to albino rabbits for 7 days. Assessment of liver injury was done by estimating levels of alanine transaminase (ALT) and argininosuccinic acid lyase (ASAL) in serum and by histopathological examination of liver. Results revealed that pretreatment with high dose of tocopherol (100 mg/kg) prevented both biochemical as well as histopathological evidence of hepatic damage induced by INH and RMP combination. Moreover, tocopherol (100 mg/kg) was found to be a more effective hepatoprotective agent as compared to cimetidine.

Keywords: Hepatoprotection, Hepatotoxicity, Isoniazid, Rifampicin, Tocopherol

Antitubercular drug induced hepatotoxicity is an important and commonly encountered adverse effect1,2. Incidence of hepatotoxicity is 1-2 % with INH alone and increases to 8-10 % when it is combined with rifampicin3. The rate of hepatotoxicity has been reported to be much higher in developing countries like India (8-30%) compared to advanced countries (2-3%) with a similar dose schedule4.

Occurrence of antitubercular drug induced hepatotoxicity is unpredictable and its pathogenesis is unknown. Toxic reactive metabolites generated during hepatic bio-transformation of some of the anti-tubercular drugs are thought to covalently bind with cellular macro-molecules to generate free radicals, which in turn bring about cellular injury1,2. Isoniazid metabolites, acetylhydrazine and hydrazine, have been implicated as the causative hepatotoxins. Oxidative activation of these metabolites in liver by cytochrome P450 (Cyt P450) monooxygenase system generates electrophilic intermediates and free radicals which are capable of causing liver injury in animals5,6. Thus, Cyt P450 enzymes are critical in hepatotoxicity that leads to reactive, toxic metabolites. Rifampicin, a powerful inducer of mixed function oxidase, contributes to hepatotoxicity of INH by enhancing the production of toxic metabolites7,8.

Oxidative stress has been found to be an important mechanism in hepatotoxicity of anti-tubercular drugs in young rats9,10. The altered profile of antioxidant enzymes along with increased lipid peroxidation indicated enhanced oxidative stress in treatment with combination of INH and RMP9,10.

Alpha-tocopherol (Vitamin E) is a potent antioxidant that provides hepatoprotection by scavenging free radicals. In a recent study, we have shown that cimetidine serves as a hepatoprotective agent against INH and RMP induced hepatotoxicity in rabbits11. Keeping this in view, the present study was undertaken to determine the efficacy of α-tocopherol (an antioxidant) in preventing INH and RMP induced liver injury in albino rabbits and to compare it with that of cimetidine (cytochrome-P450 enzyme inhibitor).

Materials and Methods

Animals — Albino rabbits (2-3 kg) of either sex were selected in the present study after prior approval from institutional animal ethical committee and committee for purpose of control and supervision of experiments on animals. The animals were allowed to acclimatize for 7-10 days before start of experiment and were provided with standard diet and free access.
to water throughout the study. Rabbits were chosen for the study because isoniazid induced hepatotoxicity in rabbits is more close to that in human beings. It is a reliable, reproducible, clinically relevant and useful model of isoniazid induced hepatotoxicity\textsuperscript{12,13}.

**Drugs used and experimental design**—Isoniazid, rifampicin (both provided by Lupin Research Lab), cimetidine (Symmedics lab Pvt. Ltd) and tocopherol (Franco Indian Pharmaceuticals Ltd.) were used in the present study. Dose of each drug was given according to the body weight of animals. Isoniazid (50mg/kg) was dissolved in distilled water (1ml/kg). Rifampicin (100mg/kg) was dissolved in distilled water (1ml/kg) and then $p$H was adjusted to 3.0 with 0.1 N HCl to have a clear solution\textsuperscript{14}. Cimetidine (120 mg/kg) was also dissolved in distilled water (1ml/kg) and dilute HCl was added drop by drop till the solution became clear. Tocopherol (50; 100mg/kg) was dissolved in olive oil and the solution was made up to a volume of 1ml/kg. Animals were divided into 5 groups of six animals each and were subjected to either treatment for 7 days. The treatment given to Group I: normal saline (2 ml/kg, po); Group II: INH (50mg/kg, po) + RMP (100 mg/kg, po); Group III: INH (50mg/kg, po) + RMP (100 mg/kg, po) + cimetidine (120 mg/kg, ip); Group IV: INH (50mg/kg, po) + RMP (100 mg/kg, po) + low dose of tocopherol (50 mg/kg, po); and Group V: INH (50mg/kg, po) + RMP (100 mg/kg, po) + high dose of tocopherol (100 mg/kg, ip)

In groups III, IV and V, respective drug injections (ip) were given 30 min before oral administration of INH and RMP.

Blood samples (2 ml) were drawn from lateral ear vein (after mopping the ear with xylene) before drug administration on day 1 in each group and then after completion of drug, administration ie after 7 days. Serum was separated from the blood samples by centrifugation at 2500 rpm and stored at -80°C till analysis (2–4 weeks).

**Assessment of liver damage**

*Biochemical investigations*—Serum was assayed for liver injury by estimating levels of alanine transaminase (ALT or SGPT) by method of Reitman & Frankel\textsuperscript{15} and argininosuccinic acid lyase (ASAL) by method of Campinini et al\textsuperscript{16} with modification as described by Sarich et al\textsuperscript{13}.

*Histopathology*—After 7 days of drug administration, animals were sacrificed by cervical dislocation followed by exsanguination. Livers were dissected out through abdominal incision, weighed and examined for any gross morphological changes. Livers were preserved in buffered formalin (10%) for histopathological examination. Light microscopy was performed after slides were routinely stained with hematoxylin and eosin (H&E). Mainly 4 parameters were taken into consideration–ballooning degeneration, fatty changes, portal inflammation, and necrosis.

**Statistical analysis**—Results are presented as mean ± standard deviation. Within-the-group comparison, (i.e. change from the pretreatment values), for all parameters was done using Wilcoxon matched-pairs test. Comparison among different groups for various parameters was done using Mann Whitney-U test. Statistical analysis of histopathological parameters was done by using Fischer’s Exact test. Karl Pearsons rank correlation coefficient was used to determine the correlation of ASAL and ALT with the extent of liver injury. $P<0.05$ was considered significant.

**Results**

Administration of INH and RMP combination only, showed a significant derangement of liver function as assessed by change in serum enzymes (ASAL and ALT) as well as liver histopathology.

*Effect on food intake and body weight of animals*—INH and RMP treated group showed a significant decrease in food intake and body weight in comparison to control group. Similarly, rabbits pretreated with low dose of tocopherol showed a significant reduction in body weight when compared to control group (Table 1). However, in groups III and V, no significant ($P>0.05$) change in food intake or body weight was found.

*Effect on liver enzymes*—When compared to pretreatment values, INH and RMP treated group showed a significant increase in mean ALT and ASAL levels ($P$=0.02; Table 2). Similarly, in groups III and IV, administration of cimetidine and low dose of tocopherol (50 mg/kg), respectively along with INH and RMP showed increase in ALT and ASAL levels. However, this elevation was significantly less when compared with INH & RMP group. In group V, concomitant administration of tocopherol in high dose (100 mg/kg) along with INH and RMP significantly prevented an increase in the levels of ASAL and ALT as compared to pretreatment levels (Table 2). The biochemical markers also correlate with the results of the morphological changes occurring in hepatocytes. When ASAL and ALT levels were correlated with liver histopathology, it was found that level of serum
ASAL in all the treatment groups was more closely indicative of liver damage (correlation value of 0.8457 for ASAL vs 0.8426 for ALT).

**Histopathological study of liver**

Essentially normal liver morphology was observed in control animals (100%) with no evidence of portal inflammation, ballooning degeneration or fatty change and necrosis. However, histopathological changes were observed in all other treatment groups after one week.

Portal inflammation — In the present study, INH and RMP treated groups showed presence of portal inflammation (Table 3). However, the group treated with high dose of tocopherol showed a significant reduction ($P=0.04$) in inflammation compared to INH and RMP treated group.

Ballooning degeneration — Moderate to severe ballooning degeneration was observed in all the rabbits in INH and RMP group. In other treated groups, all rabbits showed ballooning degeneration, but with lesser severity (Table 3). However, reduction in severity compared to INH and RMP group was statistically significant ($P=0.03$) only in high dose tocopherol treated group.

**Table 1** — Effect of tocopherol and cimetidine on body weight and food intake in isoniazid (INH) and rifampicin (RMP) induced sub acute liver injury in rabbits

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>Body weight (kg)</th>
<th>Food intake (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 8</td>
</tr>
<tr>
<td>Control (normal saline)</td>
<td>2.35 ± 0.16</td>
<td>2.29 ± 0.15</td>
</tr>
<tr>
<td>INH (50) + RMP (100)</td>
<td>2.33 ± 0.26</td>
<td>2.0 ± 0.19*</td>
</tr>
<tr>
<td>INH (50) + RMP (100) + cimetidine (120)</td>
<td>2.55 ± 0.15</td>
<td>2.42 ± 0.17*</td>
</tr>
<tr>
<td>INH (50) + RMP (100) + low dose of tocopherol (50)</td>
<td>2.47 ± 0.12</td>
<td>2.32 ± 0.15*</td>
</tr>
<tr>
<td>INH (50) + RMP (100) + high dose of tocopherol (100)</td>
<td>2.37 ± 0.10</td>
<td>2.28 ± 0.075</td>
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*P values: *=0.02 vs. baseline values (day 0); $a=0.002$, $b>0.05$, $c=0.02$ vs. control group

**Table 2** — Effect of tocopherol and cimetidine on serum ALT and ASAL levels in isoniazid (INH) and rifampicin (RMP) induced sub acute liver injury in rabbits

<table>
<thead>
<tr>
<th>Treatment (mg/kg)</th>
<th>ALT(IU/l)</th>
<th>ASAL(µmole/100ml/hr)</th>
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<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 8</td>
</tr>
<tr>
<td>Control (normal saline)</td>
<td>25.67 ± 5.38</td>
<td>28.93 ± 3.77</td>
</tr>
<tr>
<td>INH (50) + RMP (100)</td>
<td>22.89 ± 5.67</td>
<td>$107.17 ± 45.54*$</td>
</tr>
<tr>
<td>INH (50) + RMP (100) + cimetidine (120)</td>
<td>22.7 ± 4.82</td>
<td>$51.12 ± 25.48*$</td>
</tr>
<tr>
<td>INH (50) + RMP (100) + low dose of tocopherol (50)</td>
<td>27.58 ± 6.1</td>
<td>$60.43 ± 22.45*$</td>
</tr>
<tr>
<td>INH (50) + RMP (100) + high dose of tocopherol (100)</td>
<td>25.08 ± 5.51</td>
<td>$33.15 ± 9.64$</td>
</tr>
</tbody>
</table>

*P values: *=0.02 vs. baseline values (day 0); $a=0.002$, $b<0.05$, $c>0.05$ vs. control group, $d=0.04$, $e=0.002$ vs. INH + RMP + high dose tocopherol group

Fatty change — All the treated groups showed moderate to severe fatty changes except for high dose tocopherol (100 mg/kg) treated group in which none of the rabbits showed fatty changes and this difference was highly significant ($P=0.001$) compared with INH and RMP group.

Necrosis — Moderate to severe necrosis was observed in 5 out of 6 rabbits in INH and RMP group (Fig. 1). However, necrosis was significantly ($P=0.007$) absent in all the rabbits treated with cimetidine and high dose of tocopherol as compared with INH and RMP group (Table 3).

**Discussion**

It is becoming increasingly apparent that many reactive intermediates formed during isoniazid metabolism are free radicals$^{6, 17-19}$. Few studies have shown that oxidative stress is the mechanism of INH and RMP induced hepatotoxicity in experimental animals$^{9, 10}$.

Vitamin E (tocopherol) is a natural antioxidant and extremely effective in detoxifying harmful free radicals in various tissues. It has been shown to inhibit hepatocyte lipid peroxidation caused by free radical forming agents like carbon tetrachloride$^{50}$ and
In the present study, when high dose of tocopherol (100 mg/kg) was given half an hour prior to INH and RMP administration, the levels of ALT and ASAL remained near baseline. Moreover, both reversible as well as irreversible liver changes were prevented (Fig. 2). This reflects the ability of tocopherol to scavenge reactive species within the lipid region of the membrane and thereby, prevent cell injury.

The present study is in agreement with the earlier reports that tocopherol reduced the hepatotoxicity of antitubercular drugs in animals\textsuperscript{22,23}. Skakun\textit{et al.}\textsuperscript{22} have investigated the effectiveness of tocopherol and antihypoxic agents in INH, RMP and pyrazinamide induced hepatotoxicity in rats, and observed hepatoprotection with tocopherol. Kothekar\textit{et al.}\textsuperscript{23} have also revealed that tocopherol was effective in reversing the hepatotoxicity due to INH, RMP and pyrazinamide combination in rats.

Few safe inhibitors of drug metabolism are available for use in man, one of which is cimetidine. In the present study, pretreatment with cimetidine half an hour prior to INH and RMP administration effectively prevented necrosis. Out of all other aforementioned histopathological parameters, necrosis is the most relevant and dependable one, because it is an irreversible change (cell death). Similar findings have been reported with cimetidine in an earlier study by Kalra\textit{et al.}\textsuperscript{11} who have reported absence of necrosis when cimetidine (120 mg/kg) is given to rabbits along with INH and RMP. Similarly, Lauterburg\textit{et al.}\textsuperscript{24} have also reported significant absorption of INH, RMP and pyrazinamide

<table>
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<th>Table 3—Histopathological changes in liver of rabbits after 7 days of treatment in different groups</th>
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<tr>
<td><strong>Histopathological changes</strong></td>
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<tr>
<td><strong>Portal inflammation</strong></td>
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<tr>
<td><strong>Ballooning degeneration</strong></td>
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<tr>
<td><strong>Fatty changes</strong></td>
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<tr>
<td><strong>Necrosis</strong></td>
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Values in parenthesis indicate percentage of rabbits affected [16.6% - 1 out of 6 rabbits; 33.3% - 2 out of 6 rabbits; 50% - 3 out of 6 rabbits; 66.6% - 4 out of 6 rabbits; 83.3% - 5 out of 6 rabbits]

Group I-(control group :saline, 2 ml/kg); Group II (INH, 50 mg/kg + RMP 100 mg/kg); Group III (INH, 50mg/kg + RMP, 100 mg/kg + cimetidine, 120 mg/kg); Group IV (INH, 50mg/kg + RMP, 100 mg/kg + low dose tocopherol, 50 mg/kg); Group V (INH, 50mg/kg + RMP, 100 mg/kg + high dose tocopherol, 100 mg/kg)
decrease in the number of acetylhydrazine induced necrotic hepatocytes in rats, when cimetidine (150 mg/kg) is given 1 h prior to acetylhydrazine administration. However, findings from our study revealed that cimetidine was not effective in preventing reversible liver changes (ballooning degeneration, fatty changes and inflammation) and elevations in the liver enzymes (ALT, ASAL). The present results indicated that cimetidine was as effective as tocopherol (100mg/kg) in preventing liver damage (necrosis), but was less effective in preventing other histopathological and biochemical parameters of liver injury.

Since antioxidants directly target free radicals formed during INH and RMP metabolism which are ultimately responsible for liver cell necrosis, this might explain superior efficacy of tocopherol in comparison to cimetidine (cytochrome P-450 inhibitor) which acts at an earlier step in INH and RMP metabolism pathway.

The enzyme ASAL, which was estimated in the present study, is a very specific and sensitive marker of liver injury. There are very few studies that have correlated ASAL levels with the extent of liver injury. Overall, ASAL levels in different groups correlated positively with ALT levels which concur with the findings of Sarich et al. The major usefulness of ASAL assay for detecting liver disease is based on the fact that its elevation correlates only with the extent of liver disease (whereas ALT levels may also increase during injury to other tissues) and therefore, more closely follows the course of the hepatic disease process compared to ALT levels.

In addition, the plasma ASAL activity assay is especially useful in INH and RMP induced hepatotoxicity in rabbits because plasma samples of INH-treated rabbits often become cloudy, which can interfere with the ALT assay, but not ASAL assay.

It is concluded from the present study that high dose of tocopherol (100 mg/kg) administered along with INH and RMP effectively prevented the hepatotoxicity induced by antitubercular drugs and was more effective than cimetidine (120mg/kg) and low dose of tocopherol (50 mg/kg) in rabbits.

Acknowledgement

We are thankful to Lupin research lab. for providing isoniazid and rifampicin and Synmedics lab Pvt. Ltd. for providing cimetidine. We also extend our gratitude to Franco Indian Pharmaceuticals Ltd. for providing us tocopherol.

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