JKKI
SCIENTIA EST BASIC VITAE

JKKI: Jurnal Kedokteran dan Kesehatan Indonesia
Indonesian Journal of Medicine and Health
Journal homepage: https://journal.uii.ac.id/JKKI
P-ISSN 2085-4145 | E-ISSN 2527-2950

Effects of ursodeoxycholic acid and glutathione combination in spleen TNF-α and apoptotic index in rats with cholestasis

Danu Adi Prakosa Darmawan*[1], Endang Sri Lestari[2], Sigit Adi Prasetyo[3], Muflihatul Muniroh[4], and Agung Aji Prasetyo[5]

[1]General Surgery Resident, Faculty of Medicine Diponegoro University/Kariadi Central Hospital, Semarang, Indonesia
[2]Microbiology Department, Faculty of Medicine Diponegoro University/Kariadi Central Hospital, Semarang, Indonesia
[3]Digestive Surgery Division, Surgery Department, Faculty of Medicine Diponegoro University / Kariadi Central Hospital, Semarang, Indonesia
[4]Physiology Department, Faculty of Medicine Diponegoro University/Kariadi Central Hospital, Semarang, Indonesia
[5]Pediatric Surgery Division, Surgery Department, Faculty of Medicine Diponegoro University / Kariadi Central Hospital, Semarang, Indonesia

Original Article

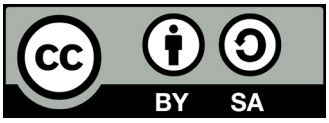
ARTICLE INFO

Keywords:
Apoptosis,
Cholestasis,
Glutathione,
TNF-α,
UDCA

***Corresponding author:**
danuadiprakosa@gmail.com

DOI: 10.20885/JKKI.Vol14.Iss2.art6

History:
Received: April 8, 2023
Accepted: August 29, 2023
Online: August 31, 2023



ABSTRACT

Background: Cholestasis is a disorder of the formation or flow of bile. Among its contributors, tumour necrosis factor (TNF)-α stands out as the most influential inducer of apoptosis. Meanwhile, ursodeoxycholic acid (UDCA) is a valuable agent with choleretic properties, protecting the hepatobiliary system. Glutathione (GSH) enhances endothelial response and prevents liver fibrosis

Objectives: This study evaluates the effect of a combination of GSH-UDCA on splenic TNF-α expression and apoptosis index in Sprague Dawley (SD) rats with cholestasis

Methods: This experiment with a post-tests-only control group design involving 28 male SD rats. They were randomly into four groups: group (K) with 20 mg UDCA, group 1 (P1) with 10 mg UDCA + 10 mg GSH, group 2 (P2) was given UDCA 20 mg + GSH 15 mg, and group 3 (P3) was given UDCA 30 mg + GSH 20 mg. Cholestasis was obtained by ligation of the common bile duct through a laparotomy. During three weeks of trial, rats were administered daily with UDCA orally and GSH intramuscularly. On day 22, rats were sacrificed and spleen samples were taken for anatomical pathology examination.

Results: There were significant differences in TNF-α expression between groups K vs P3; P1 vs P3, and P2 vs P3 ($p=0.002$). There was a significant difference in the apoptotic index between groups K vs P1 ($p<0.001$); K vs P2 ($p=0.004$), and K vs P3 ($p=0.005$).

Conclusions: The UDCA-GSH combination demonstrated a prophylactic effect in SD rats with cholestasis and might be an effective supplemental therapy with UDCA for cholestatic diseases. The difference in TNF-α expression and apoptotic index was lower in SD rats UDCA-glutathione combination group than single dose UDCA. Between TNF-α and the apoptotic index, there is a moderate positive relation.

Latar Belakang: *Kolestasis adalah gangguan pembentukan atau aliran empedu. Di antara kontributornya, tumour necrosis factor (TNF)-α berperan sebagai penginduksi apoptosis yang paling berpengaruh. Sementara itu, asam ursodeoxycholic (UDCA) adalah agen koleretik yang melindungi sistem hepatobilier. Glutathione (GSH) meningkatkan respons endotel dan mencegah fibrosis hati.*

Tujuan*: Studi ini mengevaluasi efek kombinasi GSH-UDCA terhadap ekspresi TNF-α limpa dan indeks apoptosis pada tikus Sprague Dawley (SD) dengan kolestasis*

Metode: *Eksperimen ini dengan desain kelompok kontrol post-test-only yang melibatkan 28 tikus SD jantan.*

Mereka dibagi secara acak menjadi empat kelompok: kelompok (K) dengan 20 mg UDCA, kelompok 1 (P1) dengan 10 mg UDCA + 10 mg GSH, kelompok 2 (P2) diberi UDCA 20 mg + GSH 15 mg, dan kelompok 3 (P3) diberi UDCA 30 mg + GSH 20 mg. Kolestasis diperoleh dengan ligasi saluran empedu melalui laparotomi. Selama tiga minggu percobaan, tikus diberikan UDCA setiap hari secara oral dan GSH secara intramuskular. Pada hari ke 22, tikus dikorbankan dan diambil sampel limpa untuk pemeriksaan patologi anatomi

Hasil: *Terdapat perbedaan signifikan pada ekspresi TNF-α antara kelompok K vs P3; P1 vs P3, dan P2 vs P3 (p=0,002). Terdapat perbedaan yang signifikan pada indeks apoptosis antara kelompok K vs P1 (p<0,001); K vs P2 (p=0,004), dan K vs P3 (p=0,005).*

Kesimpulan: *Kombinasi UDCA-GSH menunjukkan efek profilaksis pada tikus SD dengan kolestasis dan mungkin merupakan terapi tambahan yang efektif dengan UDCA untuk penyakit kolestatik. Perbedaan ekspresi TNF-α dan indeks apoptosis lebih rendah pada tikus SD kelompok kombinasi UDCA-glutathione daripada UDCA dosis tunggal. Antara TNF-α dan indeks apoptosis terdapat hubungan positif yang sedang.*

INTRODUCTION

Cholestasis is a disorder of the formation or flow of bile caused by various factors, one of which is primary biliary cirrhosis (PBC). Primary biliary cirrhosis is a slowly progressive cholestatic liver disease culminating in cirrhosis and liver failure.[1] The incidence of PBC in Southeast Asia reaches.[2] 3 cases per 100,000 and tends to increase.[2] Cholestasis occurrence prompts an elevation in bile acids. These bile acids will also infiltrate the hepatic sinusoids, thereby inducing hepatocyte toxicity.[3] This cascade culminates in the development of liver cirrhosis, wherein an increase in intrahepatic vascular resistance causes heightened portal inflow. Consequently, portal pressure rises, leading to the onset of portal hypertension. This condition alters the extrahepatic vasculature within the splanchnic and systemic circulatory systems, prompting the creation of collateral vessels and arterial vasodilatation. A direct consequence of portal hypertension is spleen enlargement, known as splenomegaly. This phenomenon triggers the initiation of apoptosis in splenic cells.[4,5] According to Brenner et al., tumour necrosis factor (TNF)-α could be the most potent initiator of apoptosis, simultaneously activating cell death and survival mechanisms.[6]

Glutathione (GSH) is an antioxidant protein consisting of three primary amino acids: L-glutamic acid, L-cysteine, and L-glycine. It increases the responsiveness of the endothelium and prevents liver fibrosis. Glutathione destroys TNF-α and interleukin (IL)-1β, proinflammatory cytokines that promote the process of liver fibrosis.[7,8]

Ursodeoxycholic acid (UDCA) is a secondary bile acid produced by intestinal bacteria as a metabolic by-product, which is effective in the non-surgical treatment of cholesterol gallstones. Administration of UDCA increases the amount of non-toxic hydrophilic bile acids in the liver and functions as a choleretic agent, immunomodulatory agent and protective against the hepatobiliary system.[1,9,10] The implementation of UDCA therapy, alongside its role in protecting the liver from cholestasis-induced harm, exhibited an enhancement in the synthesis of GSH. However, this improvement was noted at a level lower than when UDCA was administered in combination with another agent. A separate study conducted by our team exhibited that the concurrent usage of UDCA and GSH in cholestatic rats led to a reduction in TNF-α expression within the terminal ileum.[3]

While the potential beneficial impact of UDCA on liver diseases and the potent antioxidative attributes of GSH in addressing cholestasis have been suggested, further investigation is needed to comprehensively assess the combined effect of GSH and UDCA on cholestasis management. This evaluation particularly concerns the expression of TNF-α and the apoptotic index within the spleen, which are closely connected to cholestasis.11 Distinguishing itself from previous studies, this study introduces an innovative independent variable: the combination of glutathione and ursodeoxycholic acid as antioxidants to enhance TNF-α expression. Furthermore, UDCA, an FDA-recommended drug, is incorporated. Meanwhile, the dependent variables were the spleen's TNF-α levels and the spleen tissues' histopathology to assess its apoptotic index. The study employs rats that have been induced with cholestasis as its subjects. This study aims to evaluate the impact of the GSH-UDCA combination on the expression of TNF-α within the spleen, as well as the ensuing apoptotic index of the spleen.

METHODS

Study design

This experimental study employs a post-test-

only control group design. The study subjects were cared for in the Bioscience Laboratory of the Faculty of Medicine at Brawijaya University, Malang. The experiments were conducted following the institutional guidelines, and the protocol was approved by the Health Research Ethics Committee of the Faculty of Medicine Diponegoro University (Permit Numbers: 32/EC/H/FK-UNDIP/IV/2022).

UDCA and GSH dosage

This study utilised graded doses of UDCA and GSH. The doses of UDCA and GSH used were obtained from the range of doses used in humans, which were then converted to experimental animals using Laurence and Bacharach conversion.[12] The dosage range of UDCA in humans is established at 8-25 mg/kgBW. For this study, the administered doses in humans were set at 8 mg/kg, 17 mg/kg, and 25 mg/kg. Upon conversion for rats weighing 200 g, the corresponding doses were determined to be 10 mg, 20 mg, and 30 mg, respectively.

The recommended dosage range for GSH in humans is 600-1200 mg. In this study, the dosages administered to humans were 600 mg, 800 mg, and 1200 mg. Following conversion for rats weighing 200 g, the resultant doses were calculated as 10 mg, 15 mg, and 20 mg, correspondingly.

Animal experiment

The study utilised Sprague Dawley (SD) rats aged 3 to 6 weeks, weighing between 100 and 200 grams, and exhibiting good health and activity. Rats with anatomical defects or cirrhotic livers were excluded from the study. Rats that passed away during the study or developed infections after surgery were considered for dropout. The sample size was determined following the World Health Organisation (WHO) guidelines, specifying five rats for each group.[13] Rats underwent acclimatisation for seven days before receiving bile duct ligation (BDL) treatment.[14] Here, 28 rats were divided into four treatment groups (7 rats each) using simple randomisation: the control group (K) was administered 20 mg UDCA; treatment group 1 (P1) was administered 10 mg UDCA and 10 mg glutathione; treatment group 2 (P2) was administered UDCA 20 mg and glutathione 15 mg; treatment group 3 (P3) was administered UDCA 30 mg and glutathione 20 mg. UDCA was administered orally, while glutathione was administered intramuscularly.15 The entire treatment protocol was administered continuously for 21 consecutive days.[16]

On day 22, rats were sacrificed humanely euthanised randomly between 9:00 and 11:00 AM following an overnight fasting period. Euthanasia was carried out by administering an overdose of an anaesthetic agent. Blood and spleen samples were collected afterwards. The spleen organs were preserved in 10% formalin and subsequently sent to the anatomical pathology department for processing into paraffin blocks and subsequent tissue staining using hematoxylin and eosin (HE) staining. Cirrhosis manifestation is characterised by the presence of extensive septa and nodules. Cirrhosis was categorised into three groups: mild cirrhosis (4a), moderate cirrhosis (4b), and severe cirrhosis (4c), with distinctions based on nodule area and the extent of septa. Additionally, a spectrum of fibrosis grades ranging from minimal (Grade 1) to moderate (Grade 3) was observed, while normal liver images (Grade 0) were absent.

BDL procedure

Prior to conducting the BDL procedure, each rat received an intramuscular injection of 19 mg of Cefotaxime, followed by Ketamine Hydrochloride as an anaesthetic agent. The BDL was performed through a laparotomy procedure. Post-surgery, all rats were given oral analgesia using 7 mg of Ibuprofen for three consecutive days.

TNF-α expression examination

Assessment of TNF-α expression was carried out by immunohistochemical staining with brown staining results in the nucleus and cytoplasm of cells. TNF-α immunoreactivity was assessed by multiplying the area score by the intensity score to obtain the TNF-α immunoreactivity score.[17] TNF-α expression examination using the ImageJ application.

Apoptotic index examination

The apoptotic index was calculated according to the method used by Aihara et al., in which cells undergoing apoptosis were counted per 100 lymphocyte cells at 400× magnification in 10 fields of view of each splenic tissue preparation stained with HE in a single paraffin block.[18]

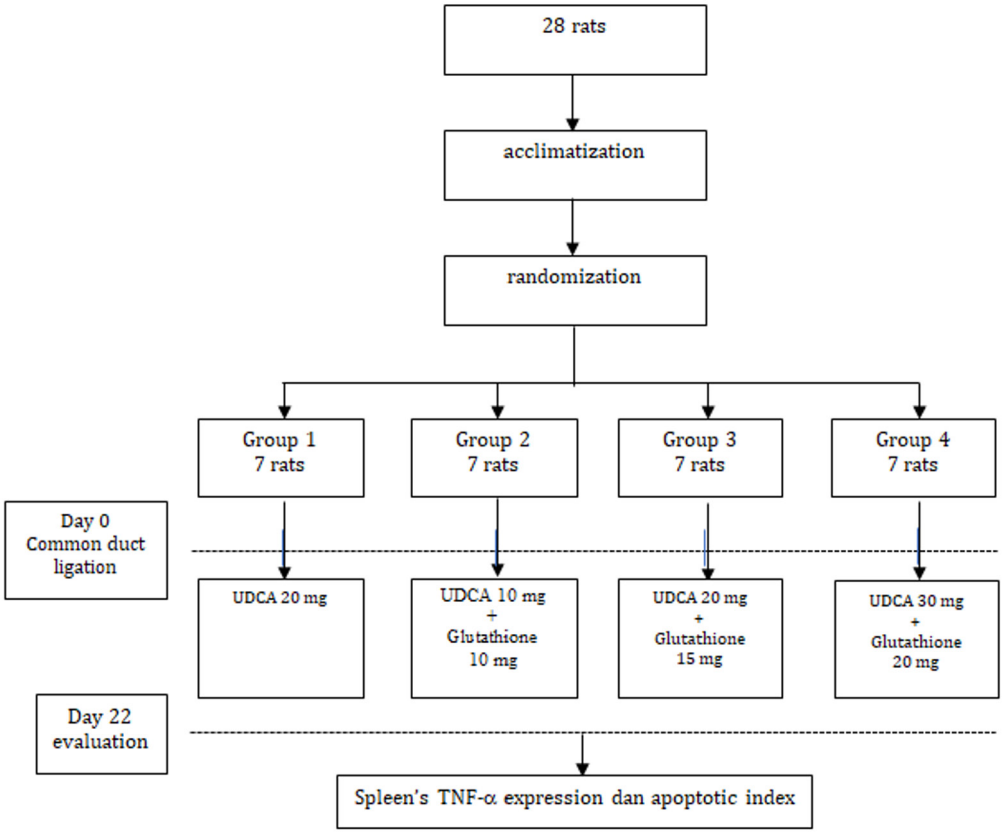


Figure 1. Flow diagram of the animal experiments

Subsequently, an average value was computed. The fields of view were examined from left to right and then progressed downward, starting anew from the left. These observations were conducted in a blinded manner by an anatomical pathology specialist.

Statistical analysis

Collected data was processed into cleaning, coding, and tabulation. The subsequent data analysis was carried out employing SPSS Ver. 26.0 for Windows. Information relating to administering the UDCA-glutathione combination concerning splenic TNF-α expression and splenic apoptosis index in cholestasis was presented in tabular format, incorporating mean, standard deviation, median values, and graphs. The normality test was performed using the Saphiro-Wilk test. Data with normal distribution were analysed using the one-way ANOVA test and continued with the post hoc test. In contrast, nonparametric data was analysed using the Kruskal-Wallis test, followed by the Mann-Whitney test. The limit of the degree of significance is if $P < 0.05$ with a 95% confidence interval. Additional statistical analysis was done for the apoptosis index to determine the suitability between two observers; the cut-off value was determined as > 0.75.

RESULTS

Hepatic fibrosis degree

The histopathological image of the liver tissue for each preparation was examined in five visual fields. This study used five rats per group, so 25 visual fields were obtained to assess the degree of fibrosis. Determination of the degree of liver fibrosis was carried out using a magnification of 100× to look for a visual field, and the degree of liver fibrosis was determined based on the Laennec system.

Microscopic observation of the liver in the control group (K) revealed that most (52%) of the visual field demonstrated severe liver damage that reached cirrhosis (Grade 4). Liver microscopic observation in the UDCA-glutathione combination treatment group demonstrated better results when compared to the other two treatments. There was a normal liver picture or grade 0, which was not found in the other treatment groups and cirrhosis or grade 4 with a lower number. In addition, there are still images of fibrosis in grades

1 to 3 (Figure 2).

TNF-α expression

The mean expression of TNF-α in the cholestatic spleen after BDL and the results of the normality test from the highest to lowest was in group K (control group), followed by groups P2 (UDCA 20 mg + glutathione 15 mg), P1 (UDCA 10 mg + glutathione 10 mg), and P3 (UDCA 30 mg + glutathione 20 mg).

Table 1 represents the results of TNF-α expression. These results demonstrated a significant difference in TNF-α between the treatment groups ($P < 0.05$). From the results of the Mann-Whitney test, it was found that there was a significant difference in the expression of TNF-α between group K and P3 ($P < 0.002$), groups P1 and P3 ($P < 0.002$), and groups P2 and P3 ($P < 0.002$). Using the ImageJ application, the results of the brown streaks are converted to black and white to be calculated as the presentation ratio to the black part. There was a difference in the ratios of the K, P1, P2, and P3 groups. The P3 group demonstrated the lowest ratio compared to the other groups (Figure 3)

Apoptotic index

According to the one-way ANOVA test, there was a significant difference in apoptotic index between all groups ($P < 0.05$) (Table 2). Subsequently, the test was continued with a post hoc LSD test.

The post hoc test revealed significant differences in the apoptotic index among the treatment groups. Specifically, a substantial difference was noted between K and P1 groups ($P < 0.001$), as well as the P2 group ($P < 0.004$), and the P3 group ($P < 0.005$). This result indicates varying quantities of cells undergoing apoptosis. Notably, the apoptotic index exhibited a marked contrast between group K and P1, P2, and P3. The latter groups (P1, P2, and P3) displayed a noticeable reduction in the number of cells undergoing apoptosis (Figure 4).

A reliability test was conducted for the measurements of the apoptotic index, resulting in a correlation coefficient (r) of $0.906 > 0.75$. So it can be concluded that the reliability of the apoptosis index between the two observers was very good

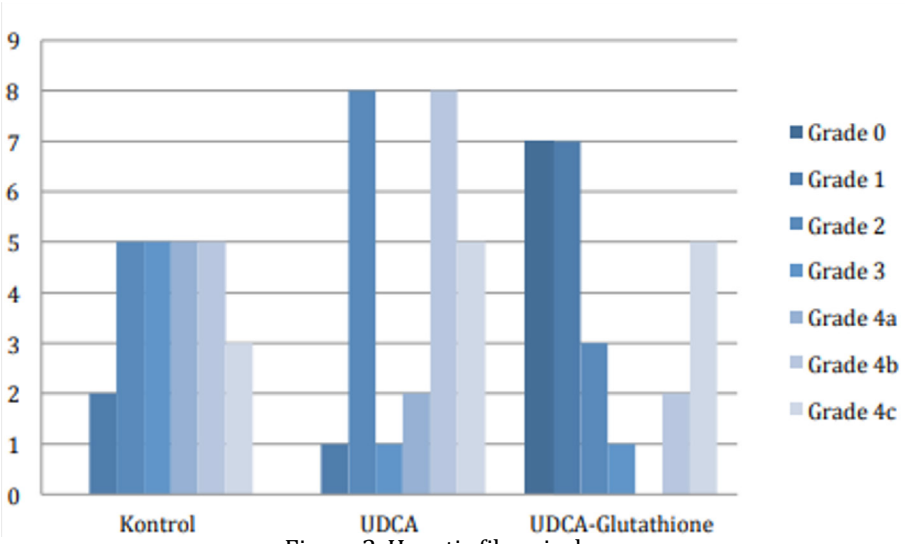


Figure 2. Hepatic fibrosis degree

Table 1. TNF-α and apoptosis index after BDL

	Treatment groups				p
	K (n=7)	P1 (n=7)	P2 (n=7)	P3 (n=7)	
TNF-α @	23.4 (4.0–41.0)	6.2 (5.1 – 51.5)	20.5 (2.8 – 46.1)	0.3 (0.1 – 0.8)	0.002*
Apoptosis index #	4.51 ± 0.92	2.41 ± 0.96	2.84 ± 0,98	2.74 ± 0.53	0.002*

*P < 0.05; @ Kruskal-Wallis test; # One-way Anova; K: UDCA only; P1: UDCA 10 mg + glutathione 10 mg; P2: UDCA 20 mg + glutathione 15 mg; P3: UDCA 20 mg + glutathione 15 mg

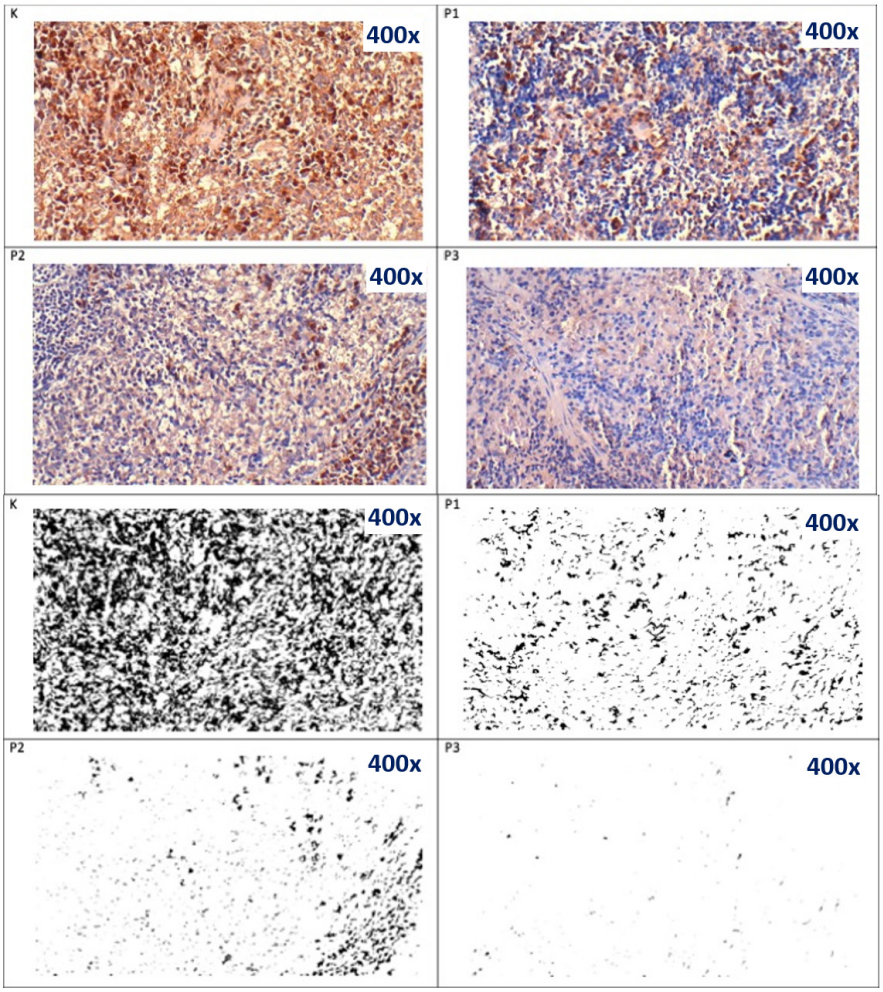


Figure 3. Anatomical pathology of TNF-α expression TNF immunohistochemical staining of spleen tissue at 400x magnification. UDCA 20 mg (K), UDCA 10mg and Glutathione 10mg (P1), UDCA 20mg and Glutathione 15mg (P2), UDCA 30mg and Glutathione 20mg (P3) groups.

Table 2. TNF-α and apoptosis index comparison between groups

Groups		Mean difference	p value
K	P1	0.848m	<0.001*L
	P2	0.848m	0.004*L
	P3	0.002*m	0.005*L
P1	P2	0.749m	0.334L
	P3	0.002*m	0.316L
P2	P3	0.002*m	0.970L

*P < 0.05; mMann-Whitney; LPost-hoc LSD; K: UDCA only; P1: UDCA 10 mg + glutathione 10 mg; P2: UDCA 20 mg + glutathione 15 mg; P3: UDCA 20 mg + glutathione 15 mg

DISCUSSION

In this study, three main results were obtained. Firstly, the combination of UDCA and glutathione reduced the expression of TNF-α in rats treated with the UDCA-glutathione combination, which was lower than that of UDCA alone. Second, the UDCA-glutathione combination could reduce the apoptotic index compared with the single UDCA administration in rats with their bile ducts ligated. Third, a moderate positive relationship existed between TNF-α and apoptosis in rats with ligated bile ducts.

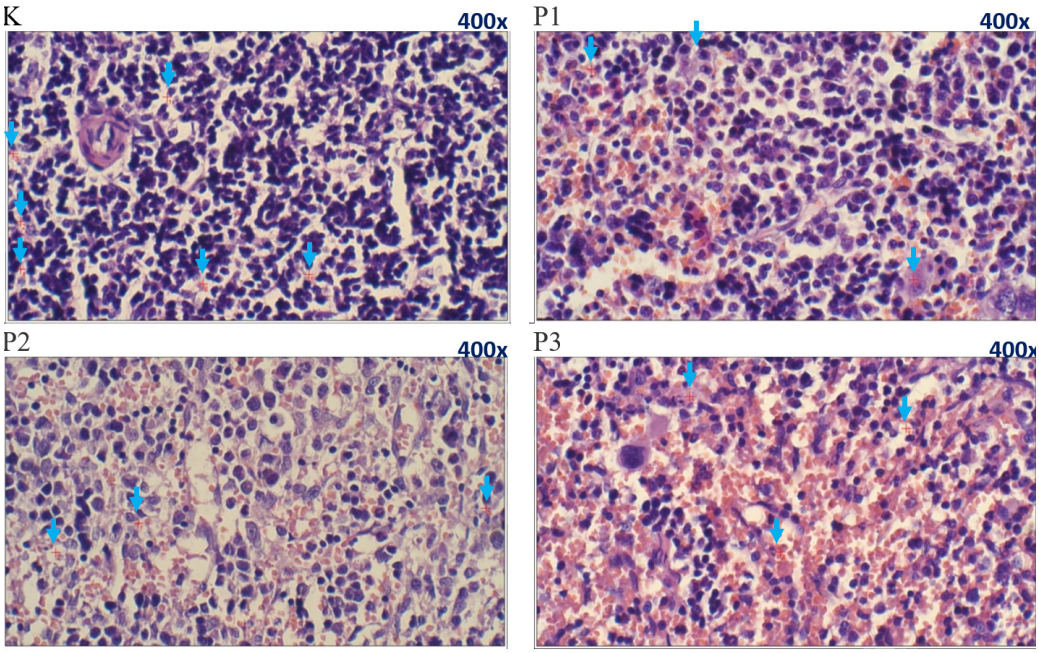


Figure 4. Anatomical pathology of apoptosis lien at 400x magnification. UDCA 20 mg (K), UDCA 10mg and Glutathione 10mg (P1), UDCA 20mg and Glutathione 15mg (P2), UDCA 30mg and Glutathione 20mg (P3) groups.

kB-dependent genes regulates the survival and proliferative effects of pf TNF, whereas activation of caspases regulates apoptotic effects. TNF-α induced apoptosis is mediated primarily through activation of the type I receptor, a death domain that recruits several different signalling proteins, which together are thought to be part of the apoptotic cascade.[15]

This study did not examine other inflammatory mediators released by liver cells, such as IL-1β and IL-6, which could cause bile secretion failure and affect the spleen. This study also did not examine the dose of UDCA and glutathione, which could significantly reduce TNF-α and splenic cell apoptosis.

This study has important implications for the treatment of patients with cholestasis. This in vivo study has proved glutathione's potential as a combination therapy for cholestasis. Our findings reveal that UDCA and glutathione can yield a potent antioxidant effect, enhancing the condition of cholestasis patients. These outcomes represent a substantial advancement in novel treatment approaches for cholestasis. However, it is important to note that further comprehensive research, encompassing additional inflammatory cytokines, as well as translational and clinical studies, is necessary to establish the robustness of this combination therapy approach.

CONCLUSION

In conclusion, combining UDCA and glutathione decreased TNF-α expression and apoptotic index in SD rats. However, the exact mechanisms explaining the advantage of combining UDCA and glutathione are to be established.

CONFLICT OF INTEREST

The authors have no conflicts of interest to declare.

ACKNOWLEDGEMENT

We want to thank Fitria Novitasari from Institut Biosains Malang and Fikar Arsyad Hakim from the Anatomical Pathology Department, Universitas Negeri Surakarta, for the support given during the study.

REFERENCES

1. Shah R, John S. Cholestatic jaundice. StatPearls [Internet]. 2021 Jul 19 [cited 2022 Jun 16]; Available from: https://www.ncbi.nlm.nih.gov/books/NBK482279/
2. Sarin S, Kumar M, Eslam M. Liver diseases in the Asia-Pacific region: a Lancet Gastroenterology & Hepatology Commission. Lancer Gastroenterol Hepatol. 2020;5:167–228.

A significant difference was found in the reduction of TNF-α in the group of SD rats given UDCA 30 mg and glutathione 20 mg. The effect provided by UDCA and glutathione aligns with a previous study that reported that the administration of UDCA and glutathione will influence TNF-α expression and apoptotic index.[1] The administration of UDCA led to an enhancement in glutathione status and a reduction in inflammatory markers, quantifiable through parameters such as C-reactive protein (CRP), aspartate transaminase (AST), and alanine transaminase (ALT). This outcome suggests a potential slowdown in the inflammatory process, thereby diminishing the risk of cellular damage. A similar result was also reported by Rodriguez et al.[9] that liver perfusion with UDCA resulted in notably elevated glutathione levels and increased activity of methionine adenosyl transferase, an enzyme critical to glutathione biosynthesis, when compared to liver perfusion with other bile salts.

In a state of cholestasis, there is a disruption of hepatocyte secretion, resulting in impaired bile formation. If this progresses chronically, fibrosis and necrosis of the liver may occur. Apart from impaired secretion, liver damage is also caused by the activation of several inflammatory mediators, macrophage activity, dysregulation of the immune response, and oxidative stress. One of the proinflammatory mediators macrophage produces during this cholestatic process is TNF-α.[19] TNF-α is the most powerful inducer of apoptosis, activating mechanisms that regulate cell survival and death. Activation of TNF-α in NF-kB regulates its survival and proliferative effects, while caspases regulate the effects of apoptosis. In liver damage, the apoptotic pathway is mostly activated by TNF-α, so a possible way to reduce the apoptosis impact is to inhibit the action of TNF-α.[19]

UCDA, a constituent of human bile salts, exhibits promising therapeutic potential in addressing liver fibrosis and cirrhosis.[15] Its mechanism involves stimulating hepatobiliary secretion, which aids in resolving cholestasis-related issues by promoting the excretion of bile from the liver. Additionally, UCDA contributes to the enhancement of glutathione synthesis. This glutathione then counteracts the chain reaction of oxidative stress and facilitates the reduction of H2O2.[20] Furthermore, the elevation of endothelial response attributed to glutathione leads to a decrease in TNF-α. This decrease affects the activity of liver stellate cells, subsequently decelerating the liver fibrosis process. When the functionality of liver cells is preserved, it positively impacts spleen function, preventing the occurrence of apoptosis in the spleen.

The outcomes of this study revealed a noteworthy discrepancy in the apoptotic index when comparing the control group to the treatment groups. These findings align with the study's hypothesis, which posits that the joint administration of UDCA and glutathione can yield a more pronounced reduction in TNF-α expression and splenic apoptotic index than the administration of singular UDCA in SD rats treated with common BDL.[21] This notion finds support in the work of Khairunnisa et al.[22] who demonstrated a significant divergence in fibrosis levels between the treatment group receiving combined UDCA-glutathione therapy and both the control group and the single UDCA group, thus corroborating the present study's outcomes.

Glutathione is among the endogenous antioxidants present within the human body. During the onset of cholestasis, the body experiences diminished antioxidant levels, leading to an inflammatory response and an escalation in reactive oxygen species (ROS). To mitigate the incidence of apoptosis, augmenting antioxidant levels is imperative. Achieving this augmentation involves upregulating GSH levels, which can be facilitated through combination therapy involving UDCA and GSH. Such a therapeutic combination effectively reinforces the protective mechanisms against ROS and anti-apoptosis.[22]

UDCA and glutathione play an important role in the apoptosis process, as both substances exert antiapoptotic effects.[23,24,25] Liver cirrhosis gives rise to numerous complications, among which is portal hypertension. The immediate consequence of portal hypertension is splenomegaly. This condition emerges from heightened vascular pressure, thereby inducing vascular hypertension. The elevation in vascular pressure subsequently prompts apoptosis of splenic cells. Within this mechanism, UDCA and glutathione operate by impeding the progression of apoptosis.[26]

TNF-α activates cell survival and cell death mechanisms simultaneously. Activation of NF-

3. Onofrio FQ, Hirschfield GM. The pathophysiology of cholestasis and its relevance to clinical practice. Clin liver Dis. 2020 Mar 1;15(3):110–4. doi:10.1002/CLD.894
4. Chapman J, Goyal A, Azevedo AM. Splenomegaly. 5-minute pediatr consult 8th Ed. 2021 Aug 11;870–1.
5. Bloom S, Kemp W, Lubel J. Portal hypertension: pathophysiology, diagnosis and management. Intern Med J [Internet]. 2015 Jan 1 [cited 2022 Jun 22];45(1):16–26. doi:10.1111/IMJ.12590 Available from: https://pubmed.ncbi.nlm.nih.gov/25230084/
6. Brenner D, Blaser H, Mak T. Regulation of tumour necrosis factor signalling: live or let die. Nat Rev Immunol. 2015;15(6):362–74.
7. Asrani SK, Larson JJ, Yawn B, Therneau TM, Kim WR. Underestimation of liver-related mortality in the United States. Gastroenterology. 2013 Aug 1;145(2):375-382.e2. doi:10.1053/j.gastro.2013.04.005
8. Aquilano K, S CB. Glutathione: New roles in redox signalling for an old antioxidant. Front Pharmacol. 2014;5:1–13.
9. Gould R, Pazdro R. Impact of supplementary amino acids, micronutrients, and overall diet on glutathione homeostasis. Nutrients. 2019;11(5):1056.
10. Cholestasis - Liver and Gallbladder Disorders - MSD manual consumer version [Internet]. [cited 2022 Jun 22]. Available from: https://www.msdmanuals.com/home/liver-and-gallbladder-disorders/manifestations-of-liver-disease/cholestasis
11. Farashbandi A, Shariati M, Mokhtari M. Comparing the protective effects of curcumin and ursodeoxycholic acid after ethanol-induced hepatotoxicity in rat liver. Ethiop J Heal Sci. 2021;31(3):673–82.
12. Paget G., Barnes J. Toxicity Test. In: Evaluation of drug activities. Massachusetts: Academic Pres; 1964. p. 135–66.
13. Office of Laboratory Animal Welfare. Care and use institutional animal care and use. Sect B. 2002;43–9.
14. Tag C, Sauer-Lehnen S, Weiskirchen S, Borkham-Kamphorst E, Tolba R, Tacke F. Bile duct ligation in mice: induction of inflammatory liver injury and fibrosis by obstructive cholestasis. J Vis Exp. 2015;96.
15. Halim J, Lestari E, Prasetyo S, Muniroh M, Prasetyo A. Combination of ursodeoxycholic acid and glutathione improves intestinal morphology in cholestasis by downregulating TNF-α expression. Indones Biomed J. 2022;14(4):429–35.
16. Al Shoyaib A, Archie SR, Karamyan VT. Intraperitoneal route of drug administration: should it be used in experimental animal studies? Pharm Res. 2020;37(1):1–30. doi:10.1007/s11095-019-2745-x
17. Zhou XL, Fan W, Yang G, Yu MX. The clinical significance of PR, ER, NF- B, and TNF-α in breast cancer. Dis Markers. 2014;2014. doi:10.1155/2014/494581
18. Barichello T, Generoso JS, Singer M, Dal-Pizzol F. Biomarkers for sepsis: more than just fever and leukocytosis—a narrative review. Crit Care. 2022;26(1):1–31. doi:10.1186/s13054-021-03862-5
19. Cholestasis - Liver and Gallbladder Disorders - MSD manual consumer version [Internet]. [cited 2022 Aug 18]. Available from: https://www.msdmanuals.com/home/liver-and-gallbladder-disorders/manifestations-of-liver-disease/cholestasis
20. Tang N, Zhang Y, Liang Q, Liu Z, Shi Y. The role of ursodeoxycholic acid on cholestatic hepatic fibrosis in infant rats. Mol Med Rep. 2018;17(3):3837–44.
21. Rahantaniaina M, Li S, Chatel-Innocenti G, Tuzet A, Mhamdi A, Vanacker H. Glutathione oxidation in response to intracellular H 2 O 2 : Key but overlapping roles for dehydroascorbate reductases. Plant Signal Behav. 2017;12(8):e1356531.
22. Khairunnisa NI, Prasetyo AA, Miranti IP. Perbedaan derajat fibrosis hepar tikus Wistar yang dilakukan ligasi duktus koledokus antara kelompok pemberian kombinasi Udca-Glutathione dengan pemberian tunggal Udca. Diponegoro Med J (Jurnal Kedokt Diponegoro). 2016;5(4):1378–88.
23. VanderWall K, Lu B, Alfaro J, Allsop A, Carr A, Wang S, et al. Differential susceptibility of retinal ganglion cell subtypes in acute and chronic models of injury and disease. Sci Rep. 2020;10(1):17359.
24. Wiegand J, Berg T. The Etiology, Diagnosis and prevention of liver cirrhosis: Part 1 of a series on liver cirrhosis. Dtsch Arztebl Int. 2013 Feb 8;110(6):85. doi:10.3238/ARZTEBL.2013.0085
25. Perez MJ, Macias RIR, Duran C, Monte MJ,

Gonzalez-Buitrago JM, Marin JJG. Oxidative stress and apoptosis in fetal rat liver induced by maternal cholestasis. Protective effect of ursodeoxycholic acid. J Hepatol. 2005 Aug;43(2):324–32. doi:10.1016/J.JHEP.2005.02.028

26. Suwanti LT, M M. Peningkatan TNF-α dan indeks apoptosis pada tulang mencit yang diinfeksi Toxoplasma gondii. J Kedokt Hewan - Indones J Vet Sci. 2015 Sep 1;9(2). doi:10.21157/J.KED.HEWAN.V9I2.2808