Research Article

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Antituberculosis Drug-Induced Hepatotoxicity: Preclinical Benefit of Glutamine

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Abstract

Introduction: The use of rifampicin/isoniazid/pyrazinamide/ethambutol (RIPE) for the treatment of tuberculosis may cause hepatotoxicity. Glutamine (Gln) is an important amino acid with potential cell-regulatory and cytoprotective capabilities.

Objective: This study assessed the ability of Gln to prevent RIPE-induced hepatotoxicity in adult Wistar rats.

Materials and Methods: Thirty adult Wistar rats (both sexes) weighing 200-250 g were used. The rats were randomized into 6 groups of n=5/group and were orally administered with the experimental agents daily for 30 days as follows: Groups 1-3 were administered with ([Control] normal saline, 0.2mL), Gln (80mg/kg) and RIPE (Rifampicin 150, isoniazid/75, pyrazinamide 400 and ethambutol 275 mg/kg), respectively. Groups 4-6 were supplemented with Gln (20mg/kg, 40mg/kg and 80mg/kg) prior to the administration of RIPE, respectively. On day 31, the rats were weighed, anesthetized and blood samples were collected and assessed for biochemical markers. Liver samples were weighed and examined for histology and oxidative stress markers.

Results: RIPE significantly ($p<0.001$) decreased body weight, liver superoxide dismutase, glutathione peroxidase, catalase and glutathione levels when compared to the control. Liver weight, serum lactate dehydrogenase, gamma glutamyl transferase, aminotransferases, alkaline phosphatase, total bilirubin and liver malondialdehyde levels increased significantly ($p<0.001$) in RIPE -administered rats when compared to the control. RIPE caused hepatocelluar necrosis and steatosis in the liver of rats. However, the aforementioned RIPE-induced changes were mitigated in a dose-related fashion by Gln (20, 40 and 80 mg/kg) supplementation. Also, Gln supplementation restored liver histology.

Conclusions: Gln may be effective for the treatment of RIPE related hepatotoxicity.

Keywords: Antituberculosis drug, glutamine, liver, mitigation, toxicity

Introduction

The liver is a vital and primary organ involved in numerous functions including the detoxification and removal of waste products. [1] The metabolism of drugs takes place largely in the liver, which accounts for the organ's susceptibility to metabolism-dependent, drug-induced hepatotoxicity. Drug associated hepatotoxicity accounts for about one-half of the cases of acute liver failure and mimics all forms of acute and chronic liver diseases. [2 3] An estimated 1000 clinically used drugs have hepatotoxic potential. [4] The pathogenesis of drug-induced hepatotoxicity usually involves the participation of a drug and/or its metabolites, which affects cell biochemistry or stimulates an immune response. Each hepatotoxic drug is associated with a characteristic signature concerning the pattern of injury and latency. [5] However, some drugs may have more than one signature. Drug-induced hepatotoxicity could be characterised by unpredictable and idiosyncratic reactions, which may occur on a background of an increased rate of mild asymptomatic liver injury that may be difficult to recognize. [5]

Isoniazid/rifampicin/pyrazinamide/ethambutol (RIPE) is a frequently used antituberculosis drug combination. It has significantly reduced the health menace associated with TB infection in endemic regions .[6] However, RIPE is one of the known groups underlying idiosyncratic hepatotoxicity worldwide. [7] RIPE may cause hepatotoxicity in 5%–28% of tuberculosis (TB) patients on therapy. [8] Hepatotoxicity related to RIPE has been attributed to isoniazid [9] which can be aggravated by rifampicin. [10] Also, pyrazinamide intermediaries (pyrazinoic acid and 5- hydroxy pyrazinoic acid) can potentiate isoniazid associated hepatotoxicity. [11] Hepatotoxicity caused by RIPE is often accompanied by notable changes in serum liver markers [6] and hepatocellular changes such as necrosis, apoptosis and steatosis. [7] The precise mechanism of RIPE-induced hepatotoxicity is not clear, but may involve oxidative stress marked by lipid peroxidation and inflammation. [7]

L-glutamine (Gln) is an important amino acid that accounts for 60% of free amino acids in the body. [12] It has been associated with lots of vital physiological functions. It is a primary energy supply substance for mitochondria to produce adenosine triphosphate. [13]Gln plays essential functions in nitrogen and carbon skeleton exchange in different tissues, where it regulates many biochemical functions.[14] It has immunoregulatory and cell-regulatory

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capabilities, as reported in recent investigations. [15] Gln is essential for glutathione synthesis in tissues including the liver where it functions as a rate-limiter. [16] Glutathione is a potent ubiquitous antioxidant, which inhibits oxidative stress and is vital for drugs and endogenous substance metabolisms. [17, 18] In addition to the aforementioned activities, Gln has shown beneficial effects in diabetes, cancer, and neurodegenerative diseases. [13] It has been shown to reduce liver ischemia, reperfusion injury and alcohol-induced liver damage in experimental studies. [12] This study assessed the protective ability of Gln against RIPE-induced hepatotoxicity in Wistar rats.

Materials and Methods

Animals, drugs and experimental design

Animals: Adult Wistar rats (both sexes) weighing 200-250 g were procured from the animal house of the Department of Pharmacology, Faculty of Clinical Sciences, University of Port Harcourt, Rivers State, Nigeria. The rats were acclimated for 2 weeks under 12 h light: 12 h dark cycle at 25±2°C in the Department of Pharmacology/Toxicology, Faculty of Pharmacy, Niger Delta University, Nigeria where the study was performed.

Drugs/Chemicals: Isoniazid/rifampicin/pyrazinamide/ethambutol tables (RIPE) used were manufactured by Lupin Limited Chikalthana, Aurangabad India. Gln used was purchased from Qualikems Fine Chemical Private Limited, Gujarat, India. All other chemical compounds used were of analytical grades. RIPE (Rifampicin 150, isoniazid 75, pyrazinamide 400 and ethambutol 275 mg/kg) [19] and modified doses of Gln (20, 40, 80 mg/kg) [20] were used for the study.

Experimental design: Thirty adult Wistar rats (both sexes) weighing 200-250 g were randomized into 6 groups of n=5/group. The rats were orally administered with the experimental agents daily for 30 days as follows: Groups 1-3 were adminstered with the vehicle ([Control] normal saline, 0.2mL), Gln (80mg/kg) and RIPE (Rifampicin 150, isoniazid/75, pyrazinamide 400 and ethambutol 275 mg/kg), respectively. Groups 4-6 were supplemented with Gln (20mg/kg, 40mg/kg, 80mg/kg) prior to the administration of RIPE, respectively. On day 31, the rats were weighed and subjected to light diethyl ether anaesthesia and blood samples (5mLs) were collected in non-heparinized tubes. The blood samples were (centrifuged at 3000 rpm for 15 minutes) and sera were collected and assessed for biochemical markers. Subsequently, the rats were dissected, liver tissues were collected cleaned and weighed. Liver tissues were rinsed in cold saline and homogenized in phosphate buffer. The homogenates were centrifuged (3000 rpm for 15 minutes) and the supernatants were decanted and assayed for oxidative stress markers. Also, liver tissues were collected and fixed in 10% neutral buffered formalin for histological study.

Ethical consideration

This study was approved with the number NDU/PHARM/PCO/AEC/078 by the Research Ethics Committee of the Department of Pharmacology/Toxicology, Faculty of Pharmacy, Niger Delta University, Nigeria. The guide for the care and use of laboratory animals, 8th edition was used for the study.

Biochemical and histological evaluations

Biochemical markers: Serum lactate dehydrogenase (LDH), gamma glutamyl transferase (GGT), aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), and total bilirubin (TB) were measured using standard laboratory reagents.

Liver oxidative stress maker assay: Malondialdehyde (MDA) was estimated using the procedure described by Buege and Aust, 1978. [21] Superoxide dismutase (SOD) was assayed as explained by Sun and Zigman, 1978. [22] Glutathione peroxidase (GPx) was assessed using the protocol described by Rotruck *et al*., 1973. [23] Catalase (CAT) was assayed as described by Aebi 1984. [24] Glutathione (GSH) was measured using the method reported by Sedlak and Lindsay 1968. [25]

Liver histology: The liver tissues were fixed in 10% neutral buffered formalin for 24h and dehydrated in ascending concentrations of ethyl alcohol solution. Liver tissues were processed and imbedded in paraffin block. Liver sections (3-4µm) were obtained from the paraffin blocks, mounted on slides and stained (hematoxylin-eosin). Stained sections were examined using a microscope (Nikon, Eclipse E200-LED, Tokyo, Japan).

Statistical analysis

Data as mean values with standard error of mean. Analysis of variance (ANOVA), complimented by Tukey's *post-hoc test* were used for data analysis with the aid of GraphPad Prism version 4.03 (GraphPad software Inc., San Diego, CA, USA). *P* values <0.05, <0.01 and <0.001 were considered significant.

Results

Effect of glutamine on body and liver weights of RIPE-administered rats

Administered Gln (80mg/kg) had no significant ($p>0.05$) effects on the body and liver weights whereas RIPE decreased body weight and increased liver weight significantly at $p<0.01$ when compared to the control **(Table 1).** But Gln supplementation significantly restored the body and liver weights at 20mg/kg ($p<0.05$), 40mg/kg ($p<0.01$) and 80 mg/kg ($p<0.01$) when compared to RIPE **(Table 1).**

Table 1: Effects of glutamine on the body and liver weights of RIPE-administered rats

Treatment (mg/kg)	FBW (g)	ALW(g)	RLW (%)
Control	290.9±20.2	6.56±0.54	2.26±0.12
Gln 80	297.6±20.4	6.37±0.32	2.14±0.27
RIPE	162.1±17.5*	10.01±0.45*	6.18±0.43*
Gln 20 + RIPE	200.3±19.4 [a]	8.21±0.32[a]	4.10±0.16 [a]
Gln 40 + RIPE	260.6±20.8 [b]	6.88±0.56 [b]	2.64±0.09[b]
Gln 80 + RIPE	271.5±17.8 [b]	6.56 ±0.98[b]	2.42±0.41[b]

Gln: Glutamine, RIPE: Rifampicin/isoniazid/pyrazinamide/ethambutol, FBW: Final body weight ALW: Absolute liver weight, RLW: Relative liver weight, Data as mean ± SEM, n=5, *p<0.01 Significant difference when compared to control, a p<0.05 and bp<0.01 Significant difference when compared to RIPE. SEM: Standard error of mean, ANOVA (Analysis of variance)

Effect of glutamine on serum biochemical markers of RIPE-administered rats

Serum AST, GGT, ALP, TB, ALT and LDH levels did not differ (p>0.05) from the control in Gln (80 mg/kg) administered rats, but were increased significantly (p<0.001) in RIPE- administered rats when compared to the control **(Table 2).** However, AST, GGT, ALP, TB, ALT and LDH levels were restored by Gln supplementation at 20 mg/kg (p<0.05), 40 mg/kg (p<0.01) and 80 mg/kg (p<0.001) when compared to RIPE **(Table 2).**

Effect of glutamine on liver oxidative stress markers of RIPE-administered rats

The administration of Gln (80 mg/kg) had no significant (p>0.05) effects on liver GPx, CAT, GSH, SOD and MDA levels when compared to the control. Administered RIPE decreased GPx, CAT, GSH, and SOD, but increased MDA levels significantly at p<0.001 when compared to the control (**Table 3).** Nonetheless, Gln supplementation restored liver GPx, CAT, GSH, SOD and MDA levels at 20mg/kg (p<0.05), 40mg/kg (p<0.01) and 80 mg/kg (p<0.001) when compared to RIPE **(Table 3).**

Effect of glutamine on liver histology of RIPE-administered rats

Normal liver histology was observed in the control rats **(Figure 1a),** but hepatocyte necrosis, inflammatory cell infiltrations **(Figure 1b)** and steatosis **(Figure 1c)** were noted in RIPE- administered rats. Steatosis **(Figure 1 d)** and inflammatory cells infiltration **(Figure 1e)** were observed in rats supplemented with Gln (20 mg/kg), and (40 mg/kg), respectively whereas normal liver histology **(Figure 1f)** was observed in rats supplemented with Gln (80 mg/kg).

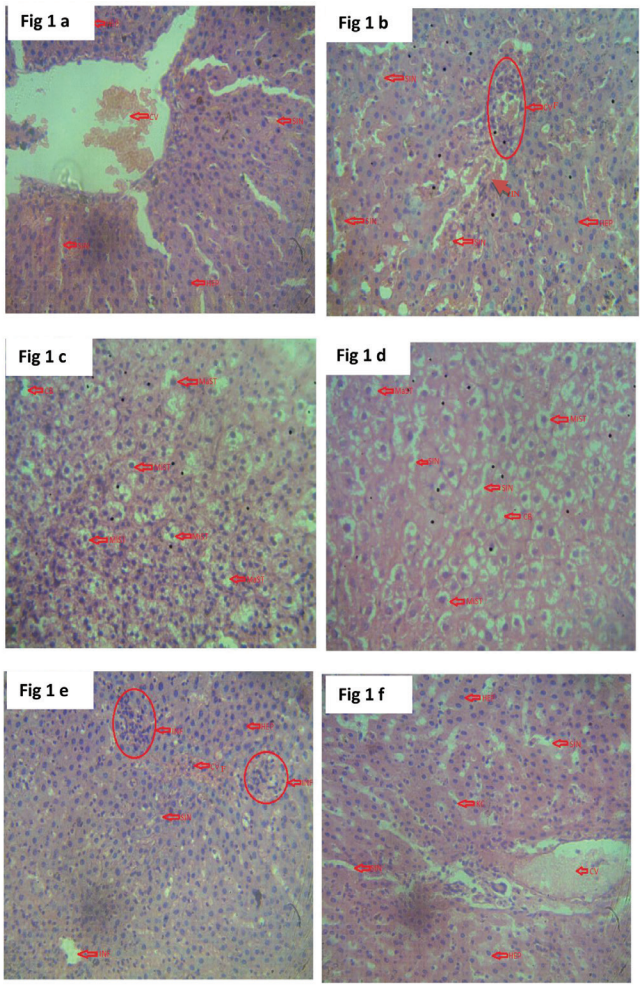

Figure 1 a-f: are liver micrographs. **Figure 1a:** Control, Figures b and c: RIPE administered rats, **Figures d-e:** Supplemented with glutamine (20 mg/kg), glutamine (40 mg/kg) and glutamine (80 mg/kg). **SIN:** Normal Sinusoids, **HEP:** Normal hepatocytes, **HN:** Hepatocyte necrosis, **CVF:** Central vein with inflammatory cells, **HV:** Hepatic vein, **CV:** Normal central vein. **INF:** Inflammatory cells, **MIST:** Microvesicular steatosis, **MAST:** Macrovesicular steatosis, **CB:** Councilman body, KC; Kuffer cells. X 400 (Hand E)

Table 2: Effect of glutamine on serum liver biomarkers of RIPE-administered rats

Treatment (mg/kg)	AST (U/L)	ALT(U/L)	ALP(U/L)	TB(g/dL)	LDH(U/L)	GGT(U/L)
Control	38.20±2.86	35.80±2.07	45.82±4.86	8.40±0.60	25.45±2.34	0.65±0.01
Gln 80	37.81±3.87	35.61±3.87	43.00±5.21	7.71±0.45	23.72±3.62	0.68±0.06
RIPE	141.02±12.7*	149.00±12.0*	184.20±13.7*	26.43±3.41*	110.66±18.1*	2.23±0.04*
Gln 20 + RIPE	95.22±9.12[a]	99.31±10.6[a]	123.21±17.8[a]	17.80±1.54[a]	70.05±7.21[a]	1.61±0.09[a]
Gln 40 + RIPE	71.20±8.13[b]	72.20±7.13[b]	80.00±6.35[b]	13.41±1.51[b]	48.10±5.72[b]	1.13±0.05[b]
Gln 80 + RIPE	44.12±4.09[c]	38.27±4.73[c]	56.20±6.99[c]	9.00±0.37[c]	27.52±3.54[c]	0.71±0.08[c]

Gln: Glutamine, RIPE: Rifampicin/isoniazid/pyrazinamide/ethambutol, AST: Aspartate aminotransferase, LDH: Lactate dehydrogenase, ALT: Alanine aminotransferase, GGT: Gamma-glutamyl transferase, ALP: Alkaline phosphatase, TB: Total bilirubin, n=5, Data as mean± SEM, *p<0.001 Significant difference when compared to control, ap<0.05, bp<0.01 and c p<0.001 Significant difference when compared to RIPE. SEM (Standard error of mean), ANOVA (Analysis of variance).

Table 3: Effect of glutamine on liver oxidative stress markers of RIPE-administered rats

Treatment (mg/kg)	CAT (µg/mg protein)	SOD (u/mg protein	GSH (u/mg protein)	GPx (u/mg protein)	MDA (nmol/mg protein)
Control	33.91 ± 3.05	25.42 ± 2.01	23.20± 3.83	35.41 ± 3.54	0.16 ± 0.07
Gln 80	34.21 ± 3.30	27.69 ± 3.12	23.41 ± 2.91	37.05 ± 2.93	0.15 ± 0.01
RIPE	12.60 ± 2.28*	7.01 ± 0.68*	5.60 ± 0.29*	13.61 ± 0.37*	0.98 ± 0.03*
Gln 20 +RIPE	16.81 ± 1.58a	11.20 ± 1.64a	8.41 ± 0.43a	18.86 ± 1.33a	0.58 ± 0.07a
Gln 40+ RIPE	22.80 ± 3.66b	15.31 ± 1.37b	12.60 ± 0.24b	25.01 ± 3.25b	0.32 ± 0.06b
Gln 80+ RIPE	30.89 ± 4.37c	23.22± 3.67c	20.98 ± 3.71c	32.21 ± 4.74c	0.19 ± 0.06c

Gln: Glutamine, RIPE: Rifampicin/isoniazid/pyrazinamide/ethambutol, CAT: Catalase, SOD: Superoxide dismutase, GSH: Glutathione, GPx: Glutathione peroxidase, MDA: Malondialdehyde, n=5, Data as mean ± SEM (Standard error of mean), *p<0.001 Significant difference when compared to control, a p<0.05, bp<0.01, and c p <0.001 Significant difference when compared to RIPE, ANOVA (Analysis of variance)

Discussion

Drug-induced hepatotoxicity is the leading cause of liver injury and acute liver failure in the world. [26] In TB therapy, RIPE associated hepatotoxicity is a significant adverse effect, which manifests with a broad signs of clinical features, from altered serum liver biochemistry to liver failure. [7] Gln, an essential amino acid used by several cell types, including hepatocytes showed cytoprotective activity in animal models. [27] The current research assessed the protective effect of Gln on RIPE-induced hepatotoxicity in adult rats. In toxicity studies, the measurements of organ and body weights are imperative. [28] In this study, RIPE notably decreased body weight and increased liver weight in the treated rats. This is consistent with the reports by Naji *et al.* [29] on altered organ weight in anti-tuberculosis drug administered rats. The decreased body weight might be due to decreased appetite whereas increased liver weight might be due to inflammation induced by RIPE. [29] However, Gln supplementation restored body and liver weights in dose-related manner. This might be due to increased appetite [30] and decreased liver inflammation. [27] Hepatotoxicity is recognized by abnormal liver biochemistry characterized by altered serum AST, ALT, ALP, GGT, TB and LDH with or without clinical symptoms. [31] In the RIPE-administered rats, this study observed elevated serum AST, ALT, ALP, GGT, TB and LDH levels. This is in agreement with the observations by Naji *et al.,* [29] who reported elevated levels of the aforementioned biochemical markers in rats administered with anti-tuberculosis drug. The elevated serum biochemical markers may be related to enhanced susceptibility and damage of the hepatocyte cell menbrane caused by RIPE leading to increased activities of serum biochemical markers. [29] But Gln supplementation, in a dose-related fashion restored serum biochemical markers. It can be suggested that Gln might have restored serum biochemical markers by maintaining liver plasma membrane integrity thus inhibiting the leakage of biochemical markers via the membrane. Recognizing the pattern of liver injury is vital. It helps establish a differential diagnosis and guide diagnostic evaluation. Assessing the pattern of liver injury is based on which liver enzyme elevation predominates. In this study, elevated ALT and AST levels were more prominent than ALP, which suggests hepatocellular injury. Similarly, Nagvi and others reported hepatocellular injury as the prominent pattern of liver injury associated with anti-tuberculosis drugs. [32]

Reactive oxygen species (ROS) which are by-product of normal metabolism have functions in cell signalling and homeostasis and are regulated by antioxidants. The regulation of cellular levels of ROS by antioxidants prevents their reactive nature from causing damage to key cellular components (DNA, protein, and lipids). But when the cellular antioxidant capacity is overwhelmed by ROS, oxidative stress occurs. Oxidative stress impacts have been linked with drug-induced toxicities including hepatotoxicity. [33] In this study, RIPE caused remarkable oxidative stress heralded by depleted liver antioxidants (GSH, CAT, GPx and SOD). The observation agrees with the findings by Sahu *et al.* [19] who reported depleted liver antioxidants in anti-tuberculosis drug administered rats. Studies have related depleted liver antioxidants to the metabolites of anti-tuberculosis drugs. Rifampicin induces CYP2E1, a member of the cytochrome P450 family, which facilitates the biotransformation of isoniazid to hydrazine its toxic metabolite. Hydrazine then reacts with the sulfhydryl content of GSH, depleting GSH and other antioxidants, thus exposing the liver to oxidative stress. [34] Rifampicin can increase isoniazid biotransformation to isonicotinic acid which is hepatotoxic. [35] Also, the intermediaries (pyrazinoic acid and 5- hydroxy pyrazinoic) of biotransformed pyrazinamide have been shown to cause oxidative stress. [11] Nonetheless, Gln supplementation restored liver antioxidant levels in a dose-related fashion. This may be related to the ability of Gln to inhibit the induction of oxidative by RIPE and/or its metabolites. Gln may have also increased liver antioxidant capacity especially GSH, because it is the rate-limiter for the production of GSH. [16]

Lipid peroxidation is a product of ROS action on polyunsaturated fatty acids. It is a chain phenomenon that begins with ROS giving an electron to a methylene

carbon in a polyunsaturated fatty acid, which reacts with molecular oxygen to form a peroxyl radical. LPO results in damage to membrane, inhibition of enzymes and cross-linking of protein-protein, which can cause cell death. [36] In this study, MDA, a primary LPO yardstick was elevated in the liver of RIPE administered rats. Similarly, Liu *et al.* [37] reported increased liver MDA level in anti-tuberculosis drug administered rats. Liu *et al.* [37] stated that this may be related to anti- tuberculosis drug-induced generation of ROS mediated by cytochrome P450 2E1 (CYP2E1) causing the oxidation of liver polyunsaturated fatty acids leading to LPO. However, Gln supplementation decreased LPO marked by restored liver MDA levels in a dose-related fashion. Gln might have inhibited the generation of ROS, thus preventing the oxidation of liver polyunsaturated fatty acids by RIPE.

Histology is an essential technique for evaluating the impact of test samples at tissue level. [38] In this study, histological study of the liver showed hepatocellular necrosis and steatosis in RIPE-administered rats. This is consistent with earlier liver morphological changes reported in anti-tuberculosis drug-administered rats by Saraswathy *et al.* 1998. [39] But various doses of Gln restored liver histology. Gln restored liver histology probably by inhibiting RIPE and /or metabolites from the induction of liver oxidative stress, thus preventing the damage of cellular contents (DNA, lipids and proteins).

Conclusion

Gln supplementation, in a dose-related fashion prevents RIPE-induced hepatotoxicity by restoring serum biochemical markers, liver oxidative stress markers and histology in rats.

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