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LIVER INJURY;
PROTECTIVE EFFECT OF ISONIAZID ON THIONAMIDE INDUCED IN MICE

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ABSTRACT: Methimazole (MMI) is a widely used antithyroid drug for hyperthyroidism. However its clinical use is associated with many deleterious effects including hepatotoxicity. MMI induced liver injury is dependent upon bio-activation to toxic intermediates revealing the important role of drug metabolizing enzymes in generation of this adverse reaction. **Study design:** Randomized controlled laboratory trial. **Period:** 04 months from March 2015 to June 2015. **Settings:** Department of Pharmacology and Therapeutics, Army Medical College, Rawalpindi. **Aim of the study:** The effect of isoniazid (INH) on MMI induced hepatotoxicity was evaluated in mice. **Materials and Method:** Thirty male BALB/c mice were randomly divided into five groups. Group I served as control group (C-I). Group II (C-II) served as control for INH treated group and received plain drinking water for ten consecutive days. Hepatotoxicity was induced by single intraperitoneal injection of MMI at a dose of 1000mg/kg in Group III (MMI).Group IV (INH) received isoniazid (0.1%w/v) in drinking water for ten consecutive days. A separate group V (INH +MMI) of isoniazid pretreated mice was given MMI at eleventh day for determination of combined effect of both drugs. The extent of hepatic damage was determined by estimation of serum ALT and ALP along with histopathological analysis of liver samples. **Results:** MMI resulted in markedly elevated ALT and ALP with hepatic inflammation. INH administration produced no significant change in both serum biomarkers and histopathology appearance. Pretreatment of INH with MMI produced insignificant escalation of liver enzymes and microscopic parameters. However, biochemical and histological comparison of this group with MMI group revealed statistically consequential differences. **Conclusion:** INH has beneficial role in preventing MMI induced hepatic injury.

Key words: Hepatotoxicity, Methimazole, Isoniazid.

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INTRODUCTION

Methimazole (MMI); a thionamide antithyroid has been used for the management of hyperthyroidism since its introduction in 1954.It produces its therapeutic effects by blocking thyroid hormone synthesis through inhibition of thyroid peroxidase.[1] Its greater efficacy, better compliance and superior adverse effect profile has made it the most frequently prescribed antithyroid drug.[2] However, epidemiological studies have shown that 5 % of hyperthyroid patients taking MMI can present with extra-thyroidal toxicities including renal, splenic, pulmonary and hepatic injury.[3] Hepatotoxicity is important due to liver's role in metabolism and detoxificationof xenobiotics. Liver injury produced by MMI can present as hepatitis, cholestasis and acute liver failure. Although the precise mechanism of MMI induced hepatotoxicity requires further investigation, formation of reactive metabolites and induction of oxidative stress have been implicated in this complication. MMI is metabolized by cytochrome P450 and Flavin monooxygenase into N-methylthiourea and glyoxal. These toxic intermediates cause oxidative and carbonyl stress, impair mitochondrial functions and evoke immunological responses.[4]

Growing rates of polypharmacy due to multiple morbidities in the same individual has amplified the risk of CYP mediated drug interactions. However, the possibility of drug interactions will increase for medicines requiring CYP 450 metabolism for their bio- activation. Exploration of these

interactions between concomitantly administered medicines have been carried out by pre-treating research animals with inducers and inhibitors of drug metabolism. Enhanced metabolism based hepatotoxicity has been observed in case of enzyme inducers through increase production of activated metabolites. This is exemplified by augmented toxicity of paracetamol by phenobarbitone induced hepatic enzymes while simultaneous administration of cimetidine with paracetamol proved to be hepatoprotective.[5] However, enzyme inhibitors conferred protection against tissue damage by preventing formation of culprit hepatotoxic intermediates. Similarly portion of MMI to be converted into reactive metabolites is largely dependent upon the amount and activity of metabolizing enzymes.[6] Thus, MMI's hepatotoxic potential can be modified depending upon the nature of concomitantly used medicine (inducer or inhibitor).

Isoniazid (INH) is a first line antituberculous drug effective against organisms of the genus Mycobacterium, specifically M. tuberculosis, M. bovis and M. kansasii. It also has the ability to cause potential drug interactions with a number of drugs whose clearance is dependent on the CYP450 system. It is an established inducer of CYP 2E1 working by increasing de-novo synthesis or decreasing enzyme degradation.[7] This has been demonstrated by enhanced toxicity of acetaminophen from activated metabolites owing to hepatic enzyme induction of CYP2E1 by isoniazid.[8] Acting as a mechanism based inhibitor of CYP1A2, 2A6, 2C19 and 3A4, INH can also elevate the plasma concentration and toxicity of many co-administered drugs like phenytoin, carbamazepine and triazolam.[9]

In this study, we aimed to assess the effects of MMI on hepatic biochemical and histopathological parameters alone and in combination with INH in mice.

MATERIAL AND METHODS

Study place and duration

A randomized controlled trial was carried out from March 2015 to June 2015 at the animal house of Department of Pharmacology and Therapeutics, Army Medical College, Rawalpindi. Blood and histopathological samples were analyzed at Department of Pathology, Army Medical College, Rawalpindi.

Sampling technique and sample size

Thirty animals were initially selected through non-probability convenience method and latter randomly divided into five groups with six animals in each group.

Animals used

Adult male white albino mice weighing 35-40 grams were used in the study and housed under standard husbandry conditions (temperature 20 ± 2^0 C, humidity 40-60% and 12 hour light/dark cycle) with diet and water *ad libitum.* Animal care and research was conducted in accordance with protocols of ethical committee of "Centre of Research in Experimental and applied Medicine (CREAM)". All interventions were carried out after one week of acclimatization of all animals.

Chemicals used

MMI of analytical grade was purchased from Sigma Chemicals USA through a licensed dealer. INH was provided through courtesy of Novartis Pharmaceuticals, Karachi.

Experimental design

Five groups were intervened as follows

Group I (C-1)

Six mice of this group served as control for Group III and received normal saline intraperitoneally.

Group II (C-II)

This group received plain drinking water for ten days and served as control for Group IV

Group III (MMI)

Animals in this group received 1000mg/kg MMI dissolved in normal saline intraperitoneally.

Group IV (INH)

This group was given INH at the dose of 0.1%w/v in drinking water for ten consecutive days.[10]

Group V (INH +MMI)

Mice were pretreated with INH for ten days. At eleventh day, MMI (1000mg/kg) was injected intraperitoneally to these animals.

The study period of Group I and Group III was five hours while that of Group II, IV and V lasted for eleven days.

Markers of hepatic dysfunction

Assessment of serum ALT and ALP

Initial blood sampling of all animals at 0 hour (start of experiment) was done from tail vein[11] and 5 hours after MMI administration, 0.8 -1.5 ml of terminal blood sample was collected by cardiac puncture for Group I and III. The time for cardiac sampling of Group IV was 24 hours after the last administered dose of INH. However, in Group V final blood samples were drawn 5 hours after MMI injection at eleventh day. Collected blood samples were centrifuged to separate serum which was used for estimation of ALT and ALP by commercially available kits.

Histopathology

After cardiac puncture, liver was quickly removed, washed with PBS and stored in labeled container containing 10% formaldehyde. Paraffin embed sections were stained with hematoxylin and eosin for evaluation by histopathologist at 10X and 40X magnification. Periportal or periseptal hepatitis, confluent necrosis, focal lytic necrosis, and portal inflammation were looked for and graded according to Modified Histological Activity Index Grading (Knodells method).[12]

Statistical Analysis

Data was analyzed using SPSS 21. All data was expressed as Mean ± S.E.M. Statistical difference between serum markers at initial and final hours was calculated using students t test. One way ANOVA followed by Post hoc tukey was applied for multiple comparisons between groups. Histopathological results were assessed through Chi Square test. $p<0.05$ was considered significant.

RESULTS

MMI induced hepatotoxic model was standardized in our laboratory using different doses and time interval before animal sacrifice. A single intraperitoneal dose of 1000mg/kg of MMI followed by sacrifice after 5 hours[13], produced marked liver injury as evident by elevated serum ALT and ALP levels and significant histopathological changes.

EFFECT ON SERUM ALT and ALP

Animals of the Group I and Group II showed no signs of liver damage as revealed by an insignificant difference between initial and final levels of ALT and ALP (Table-I).

MMI administration in Group III caused serum ALT and ALP to rise significantly from their baseline values (45.32U/l and 187.5U/l) to 261.33 U/l and 251.17U/l respectively (Table-I).

Comparison of mean ALT and ALP values of MMI treated group at 5 hours with Group I produced p value < 0.05 (Table-II).

When samples of Group IV (INH treated Group) were taken and analyzed after ten days, liver

Biochemical Parameter	Group I (C-I)	Group II (C-II)	Group III (MMI)	Group IV(INH)	Group V (INH+MMI)
Mean Serum ALT(U/L) level at 0 hour	47.32	48.83	45.33	43.17	45.33
Mean Serum ALT(U/L) level at 5 hour	45.17	48.33	261.33	48.5	60.67
P value of within group comparison	0.16	0.36	**0.00***	0.07	0.09
Mean Serum ALP(U/L) level at 0 hour	186.17	182.17	187.5	182.5	182.83
Mean Serum ALP(U/L) level at 5hour	184	188.67	251.17	214.17	213.83
P value of within group comparison	0.25	0.17	**0.03***	0.124	0.06

Table-I. Plasma analysis of all treatment groups, ALT (alanine aminotransferase) ALP (alkaline phosphatase).
* statistically significant values at p < 0.05

Biochemical parameter	Comparison between groups					
	C-I	C-III	C-II	C-IV	C-III	C-V
Final mean ALT(U/L)	45.17	261.33	48.33	48.5	261.33	60.67
P-value	0.00*		0.49		0.00*	
Final mean ALP (U/L)	184	251.17	188.67	214.17	251.17	213.83
P value	0.02*		0.27		0.10	

Table-II. Comparison of ALT and ALP between Experimental Groups
*statistically significant values at $p < 0.05$

enzymes remained within normal range indicating absence of hepatic damage at the administered dose for given duration. The lack of liver injury was further confirmed by an inconsequential result ($p>0.05$) obtained after comparison of biochemical parameter between Group II and Group IV (Table-II). INH pretreatment following MMI single dose prevented the hepatotoxic manifestation as evidenced by an insignificant increase of ALT and ALP from initial values of 45.33 U/l and 182.83 U/l to 60.67 U/l and 213.83U/l (Table-I).

Comparison of final liver enzymes between Group III and Group V produced statistically eloquent difference ($p < 0.05$) unveiling the protective effect of INH against MMI induced hepatotoxicity (Table-II).

HISTOPATHOLOGICAL ANALYSIS

Microscopic examination of Group I and II showed normal lobular organization with hepatic triad consisting of portal vein, branch of hepatic artery and bile duct. Cords of hepatocytes were seen radiating from central venule (Figure-III).

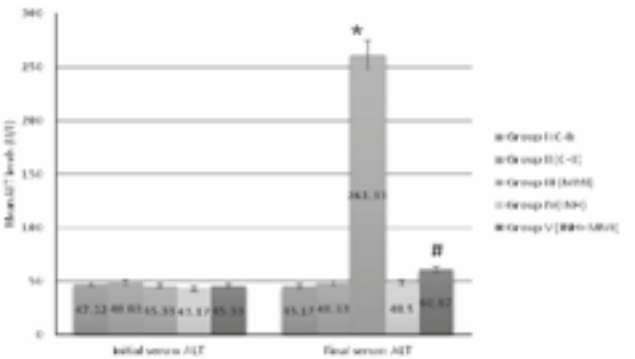

Figure-1. Initial serum ALT (Start of Experiment) and final serum ALT (End of Experiment).
*Significantly ($p< 0.05$) Higher Than Control Group (Group I);
#Significantly ($p< 0.05$) Lower Than MMI Group (Group III)

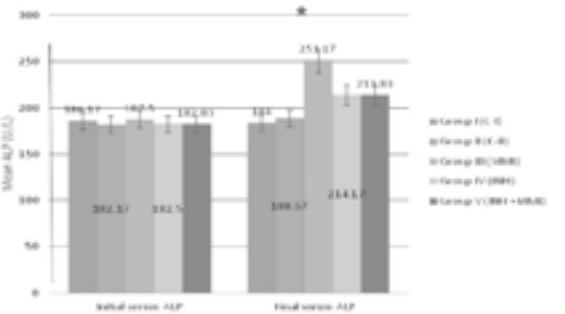

Figure-2. Initial serum ALP (Start of Experiment) and final serum ALP (End of Experiment).
*Significantly ($p< 0.05$) Higher Than Control Group (Group I)

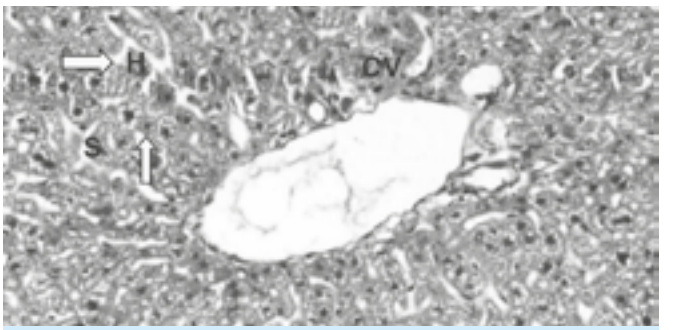

Figure-3. Normal liver specimen of control groups at 40X showing central vein (CV), hepatocyte (H) and sinusoid space (S)

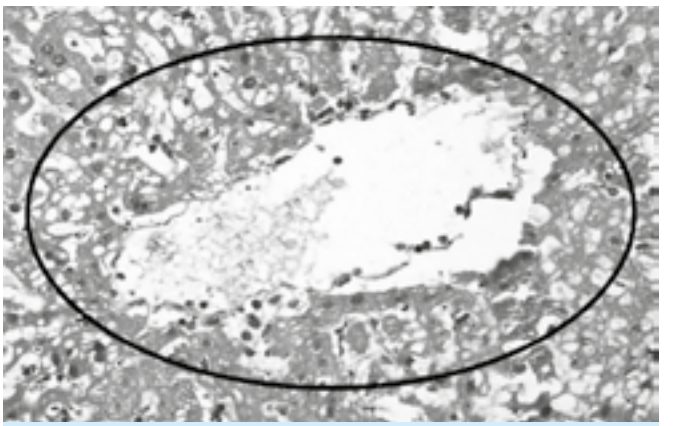

Figure 4. MMI treated group at 40X revealing cellular discontinuity and loss of radial distribution of hepatocytes

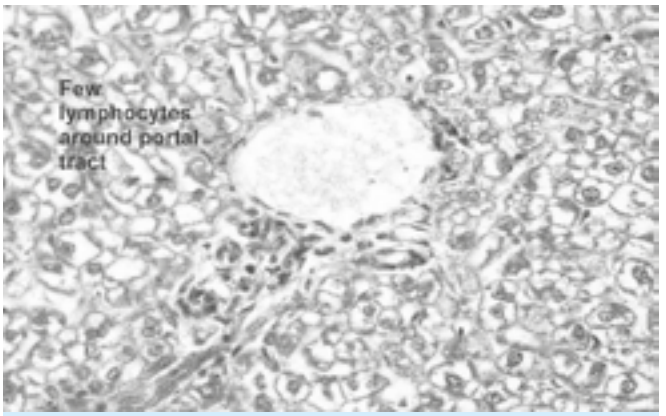

Figure-5. Group IV (INH) liver histology at 40 X showing minimal inflammation around portal tract

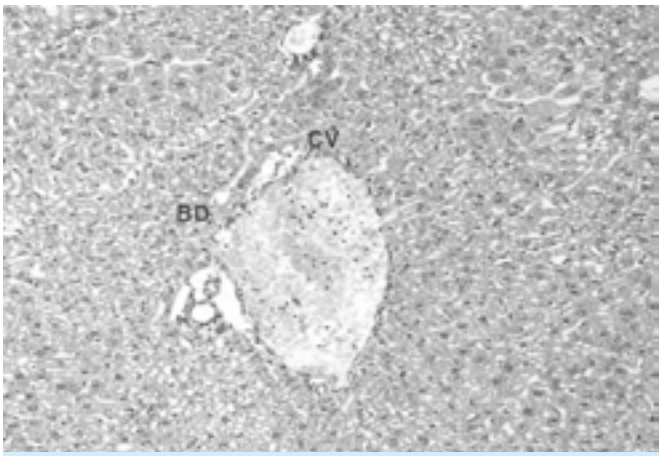

Figure-6. Group V (INH +MMI) showing preservation of hepatic architecture with few inflammatory cells around central vein (CV) and bile duct (BD)

Liver slide microscopy of MMI treated Group III exhibited cellular discontinuity, inflammatory cell infiltrates and marked to moderate portal inflammation (Figure-IV). The comparison with control group I was significant.

INH treatment of Group IV produced minimal inflammatory changes with preservation of liver architecture (Figure-V). *p* value > 0.05 was obtained after comparison with Group II.

Microscopic examination of H & E stained slides of Group V showed that INH pretreatment statistically significantly prevented the necroinflammatory changes induced by MMI (Figure-VI).

DISCUSSION

Drug induced hepatotoxicity is a major challenge for clinicians, pharmaceutical companies and drug regulatory authorities**.** It has become the most common adverse effect responsible for non-approval and withdrawal of approved drugs from market.[14] DILI can take many forms ranging from asymptomatic liver enzyme elevation to acute liver failure requiring transplantation. Eliminating the iatrogenic ''harm'' caused by a therapeutic intent is a priority in clinical care requiring identification of culprit drugs and individuals at risk for DILI.

Thionamide anti-thyroids are among thousands of drugs known to cause liver damage. Single intraperitoneal injection of MMI produced hepatic damage in Group III as indicated by deranged biochemical and histological parameters of hepatic function. Serum ALT and ALP were significantly raised from baseline as well as control Group I. This rise in liver enzymes was reflected by cellular discontinuity and marked inflammatory changes on microscopy. These findings were authenticated by researches of Koyabashi[15] and Heidari and fellows.[16] A 202% increase in ALT as compared to 191% in ALP witnessed by Tashkandi and colleagues substantiated similar preferential elevation of ALT in our study.[17]

Studies have identified CYP 450 induction and drug- drug interactions as modifiable risk factors for drug induced hepatotoxicity.[18] For exploration of impact of these factors, INH was employed for pretreatment therapy due to its CYP2E1 inducing property. A dose of 0.1% w/v for ten days was unable to raise ALT and ALP considerably from control Group II. Minimal inflammatory pathological changes also mirrored results of serum analysis. Insignificant biochemical and histopathological comparison with Group II affirmed that INH was not hepatotoxic at the selected dose and duration. Coherent with our results, Yue and his mates, demonstrated lack of derangement of liver function tests with almost 3 fold increased hepatic CYP 2E1 levels after 10 days administration of INH.[19]

Mizutani claimed evidence that metabolism of MMI by CYP 450 and FMO to N-methylthiourea and glyoxal is a prerequisite for hepatotoxicity.[20]

So role of metabolic pathways and reactive metabolites was evaluated by administration of MMI in INH pretreated mice. To our surprise, there was inconsiderable increase in both aminotransferase from baseline levels, however the level of ALT at the 11th day was eloquently different when compared with Group III (MMI group) final values. INH pretreatment significantly preserved the hepatic architecture and abated the histopathological changes caused by MMI in Group III. Thus, the protective effect of INH was disclosed which was in contrast to its potentiating effect on metabolism based hepatotoxicity of acetaminophen[8] and thioacetamide.[21] Our results also contradicted the findings of Heidari and mates in which they exhibited drastic deterioration of MMI induced liver dysfunction in phenobarbital treated mice.[13] Besides an enzyme inducer, INH can also act a mechanism based inhibitor of P450 1A2, 2A6, 2C19 and 3A4 which may have attributed to its beneficial effect by preventing formation of hepatotoxic metabolites of MMI. This is supported by literature revealing INH preventive role in N-nitrosodimethylamine[22] and halothane[23] induced hepatic damage by acting as enzyme inhibitor.

Thus, the interaction between drugs given concomitantly with MMI is complex and can influence the development and severity of DILI by modifying the level of exposure to hepatotoxic intermediates.

CONCLUSION

It can be concluded from present experimental work that INH holds protective adequacy in MMI induced hepatotoxicity**.** However, the exact underlying mechanism remains to be elucidated further.

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AUTHORSHIP AND CONTRIBUTION DECLARATION

Sr. #	Author-s Full Name	Contribution to the paper	Author=s Signature
1	Dr. Zunera Hakim	Conception of work, acqusition, analysis of data for work, drafting of work with final approval of version	
2	Dr. Akbar Waheed	Revision of drafted work for critical intellectual content	
3	Dr. Bareera Hakim	Revision for intellectual content with approval of final version	
4	Dr. Najam ul Hassan	Revision for intellectual content	