ORIGINAL ARTICLE

PROTECTIVE EFFECTS OF SILYMARIN AND CHOLCALCIFEROL ON ISONIAZID INDUCED HEPATOTOXICITY IN MICE

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Background: Hepatotoxicity is the gravest concern associated with use of anti-tuberculous drugs. The objective of this study was to evaluate the individual and combined hepatoprotective effect of silymarin and cholecalciferol in isoniazid induced hepatotoxicity. **Methods:** This animal experimental study was conducted at the Department of Pharmacology & Therapeutics, and Multidisciplinary Research Laboratory, Islamic International Medical College, in collaboration with National Institute of Health, Islamabad, Pakistan. Fifty adult Balb-C mice were included in this study. They were distributed into 5 groups. Each group contained 10 mice. Group 1 was normal control, Group 2 disease control, and Group 3, 4, and 5 were experimental groups. Except Group 1, all other groups were given isoniazid (150 mg/Kg) and only Group 2 was not fed with any drugs. Group 3 received silymarin (50 mg/Kg dissolved in physiological saline) through intragastric gavage for 28 days. Group 4 was given Vitamin D (1,000 IU/Kg) for 28 days. Group 5 was given isoniazid (150 mg/Kg) along with silymarin and Vitamin D for 28 days. Serum ALT and bilirubin levels were estimated on day 0, 14, and 28. **Results:** As compared to Group 2, Group 3 to Group 5 showed a lower rise in serum ALT and bilirubin (p<0.001). Group 4 and 5 showed significantly reduced biochemical markers (ALT and bilirubin) (p=0.001). **Conclusion:** Silymarin and cholecalciferol effectively and synergistically ameliorate hepatotoxicity induced by isoniazid. Silymarin offers better hepatoprotection than cholecalciferol in isoniazid induced hepatotoxicity.

Keywords: Silymarin, Hepatotoxicity, Isoniazid, Cholecalciferol

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INTRODUCTION

Tuberculosis (TB) is a leading cause of mortality and morbidity all over the world. Among the countries with the highest burden of TB, Pakistan is ranked 5th.[1] Multi-drug resistant (MDR) tuberculosis is one of the greatest challenges faced, and an incidence of 342 per 100,000 population has been recorded in Pakistan.[2]

Hepatotoxicity is the gravest concern associated with anti-tuberculous drug usage. They remain the leading cause of idiosyncratic hepatotoxicity worldwide. Factors that determine the development of hepatotoxicity depend on the drug regimen used, properties of the cohort under study and the threshold used to define hepatotoxicity along with its reporting and monitoring methodology.[3] Hepatotoxicity secondary to anti-TB drugs has been reported in 5–28% of people treated with anti-TB drugs. Standard treatment of TB is a combination regimen consisting of isoniazid, rifampin, pyrazinamide and ethambutol. The first three drugs are known hepatotoxic agents and drug induced liver injury (DILI) is the most serious adverse effect seen in patients taking anti-TB treatment (ATT).[4] Anti-TB drug induced hepatotoxicity has been defined by the Japanese Society of TB based on levels of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total bilirubin values. DILI is defined as elevation of peak serum aspartate aminotransferase (AST) and/or alanine aminotransferase (ALT) of more than twice the upper limit of normal.[5] Even though the global incidence of tuberculosis is on the decline, the global disease burden is still considerable (~9 million cases and ~1.5 million deaths in 2018), incidence of tuberculosis and drug resistance are rising in some parts of the world like Africa.[3]

Isoniazid and pyrazinamide are well-known hepatotoxic drugs often used in combination. Pyrazinamide (PZA) induces serious liver injury, but the exact mechanism of PZA-induced hepatotoxicity remains controversial. Endoplasmic reticulum stress-caused cell apoptosis plays a critical role in the development of DILI.[6] Isoniazid is metabolized by N-acetyltransferase 2 (NAT-2) to acetylhydrazine and diacetylhydrazine.[7] Diacetylhydrazine is nontoxic and is readily eliminated from the body.[8] Vitamin D, specifically the biologically active vitamin D metabolite 1, 25-dihydroxyvitamin D [Calcitriol: 1, 25 (OH)2D], is known to exert important physiological effects in addition to well-known effects on calcium metabolism. Relation of Vitamin D to the severity of liver disease has been reported among liver disorders other than ATT-Liver Disease.[9] Patients with non-alcoholic steatohepatitis (NASH) have significantly lower levels of Vitamin D. In addition, Vitamin D deficiency is associated with the histopathological severity of

NASH.[10] Animal studies demonstrate that elevated level of Vitamin D using phototherapy results in lower severity of non-alcoholic fatty liver disease as indicated by less necroinflammation, fibrosis, and apoptosis.[11]

Silymarin, a flavonoid from 'milk thistle' (*Silybum marianum*) plant is used almost exclusively for hepatoprotection. Silymarin manifests hepatoprotection by scavenging free radicals, raising the glutathione content, inhibiting lipid peroxidation, and restoring the function of enzymes, thereby generating membrane stabilization and preventing toxic metabolic liver injury.[12] Cholecalciferol is a readily available and essential vitamin involved in a number of physiological processes. It has shown to exert hepatoprotective effects. Experimental models showed that Vitamin D stops the proliferation of main hepatic stellate cells, decreases expression of collagen, and halts thioacetamide-induced fibrosis in liver.[10] According to Hasanain *et al*[13], patients given adjuvant cholecalciferol supplement showed significantly decreased incidence of ATT-Liver Disease as opposed to those without the supplementation (13.3 *vs* 5.3%, p=0.001). The current study compared the effects of vitamin D with a known hepatoprotective agent silymarin. It also aimed to see if a combination of silymarin and vitamin D has synergistic effects.

MATERIAL AND METHODS

This study was performed from Sep 2020 to Aug 2021 in the Department of Pharmacology, Islamic International Medical College after getting approval from the Institutional Review Committee (Approval No. Riphah/IRC/20/247). This study was conducted on a total of 50 male albino Balb/C mice divided into 5 groups each having 10 mice. Sample size was calculated using Resource Equation method.[14] Mice included in study had approximately 30–50 g weight and normal LFTs pre-intervention.[15]

Mice were placed in well-aerated cages for acclimatization, at room temperature of 22±2 °C, and a 12-hour light/dark cycle was maintained. Group 1, the control group, consumed a normal diet and tap water throughout the experiment while group 2, 3, 4, and 5 were given isoniazid in dose of 150 mg/Kg. Group 3 received silymarin also in a dose of 50 mg/Kg dissolved in 0.9% physiological saline solution.[15,16] Group 4 received vitamin D 1,000 IU/Kg for 28 days.[18] Group 5 received both isoniazid and vitamin D in the aforementioned doses along with silymarin for 28 days. On day 0, blood samples were taken for a baseline evaluation, and on day 14, a second sampling was done for the confirmation of hepatotoxicity in groups 2, 3, and 4. The final sampling took place on day 28 of the experiment.[16] BALB/C mice were chosen because the characteristics that these mice possess make them an ideal model for hepatotoxicity studies. Parameters like includes induction of injury to the liver in a huge percentage of these mice, low cost of the animal, as well as easy management.[17]

ALT and serum bilirubin levels were estimated. Serum ALT was estimated by IFCC method. Total bilirubin was estimated according to Calorimetric method. The statistical analysis was done on SPSS-24. Values were expressed as Mean±SD. The statistical significance of the differences of various quantitative changes between the experimental and control groups were evaluated using one-way ANOVA followed by Tukey's Honestly Significant Difference post hoc test for multiple comparisons. The difference was regarded statistically significant at $p \leq 0.05$.

RESULTS

Table-1 and 2 show comparison of mean differences of the groups. The results of group 3, 4, and 5 were compared to group 2. In silymarin plus cholecalciferol treated group 5, there were substantially low levels of ALT and billirubin as compared to group 2, the disease control group. The results also imply that the synergistic effect of silymarin plus cholecaciferol as a treatment tool for hepatotoxicity is greater than the effect of each drug individually in groups 3 and 4 respectively. The significant results are presented and compared with other groups in the Tables below.

Table-1: Post-Hoc Tukey test showing comparison of ALT between groups on day 28

Groups	Mean difference	*p*
1 *vs* 2	119.33	0.000*
1 *vs* 3	27.33	0.000*
1 *vs* 4	33.16	0.001*
1 *vs* 5	19.83	0.965
2 *vs* 3	92.0	0.000*
2 *vs* 4	92.0	0.001*
2 *vs* 5	86.16	0.000*
3 *vs* 4	99.5	0.764
3 *vs* 5	5.8	0.000*
4 *vs* 5	13.33	0.006*

*Significant

Table-2: Post-Hoc Tukey test showing comparison of bilirubin between groups on day 28

Groups	Mean difference	*p*
1 *vs* 2	0.09	0.000*
1 *vs* 3	0.093	0.000*
1 *vs* 4	0.093	0.001*
1 *vs* 5	0.093	0.867
2 *vs* 3	0.093	0.000*
2 *vs* 4	0.093	0.001*
2 *vs* 5	0.093	0.000*
3 *vs* 4	0.093	0.564
3 *vs* 5	0.093	0.000*
4 *vs* 5	0.093	0.006*

*Significant

DISCUSSION

The use of silymarin 100 mg/Kg alone in group 3 and in combination with cholecalciferol in group 5 resulted in prevention of hepatic damage in the mice. This

protective effect was due to its membrane stabilizing function, which keeps intracellular enzymes from leaking out. Because of its antioxidant qualities, it also stimulates phase 2 detoxification pathways. There was a significant difference in the ALT and bilirubin levels between the disease control group 2 and the groups 3 and 5 which received silymarin. These findings are consistent with the study findings of Nasim Ilyas *et al*[16] who demonstrated hepatoprotective effects of silymarin and garlic on isoniazid induced hepatotoxicity.

The decreased levels of ALT and bilirubin of group 3 in our study are supported by Sude Emnizade *et al*[19] who showed that silymarin protects the liver against toxic effects of anti-tuberculosis drugs. They found that silymarin played a hepatoprotective role in hepatotoxicity induced by anti-tuberculous drugs such as isoniazid, rifampicin and pyrazinamide. This effect was demonstrated in a manner similar to our study, with a fall in ALT and bilirubin levels.

Our study showed that silymarin has superior activity in protecting liver against DILI as compared to Vitamin D, as shown by the lower levels of ALT and Bilirubin in group 3 in comparison to group 4. These findings are supported by the findings of Chote Luangchosiri *et al*[21] who conducted a double-blinded randomized controlled trial of silymarin for the prevention of anti-tuberculosis drug-induced liver injury and concluded that silymarin played a protective role demonstrated by lowered ALT levels. Our study shows hepatoprotective effects of silymarin by a fall in ALT and bilirubin levels which was also seen by Reddy MK *et al*[22]. Their study was based on comparing the hepatoprotective effects of silymarin and rutin.

Our findings in group 4 demonstrated that Vitamin D played a hepatoprotective role in INH induced hepatic insult. It also demonstrates that a combination of silymarin and Vitamin D as administered in group 5 is superior in hepatoprotective effects as compared to either silymarin or Vitamin D alone. This finding was supported by a fall in both ALT and bilirubin levels. Therefore these two compounds have a synergistic effect on hepatoprotection (p=0.006) to support these findings we can look at the study conducted by Hasnain AF *et al*[13]. It showed that cholecalciferol played a hepatoprotective role in patients given standard anti-tuberculosis therapy. They found that patients who were given concurrent Vitamin D with ATT therapy had significantly lower levels of aminotransferases as opposed to patients who received ATT alone, especially for ALT.

Wang YQ *et al*[20] recently conducted a study on the deleterious effects of Vitamin D deficiency on acetaminophen exposed mice. Their study concluded that APAP-induced elevations in ALT and AST were exacerbated in mice fed Vitamin D deficient diet. APAP-induced liver necrosis was exacerbated in mice that were fed Vitamin D deficient diet as well. These findings were proved using parameters similar to ours, namely serum ALT and bilirubin examination.

CONCLUSION

Silymarin and cholecalciferol effectively and synergistically ameliorate hepatotoxicity induced by isoniazid. Silymarin offers better hepatoprotection than cholecalciferol in isoniazid induced hepatotoxicity.

RECOMMENDATIONS

Our study is small scale study which involved only 50 mice. Study does not include total protein level, coagulation profile and prothrombin activity. AST level and oxidative stress is not measured in this study. These parameters could not be delved into because of cost and time limitation.

REFERENCES

1. Kurokawa T, Zheng YW, Ohkohchi N. Novel functions of platelets in the liver. J Gastroenterol Hepatol 2016;31(4):745–51.
2. Hasan R, Jabeen K, Mehraj V, Zafar F, Malik F, Hassan Q, *et al*. Trends in *Mycobacterium tuberculosis* resistance, Pakistan, 1990–2007. Int J Infect Dis 2009;13(6):e377–82.
3. Tariq S, Khan TS, Malik S, Anwar MS, Rashid A. Frequency of anti-tuberculous therapy-induced hepatotoxicity in patients and their outcome. J Ayub Med Coll Abbottabad 2009;21(4):50–2.
4. Dheda K, Barry CE 3rd, Maartens G. Tuberculosis. Lancet 2016;387(10024):1211–26.
5. Tahseen S, Khanzada FM, Baloch AQ, Abbas Q, Bhutto MM, Alizai AW, *et al*. Extrapulmonary tuberculosis in Pakistan –A nation-wide multicenter retrospective study. PloS One 2020;15(4):e0232134.
6. Cambau E, Viveiros M, Machado D, Raskine L, Ritter C, Tortoli E, *et al*. Revisiting susceptibility testing in MDR-TB by a standardized quantitative phenotypic assessment in a European multicentre study. J Antimicrob Chemother 2015;70:686–96.
7. Durand F, Bernuau J, Pessayre D, Samuel D, Belaiche J, Degott C, *et al*. Deleterious influence of pyrazinamide on the outcome of patients with fulminant or subfulminant liver failure during antituberculous treatment including isoniazid. Hepatology 1995;21(4):929–32.
8. Metushi IG, Cai P, Zhu X, Nakagawa T, Uetrecht JP. A fresh look at the mechanism of isoniazid-induced hepatotoxicity. Clin Pharmacol Ther 2011;89(6):911–4.
9. Holick MF, Chen TC. Vitamin D deficiency: a worldwide problem with health consequences. Am J Clin Nutr 2008;87(4):1080–6S.
10. Wang X, Li W, Zhang Y, Yang Y, Qin G. Association between vitamin D and non-alcoholic fatty liver disease/non-alcoholic steatohepatitis: results from a meta-analysis. Int J Clin Exp Med 2015;8(10):17221–34.
11. Nakano T, Cheng YF, Lai CY, Hsu LW, Chang YC, Deng JY, *et al*. Impact of artificial sunlight therapy on the progress of non-alcoholic fatty liver disease in rats. J Hepatol 2011;55(2):415–25.
12. Polyak SJ, Morishima C, Lohmann V, Pal S, Lee DY, Liu Y, *et al*, Identification of hepatoprotective flavonolignans from silymarin. Proc Natl Acad Sci USA 2010;107(13):5995–9.
13. Hasanain AFA, Zayed AAH, Mahdy RE, Nafee AMA. Cholecalciferol for prophylaxis against antituberculosis therapy-induced liver disorders among naïve patients with pulmonary tuberculosis: A randomized, comparative study. Int J Mycobacteriol 2017;6(2):149–55.
14. Arifin WN, Zahiruddin WM. Sample size calculation in animal studies using resource equation approach. Malays J Med Sci

2017;24(5):101–5.
15. Jahan S, Danish L, Khan M, Sandhu M, Abrar N, Siddiq R, *et al.* Comparative study of protective effect of silymarin and n-acetyl cysteine on isoniazid induced hepatotoxicity in mice. Iranian J Pharmacol Ther 2019;17:1–10.
16. Ilyas N, Sadiq M, Jehangir A. Hepatoprotective effect of garlic (*Allium sativum*) and milk thistle (silymarin) in isoniazid induced hepatotoxicity in rats. Biomedica 2011;27(2):166–70.
17. Gatera VA, Abdulah R, Musfiroh I, Judistiani RTD, Setiabudiawan B. Updates on the status of vitamin D as a risk factor for respiratory distress syndrome. Adv Pharmacol Sci 2018;2018:8494816.
18. Brewer CT, Kodali K, Wu J, Shaw TI, Peng J, Chen T. Toxicoproteomic profiling of hPXR transgenic mice treated with rifampicin and isoniazid. Cells 2020;9(7):1654.
19. Eminzade S, Uras F, Izzettin FV. Silymarin protects liver against toxic effects of anti-tuberculosis drugs in experimental animals. Nutr Metab 2008;5:18.
20. Wang YQ, Geng XP, Wang MW, Wang HQ, Zhang C, He X, *et al.* Vitamin D deficiency exacerbates hepatic oxidative stress and inflammation during acetaminophen-induced acute liver injury in mice. Int Immunopharmacol 2021;97:107716.
21. Luangchosiri C, Thakkinstian A, Chitphuk S, Stitchantrakul W, Petraksa S, Sobhonslidsuk A. A double-blinded randomized controlled trial of silymarin for the prevention of antituberculosis drug-induced liver injury. BMC Complement Altern Med 2015;15:334.
22. Reddy MK, Reddy AG, Kumar BK, Madhuri D, Boobalan G, Reddy MA. Protective effect of rutin in comparison to silymarin against induced hepatotoxicity in rats. Vet World 2017;10:74–80.

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