Randomised post-test-only study of glutathione and ursodeoxycholic acid combination therapy on liver function in cholestasis-induced rats

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ABSTRACT

Introduction: Cholestasis is bile flow disruption that leads to bile accumulation, which could lead to liver fibrosis. Ursodeoxycholic acid (UDCA) has a hepatoprotective effect. Glutathione (GSH) is an endogenous antioxidant that plays a role in maintaining the function and structure of liver cells. This study aimed to examine the effect of UDCA-GSH combination therapy in multiple doses on liver function in the Sprague-Dawley rats' liver fibrosis model.

Materials and Methods: This was a randomised post-test-only study. A total of 28 rats were assigned into four groups: Group 1 is control group (C), samples had bile duct ligation and UDCA monotherapy 20 mg; Group 2, bile duct ligation + UDCA 10 mg + glutathione 10 mg (P1); Group 3, bile duct ligation + UDCA 20 mg + glutathione 15 mg (P2); Group 4, bile duct ligation + UDCA 30 mg + glutathione 20 mg (P3). Serum AST, ALT, ALP activity, total, direct and indirect bilirubin were collected. Shapiro-Wilk test was used for the normality test. All groups' data were compared using Kruskall-Wallis and Mann-Whitney tests.

Results: There was a significant difference in the ALP level in all rats and between the C and P2 groups. ALP level of all groups decreased significantly compared to the control group. Combination therapy group showed lower bilirubin levels. ALT levels significantly differed between the C-P1, P1-P2, and P1-P3 groups.

Conclusion: UDCA-GSH therapy improves liver function in BDL rats' models compared to UDCA monotherapy.

KEYWORDS:
Bile duct ligation, UDCA, glutathione, liver function

INTRODUCTION

Cholestasis is bile flow disruption that leads to the accumulation of bile in the blood, and the liver could lead to liver fibrosis. Pregnancy intrahepatic cholestasis, tumours, gallbladder stones, primary sclerosing cholangitis (PSC), and biliary atresia are the most common causes of cholestasis.[1,2] Hepatocyte tissue scarring is responded with cholangiocytes and hepatocytes by inducing the fibrosis process of periductal, biliary fibrosis, and liver cirrhosis.[3]

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) are hepatocellular injury markers. ALT is a cytosolic enzyme that is found in high concentrations in the liver. ALT is usually higher than AST in most types of liver disease in which the activity of both enzymes is predominantly from the hepatocyte cytosol. Hepatocellular injury triggers the release of these enzymes into circulation.[4] Biochemical markers of cholestasis include elevated serum alkaline phosphatase (ALP) and gamma-glutamyl transferase (GGT) levels.[5] These enzymes are located in the plasma membrane of hepatocytes. As bile acids accumulate in the liver, they act as detergents, releasing enzymes from the plasma membrane of hepatocytes.[5] Highly increased ALP, GGT, ALT and AST levels indicate obstruction of cholestatic liver disease.[6,7]

It has been hypothesised that oxidative stress may play a role in liver damage through various biological pathways. Bilirubin protects against oxidative stress by inhibiting the action of NADPH oxidase, which increases superoxide production. Moreover, bilirubin can quickly clear up peroxyl radicals, singlet oxygen, and hydroxyl radicals reactive nitrogen varieties[8,9] and minimise the alpha-tocopherol radical that promotes recycling in association with vitamin E.[10] In inclusion, bilirubin may have anti-inflammatory attribution and work as the significant anti fibrogenic agent through heme oxygenase-1 (HMOX1).[11]

Management of cholestasis involved ursodeoxycholic acid (UDCA), the lowest hepatotoxic profile among endogenous bile acids. UDCA has a hepatoprotective effect and pushes down the fibrotic rate of the liver. UDCA works through choleretic, immune system modulation, and cryoprotection mechanisms.[12] Its hydrophilic properties prevent hepatocyte damage due to bile acid accumulation.[13] In vitro study showed the hepatoprotective effect of UDCA in the amoxicillin-clavulanate hepatotoxic induced rat model.[14] However, UDCA lacks antioxidant properties, which is very important due to oxidative-stress induced fibrosis which is very common in cholestasis. Therefore, UDCA-antioxidant combination therapy needs to be studied.

Glutathione (GSH) is an endogenous antioxidant that plays a role in maintaining the function and structure of liver cells. Besides its antioxidant properties, GSH modulates cell growth

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Table I : Descriptive analysis and Kruskall-Wallis test

Variable	Group	Mean ± SD	Median (Min – Max)	p
ALP	C	1134.86 ± 71.43	1154 (1009 – 1211)	0.025*
	P1	925.14 ± 384.07	936 (337 – 1450)	
	P2	578.14 ± 194.20	449 (388 – 869)	
	P3	972.86 ± 313.34	1187 (573 – 1253)	
Total bilirubin	C	1.97 ± 2.48	0.5 (0.4 – 5.6)	0.077
	P1	1.18 ± 2.16	0.4 (0.3 – 6.1)	
	P2	2.14 ± 3.11	0.4 (0.2 – 6.8)	
	P3	0.33 ± 0.09	0.3 (0.2 – 0.5)	
Direct bilirubin	C	1.45 ± 2.08	0.3 (0.1 – 4.5)	0.309
	P1	0.82 ± 1.75	0.2 (0.1 – 4.8)	
	P2	1.48 ± 2.29	0.2 (0.1 – 4.9)	
	P3	0.15 ± 0.07	0.1 (0.1 – 0.3)	
AST (SGOT)	C	200.43 ± 68.65	159 (138 – 327)	0.919
	P1	173.71 ± 42.08	165 (134 – 265)	
	P2	219 ± 130.49	135 (106 – 405)	
	P3	168.71 ± 22.67	163 (140 – 206)	
ALT (SGPT)	C	110.57 ± 9.12	112 (95 – 122)	0.01*
	P1	80.43 ± 14.36	74 (71 - 111)	
	P2	122.43 ± 46.78	98 (82 – 193)	
	P3	114.14 ± 20.7	118 (93 – 144)	

Table II : Post-hoc Mann-Whitney test

Variable	Group	C	P1	P2	P3
ALP	C	-	0.209	0.001*	0.902
	P1	-	-	0.073	1.000
	P2	-	-	-	0.053
Total bilirubin	C	-	0.128	0.318	0.007*
	P1	-	-	1.000	0.259
	P2	-	-	-	0.383
Direct bilirubin	C	-	0.318	0.535	0.053
	P1	-	-	1.000	0.456
	P2	-	-	-	0.535
AST (SGOT)	C	-	0.805	0.620	0.710
	P1	-	-	0.710	0.902
	P2	-	-	-	0.710
ALT (SGPT)	C	-	0.004*	0.535	0.902
	P1	-	-	0.011*	0.004*
	P2	-	-	-	0.710

and death as inflammatory and hepatic fibrogenesis processes. The cholestatic patient has a low GSH level, affecting the likelihood of the fibrogenesis process.[15] UDCA-Glutathione combination therapy could increase hepato-protectivity against oxidative stress. GSH is easily found in nature and has been used several times, yet the effectiveness of UDCA-GSH combination therapy on liver fibrosis is unknown. Theoretically, GSH supplementation could increase the hepatoprotective effect on the liver, thus preventing liver fibrogenesis caused by cholestasis.[16]

UDCA-GSH combination therapy is superior to UDCA single therapy in reducing fibrosis in the liver fibrosis model Wistar rat. 20 mg oral UDCA and 15 mg intramuscular injection GSH were given in the previous study.[17] Therefore, this study aims to examine the effect of UDCA-GSH combination therapy in multiple doses on the degree of fibrosis in the Sprague-Dawley rats' liver fibrosis model.

MATERIALS AND METHODS

Experimental Animals

Male Sprague Dawley rats weighing 100 to - 200 g, aged 3 to - 6 weeks were housed at 28.0 ± 2.0 0C room temperature with 12 hour light/dark cycle and were fed rodent chow and water ad libitum. All animals were acclimated for 7 days before the experiment began. Medical Research and Ethics Committee Diponegoro University approved this study (protocol number: 32/EC/H/FK-UNDIP/IV/2022) and fully compliant with ARRIVE criteria.[18]

Fibrosis Model Rats' Induction

Cholestasis was induced by ligating the common bile duct. Before surgery, the rats were given 18 mg cefotaxime (Indofarma, Jakarta, Indonesia) via intramuscular injection as a prophylaxis antibiotic. Then, an intramuscular injection 0f 0,5 ml ketamine hydrochloride (Dexa Medica, Cikarang, Indonesia) was administered as anaesthesia.A midline laparotomy was performed under sterile conditions, and the rat's common bile duct was ligated with a 3-0 silk (DemeTECH, Miami Lakes, FL, USA). 7 mg oral Ibuprofen (Pharos, Semarang, Indonesia) was given every 8 hours/3 days to create pain-free experiment rats.

Animal Groups and Study Design

This study is a randomised post-test-only study with a control group. A total of 28 rats were randomly assigned into four groups (n = 7 per group) as follows: Group 1, samples had bile

duct ligation, and UDCA (Dexa Medica) monotherapy 20 mg, is control group (C). Group 2, bile duct ligation + UDCA 10 mg + glutathione (Sigma Aldrich, St. Louis, MO, USA) 10 mg combination therapy (P1). Group 3, bile duct ligation + UDCA 20 mg + glutathione 15 mg combination therapy (P2). Group 4, bile duct ligation + UDCA 30 mg + glutathione 20 mg combination therapy (P3). The dose of UDCA and glutathione was adjusted as pharmacokinetic of the drug for rats.[19]

UDCA was administered orally once daily, and glutathione was injected intramuscularly daily. All treatments were given continuously for 21 days.

Biochemical Analysis

Blood samples collected in centrifuge tubes were centrifuged at 3000 rpm for 10 minutes. The serum is stored at -20°C until it is used for biochemical assays. The appropriate kits were used to determine serum aminotransferase enzyme activities (AST and ALT) according to the calorimetric method. The ALP activity and total bilirubin (TB), direct bilirubin (DB) and indirect bilirubin (IB) were determined by colorimetric method.

Statistical Analysis

Results data were analysed using SPSS 27.0 for Mac Software. Data were expressed as a median. The Shapiro-Wilk test was used for the normality test. Then, all groups' data were compared using the Kruskall-Wallis and Mann-Whitney tests. All data were significant if $p < 0.05$.

RESULTS

Normality Test

The Saphiro-Wilk normality test revealed that most ALP, bilirubin, and aminotransferase level data did not have a normal distribution ($p < 0.05$).

ALP Level

Non-parametric Kruskal-Wallis test showed a significant difference in ALP level in all rats ($p = 0.025$). Mann-Whitney test showed a significant difference between the C and P2 groups ($p < 0.05$). We noticed a significant decrease in the ALP level of all groups compared to the control group. In this study, we can infer that glutathione combination therapy lowers the ALP level.

Bilirubin Level

We measure two levels of bilirubin: total bilirubin and direct bilirubin. This study shows no significant difference in bilirubin levels between experiment groups ($p = 0.077$). Further, the Mann-Whitney analysis revealed a substantial difference between the C and P3 groups regarding total bilirubin level. Other comparisons in these two parameters, total and direct bilirubin, did not show significance. However, the all-glutathione combination therapy group (P1-P3) showed lower bilirubin levels. Furthermore, the P3 group showed the lowest direct (0.15 ± 0.07) and total bilirubin levels (0.33 ± 0.09).

Aminotransferase Level

We quantitatively measured two liver function markers, AST and ALT. Kruskal-Wallis test showed a significant difference between all groups in the ALT variable ($p = 0.01$), while in the AST variable, the difference was not statistically significant ($p = 0.919$). The Mann-Whitney test result showed that ALT levels significantly differed between the C-P1, P1-P2, and P1-P3 groups ($p < 0.05$). However, the results were various, we can observe that P3 has the lowest level of AST (168.71 ± 22.67), but P1 has the lowest level of ALT (80.43 ± 14.36).

DISCUSSION

The principal result of our study is the inverse relationship between liver enzymes and GSH supplementation. UDCA is widely used due to its cytoprotective mechanism to preserve liver integrity in cholestasis hepatopathies.[20] Two years of UDCA (600 mg/day) or vitamin E (800 IU/day) treatment effectively reduced liver dysfunction in Indian NAFLD patients.[21] However, the efficacy of UDCA remains controversial.[21,22] UDCA monotherapy could not alter the level ALT, AST, and bilirubin levels in liver fibrosis model infant rats.[23] The UDCA therapy lacks antioxidant properties which oxidative stress would prove to be a major problem in cholestatic liver.[13,15] Cholestasis produces oxidative stress in the liver, as increased malondialdehyde (MDA) content shows. BDL rats also demonstrated they decreased water-soluble antioxidant potential and lipid peroxidation as reflected in superoxide dismutase (SOD), catalase, glutathione peroxidase (GTPx), and MDA level.[24] Oxidative stress contributes to hepatotoxicity induced by cholestatic liver disease.[25] Oxidative stress is the overproduction of highly active molecules, such as reactive oxygen species (ROS). The liver injury occurs when liver cells are exposed to certain noxious stimuli, leading to an imbalance between the oxidative and antioxidative systems.[26] ROS released by Kupffer cells (KCs) activate the hepatic stellate cells (HSCs), leading to an increase in the proliferation and synthesis of extracellular matrix (ECM), contributing to fibrosis and cirrhosis.[27] Oxidative stress (OS) associated with inflammation causes focal or zonal necrosis, hepatocyte destruction, and architectural disarray.[28]

GSH, consisting of L-cysteine, L-glutamic acid, and glycine, is currently the most studied antioxidant due to its involvement in oxidative stress, which interacts with and forms glutathione adducts during the protection against free radicals.[29–35] These effects seem essential in regulating cell proliferation and death by mediating the cell's main redox regulatory signalling pathway.[35,36] Previous studies have also shown that the supply of GSH prevents cell damage due to oxidative stress. In contrast, reduced glutathione levels contribute to the onset and progression of many diseases, such as liver fibrosis.[30,37] Under physiological conditions, the liver can resist oxidative stress through GSH synthesis in hepatocytes. In the present study, BDL rats treated with UDCA and UDCA-GSH exhibited low AST, ALT, ALP, and bilirubin levels, which indicated a reduction of oxidative stress and was accompanied by decreased tissue injury. GSH can directly scavenge radicals and peroxides via mixed disulfide formation or oxidisation to generate oxidised glutathione.[38–40] GSH can resist oxidative stress by serving as a

substrate for antioxidative enzymes, including GSH-Px, which converts hydroperoxide into less harmful fatty acids, water, and GSH disulfide.[40] Therefore, GSH can resist cholestasis-induced oxidative stress and attenuate liver fibrosis in advance.

Combination UDCA-GSH shows lower-level AST, ALT, bilirubin, and ALP in all graded doses compared to control (UDCA monotherapy), further alleviating liver fibrosis. In the present study, ALT and AST levels in the UDCA monotherapy group did not recover within the normal range, indicating that UDCA alone is insufficient for suppressing oxidative stress caused by cholestasis in BDL rats. Therefore, although UDCA-GSH treatment exerted a significant protective effect in BDL rats in the present study, hepatic oxidative stress continues. Limitations of the present study include the short duration of modelling and treatment and the fact that no healthy rats were present; therefore, results cannot represent the anti-hepatic fibrosis effects of UDCA-GSH compared to the baseline condition. A future study will likely clarify the therapeutic impact of UDCA-GSH on patients with cholestatic liver disease by improving the modelling method and experimental design and increasing the animal sample size. Future study with more complex variable such as liver biopsy will likely clarify the effects of UDCA-GSH.

CONCLUSION

This study demonstrates a favourable outcome of UDCA-GSH therapy on cholestasis in BDL rats' models compared to UDCA monotherapy by attenuating liver fibrosis based on liver function enzymes. Future study with more complex variable will likely clarify the effects of UDCA-GSH.

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