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Original Article

Potentiation of Hepatotoxic Effects of Methotrexate by Isoniazid in Mice; an Explorative Study

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ABSTRACT

Objective: To evaluate the effect of isoniazid on methotrexate induced hepatotoxicity.

Study Design: Laboratory-based experimental study

Place and Duration of Study: Department of Pharmacology Army Medical College, from November 2015 to November 2016.

Material and Methods: Thirty mice were randomly divided into five groups (n=6). Group Ⅰ was given 0.2 ml normal saline by intraperitoneal injection. Group Ⅱ received 0.2 ml of distilled water by oral gavage for 5 days a week for 4 weeks. Group Ⅲ received single intraperitoneal injection of methotrexate 20 mg/kg. Group Ⅳ received Isoniazid 25 mg/kg oral for 5 days a week for 4 weeks. Group Ⅴ received Isoniazid 25 mg/kg oral for 5 days a week for 4 weeks and methotrexate 20 mg/kg i.p after two weeks. Blood samples for measuring serum ALT, AST and ALP along with liver samples for hepatic histological H&E examination were taken after 24 hours of last dose.

Results: Serum ALT, AST, ALP levels were significantly raised in group V (MTX + INH) higher than all other groups exhibiting significant potentiation of MTX induced hepatotoxicity by INH. Histologically group III (MTX) and group IV (INH) showed mild to moderate inflammatory changes whereas group V (MTX + INH) were graded as severe hepatotoxicity with Central vein ischemic changes

Conclusion: It was concluded from results that Isoniazid Potentiates Hepatotoxic effects of Methotrexate in mice as evidenced by histological changes and elevated hepatic enzymes.

Keywords: Isoniazid, Methotrexate, Hepatotoxicity

Introduction

Rheumatoid arthritis (RA) is an autoimmune disorder with the global prevalence of 0.3 to 1.0 percent[1]. RA, as a chronic inflammatory disease can coexist with cardiovascular diseases, depression, lymphoproliferative malignancies and infections especially tuberculosis (TB).

Infections may be responsible for 25 percent of deaths among people with RA. The increased susceptibility of infections is the consequences of immunosuppression which is either due to the disease itself or disease modifying anti-rheumatic drugs (DMARDs) that are used to treat it or both.

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Moreover, TB remains a serious global challenge that accounted for 1.5 million deaths in year 2018[2]despite the availability of effective anti-tuberculous agents and vaccines. Isoniazid (INH) is the agent of first choice for the prophylaxis of latent TB infection (LTBI) but its major adverse effect is liver damage. Methotrexate (MTX) is used along with INH in case of comorbidity of RA or any other autoimmune disease with TB. Concomitant use of INH with DMARDs like MTX poses serious threat to the liver.

Methotrexate (MTX) is a broad spectrum anti-folate that is commonly prescribed for the prophylaxis and treatment of various inflammatory, neoplastic and autoimmune disorders[3, 4]. However, its wide spread use is often limited by its liability to cause liver damage[5]. Hepatotoxicity caused by MTX is due to the enhanced cellular sensitization to reactive oxygen species either by endoplasmic reticulum stress and/or oxidative stress leading to Glutathione (GSH) depletion (ref) Moreover, MTX inhibits NADP malic enzyme and important enzymes of pentose phosphate pathway[67]which are the main sources for the NADPH

production in proliferating cells. When these enzymes are suppressed the production and availability of NADPH is also hampered (Liu et al., 2016). NADPH is normally used up by GSH reductase for the regeneration and maintenance of reduced GSH. When the availability of NADPH is compromised, the activity of GSH reductase is also restrained and hence GSH cycle is inhibited.

Isoniazid (INH) is universally used drug for the prophylaxis and treatment of both latent and active tuberculosis. It is notorious for its hepatotoxic potential leading to hepatitis and progressive liver damage. The mechanism by which INH causes liver damage is unknown, but most possible cause is metabolites of INH especially hydrazine and other metabolites like acetyldiazene, acetylonium ion, acetyl radical or ketene are responsible for oxidative damage with the consequent irreversible cellular damage. Mild elevation of aminotransferases is common, but hepatitis or aminotransferases elevated 3 to 5 times normal warns for the drug withdrawal[8].

As formation of many of hepatotoxic metabolites of INH are dependent upon CYP 450 enzyme system, INH used to interfere with large number of drugs whose metabolism is dependent upon this enzyme system. INH, by inhibiting CYP 1A2, 2A6, 2C19 and 3A4 elevates the plasma concentration and toxicity of co-administered drugs like phenytoin, carbamazepine, vincristine, acetaminophen etc. INH, in addition to enzyme inhibitor, is also the inducer of CYP 2E1 and hence potentiates hepatotoxicity of other hepatotoxins by the mechanism similar to that of ethanol [9]. Its ability to potential acetaminophen and thioacetamide hepatotoxicity by inducing CYP 2E1 has been approved by other studies in human, rodents and rabbits [10]The best marker of hepatic necrosis in murine species especially mice is assessed by considering raised ALT levels , a cytosolic enzyme of liver cells which shows distortion in membrane permeability[11].

Both INH and its toxic metabolite hydrazine deplete hepatic stores of reduced GSH, sensitizing them to oxidative damage. INH generates significant ROS along with lipid peroxidation in GSH depleted cells but not in intact hepatocytes. Hydrazine, however showed enhance ROS generation and lipid peroxidation in both intact and GSH deprived cells. INH and hydrazine both causes exaggerated mitochondrial damage in GSH depleted cells than in intact cells[12].

INH intermediate metabolite acetylhydrazine is oxidized by CYP 2E1 into hepatotoxic metabolites such as acetyldiazene, acetylonium ion, acetyl radical or ketene. INH and its metabolite hydrazine both induces the activity of CYP 2E1 and hence increase the production of hepatotoxins andhepatotoxicity[13]. Induction of this enzyme also enhances ROS production leading to antioxidant deprivation with consequent enhanced cellular sensitivity to oxidative damage. Induction of CYP2E1 induces the hepatotoxic potential of MTX in humans by using ethanol on cultured human hepatocytes[14]. The potential of INH to increase oxidative stress in GSH depleted cells and to induce CYP 2E1 mediated oxidative damage is the rationale behind using INH with MTX in the current study in mice.

The objective of this study is to investigate the potentiation of hepatotoxic effects induced by methotrexate in combination with isoniazid.

Material & Methods

Methotrexate, Isoniazid and other chemicals used were of the analytical grade from standard companies.

The study was conducted according to the principles of the Institute of Laboratory Animal Resources, Commission on Life Sciences, National Research Council (NRC, 1996)and approved by Ethical Committee of Centre for Research in Experimental and Applied Medicine (CREAM) Army Medical College, Rawalpindi. Thirty (30) Balb/c male mice, 8-12 weeks old and weighing 30-40 grams were obtained from NIH (National Institute of Health), Islamabad, Pakistan. All animals were held in the animal house of Army medical college, Rawalpindi, under controlled conditions (20-25 °C temperature and 70 ±15 % humidity). The animals were given standard diet and water *ad libitum.*

The animals were divided randomly into 5 groupscontaining 6 animals each. Group Ⅰ served as control for MTX treated mice and was given 0.2 ml normal saline intraperitoneally. Group Ⅱ served as control for INH treated mice and received 0.2 ml of distilled water by oral gavage for 5 days a week for 4 weeks. Group Ⅲreceived single intraperitoneal injection of methotrexate 20 mg/kg (Tag, 2015). Group Ⅳreceived Isoniazid 25 mg/kg oral for 5 days a week for 4 weeks (Byrne et al., 2007). Group Ⅴ received Isoniazid 25 mg/kg oral for 5 days a week for 4 weeks and methotrexate 20 mg/kg i.p after two weeks. Markers of hepatic injury, ALT, AST, ALP were checked by taking blood samples after 24 hours of last dose. Animals were then sacrificed, dissected and liver specimens were removed. Liver samples were

processed in 10% formalin and cut into 3-micron thick sections using rotary microtome. Tissue sections were then stained with Hematoxylin and Eosin dyes and observed under light microscopy for histopathological changes including cellular necrosis, fatty change, infiltration of lymphocytes and central vein ischaemia using the Ishak Histological Activity Index[15].

SPSS 23 was used for statistical analysis. Results were expressed as mean ± S.E.M. for multiple comparisons of biochemical markers between the groups. One-way ANOVA followed by Post Hoc Tukey Test was applied. The difference between two observations were considered as significant if the *p* value was $\leq$ 0.05.

Results

Markers of hepatic injury including ALT, AST, ALP were conducted by drawing blood samples 24 hours after receiving the last doses. The serum ALT, AST, ALP results were within normal limits for Group I & II (control with i.p N/S,control with oral D.W) as shown in table 1 and Figure-1.

Table-1: Comparative analysis of serum ALT, AST and ALP of different groups of mice.

Comparative Groups	ALT (IU/L)	AST (IU/L)	ALP (IU/L)
Group I (Control with Intraperitoneal Normal Saline)	31.33 ± 3.28	87.83±3.57	94.67±4.91
Group III (MTX)	73.67 ± 3.66	128.50±7.77	315.33±12.44
Between groups *p* value	0.000*	0.002*	0.000*
Group II (Control with Oral Distilled Water)	33.33 ± 2.99	90.00±4.30	100.50±6.41
Group IV (INH)	45.33 ± 3.67	110.17±8.78	131.00±14.90
Between groups *p* value	0.184	0.253	0.325
Group III (MTX)	73.67 ± 3.66	128.50±7.77	315.33±12.44
Group V (MTX + INH)	105. 60 ± 5.40	161.40±8.89	398.00±15.05
Between groups *p* value	0.000*	0.025*	0.000*

p< 0.05 (significant (*)

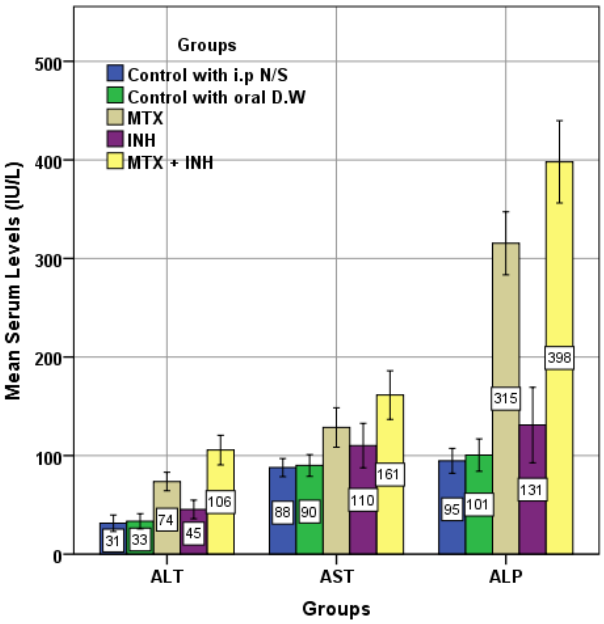

Figure-1: Graph showing comparison among different groups

In group II (MTX) there was significant rise in serum ALT, AST, ALPi.e. 73.67 ± 3.66,128.50 ± 7.77, 315.33 ± 12.44 table 1 and figure 1. In group IV (INH), ALT, AST, ALP were 45.33 ± 3.67,110.17 ± 8.78,131.00 ± 14.90 table 1 and figure 1. Serum ALT, AST, ALP levels were significantly raised in group V (MTX + INH) higher than all other groups exhibiting significant potentiation of MTX induced hepatotoxicity by INH.

Presence or absence of inflammation in hepatic tissue and the degree of severity were assessed at 40Xmagnification (figure 2). The examination of H and E stained slides of all groups were observed. The histopathological findings in all slides of control groups (I and II) remained unremarkable (Figure 2a). In group III (MTX) half of the slides were graded mild (3) and other half (3) showed moderate necro inflammatory changes. However, hepatocytes in all the slides of group III (MTX) showed fatty changes along with nuclear pleomorphism (Figure 2b). In group IV (INH) all slides showed minimum hepatic injury (5) except for the one slide which showed mild changes (Figure 2c). In group V (MTX + INH) two slides were graded as moderate while remaining three were graded as severe hepatotoxicity Central vein ischemic changes were also noted in some of the slides of this group (Figure 2d).

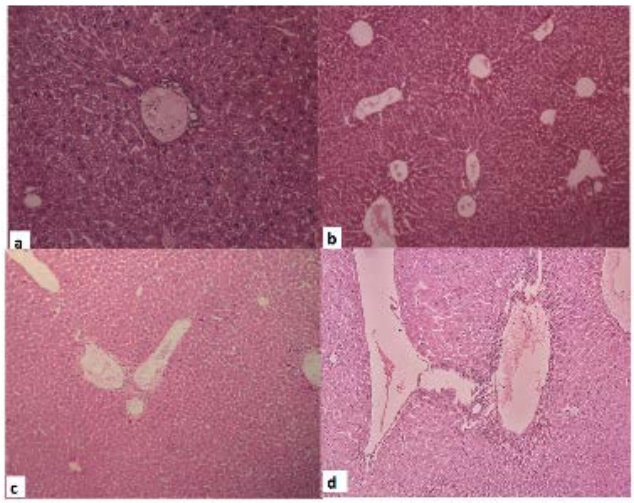

Figure2 : Photomicrograph of the liver tissue, a) group I and II showing normal hepatic architecture (H&E 10X), b) Group III (MTX) showing mild steatosis (H&E 10X), c) Group IV (INH) showing minimal inflammation around central vein (H&E 100X), d) Group V (MTX + INH) showing severe inflammation around central vein (H&E 100X)

Discussion

Hepatotoxicity induced by MTX and the feasible measures to prevent it have been the focus of researchers for many years. However, the concept of using INH with MTX was depicted by various debatable clinical studies in which INH for the prophylaxis of LTBI was given to patients with autoimmune diseases receiving MTX. Studies either favor the concomitant INH chemoprophylaxis with MTX or discourage this combination due to high incidence of hepatotoxicity with concurrent use of two potential hepatotoxins. A retrospective clinical cohort study comprising of 41 and 44 patients with autoimmune diseases especially RA that received both MTX and INH. The one out of 41 (2.44 percent) and five out of 44 patients (11.36 percent) showed raised LFTs 2-3 times ULN.On the basis of low incidence results authors claimed that occurrence of deranged liver enzymes in patients receiving MTX and INH concomitantly is same as in patients receiving MTX or INH alone[16].Vanhoof and colleagues in 2003 disputed the results demonstrating a cohort study of 88 patients depicting deranged liver enzymes in patients taking INH and TNF inhibitors in addition to receiving MTX. patients also presentedwith delayed recovery after hepatic insult in spite discontinuing INH treatment proposing MTX as the main cause for the hepatotoxicity[17,18]. Lack of preclinical trials in the form of animal studies in this regard urged us to explore the hepatic effect of these two potential hepatotoxins and our study suggested hepatic damage as all markers of liver injury were significantly raised in group III as compared to its control Group I with$p< 0.000$.

Histopathological examinationrevealed no signs of cholestasis in spite of elevated hepaticmarkers because of the single therapeutic dose of MTX along with early sample collection. Histopathological findings revealed from mild to moderate hepatic inflammatory changes consistent with other studies[19, 20] which also demonstrated elevation of LFTs in rodents treated with MTX in same dose.

Isoniazid was employed orally in usual non-hepatotoxic dose in animals of group IV. Liver enzymes were not significantly changed when compared with its control, however, minimal inflammation was evident on histopathological analysis. According to American thoracic society, hepatotoxicity with INH alone for latent TB infection (LTBI) in humans is delayed in onset and usually occurs in first 3 months (60 percent) or first 6 months (80 percent) of INH medication. Moreover, onset of symptoms of hepatic insult usually takes at least 16 weeks to appear after the initiation of INH medication [21]. In our study, as the 25 mg/kg dose of isoniazid is nearer to normal therapeutic and prophylactic dose of 5 mg/kg in adult humans, the hepatic effects were less remarkable. In group IV (INH) hepatic markers were not significantly altered as compared to its control group II with $p< 0.560$. This is also in agreement with the study[22] which reported lack of severe hepatic damage in both rats and mice with even at high dose of INH for 5 weeks.

Mice in group Ⅴ received both potential hepatotoxins i.e. MTX and INH. The aim of this group was to assess potentiationof MTX induced hepatotoxicity with INH in usual non-hepatotoxic doses. Highest values were observed for group V (MTX + INH) when it was compared with other groups with $p<0.000$*showing significant potentiation of MTX induced hepatotoxicity by INH.Moreover, to assess the extent of potentiation of MTX induced hepatotoxicity by INH, this group was also compared with group Ⅲ (MTX). The serum ALT, AST and ALP raised significantly when compared with its control and group Ⅲ (MTX). The histopathological assessment of slides on light microscope showed moderate to severe hepatic damage which was significant when compared with its control as well as group Ⅲ (MTX).

To the best of our knowledge no existing animal study and empirical research before this one has ever been

conducted in which both MTX and INH were used together in therapeutic doses for a considerable length of time to investigate the possible interaction between the two drugs. Hence, this study in mice covers this area for the first time.

Conclusion

The study helps in identifying the Potentiating hepatotoxic effects of Methotrexate by Isoniazid in mice.

Conflict of Interest:

This study has no conflict of interest to declare by any author.

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