

RESEARCH ARTICLE

Combination of Ursodeoxycholic Acid and Glutathione Improves Intestinal Morphology in Cholestasis by Downregulating TNF- α Expression

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Abstract

BACKGROUND: Cholestasis caused by obstruction of the common bile duct and may developed gut-derived sepsis due to reactive oxygen species (ROS) accumulation. Ursodeoxycholic acid (UDCA) and glutathione are widely known for their antioxidant properties, that might be beneficial against ROS. However, the effects of UDCA-glutathione combination against ROS have not been well elucidated in previous studies. Thus, this study was conducted to evaluate tumor necrosis factor (TNF)- α level and height of terminal ileal mucosal villus after UDCA-glutathione administration in cholestasis rat model.

METHODS: Twenty-eight male Sprague Dawley rats were randomly grouped into four treatment groups, each group consisted of seven rats that had previously undergone bile duct ligation. Three groups received treatment of UDCA-glutathione combination on stratified dose, while the other one only received UDCA. Each treatment was given for 21 days. Ileal samples were collected from the rats and

stained with mouse anti TNF- α antibody and hematoxylin-eosin (HE). Immunohistochemistry and histopathological examination were done using microscope and then calculated with ImageJ.

RESULTS: The combination of UDCA and glutathione treatment decreased the TNF- α expression ($p<0.05$) compared to UDCA only group, particularly in group that received 20 mg UDCA and 15 mg glutathione supplementation ($p<0.05$) and group that received 30 mg UDCA and 20 mg glutathione supplementation ($p<0.05$). The height of the mucosa villous was higher in the UDCA-glutathione combination groups for all the three dosage variations given ($p<0.05$) compared to UDCA only group.

CONCLUSION: UDCA-glutathione combination downregulates TNF- α expression and improves ileum mucosal villus height in cholestasis.

KEYWORDS: cholestasis, glutathione, intestinal villus height, TNF- α , UDCA

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Introduction

Cholestasis is commonly seen in clinical setting but it may got complicated with intestinal failure and endotoxemia, thus leading to high prevalence of morbidity and mortality. (1) The incidence of cholestasis is reported in every 5 in

1000 people in the USA.(2) About 500-600 mg bile acids are synthesized *de novo* in the liver daily, and more than 90% of bile acids are reabsorbed in the terminal ileum for reuse. This cycle is known as the enterohepatic cycle which occurs six to ten times per day under normal conditions. In cholestasis, this cycle is disrupted and causes changes in the shape and function of the terminal ileum.(3) The

digestive system carries out a number of tasks related to digestion, selective absorption, and secretion. The barrier, though, stops intraluminal bacteria and endotoxins from spreading to the organs and tissues.(4) Increased bacterial and toxin translocation from the intestinal lumen to the systemic circulation is related with intestinal barrier failure, leading to systemic infection and numerous organ failures. (5) Inadequate host immunological defences, disruption of the natural ecological balance of the gut microbiota, and physical disruption of the gut mucosal barrier can all contribute to the breakdown of the gut barrier.(6) Recent developments in the pathophysiology of intestinal failure in cholestasis have demonstrated that disturbance of the immunologic, biological, mechanical, and biochemical barriers is just one of several factors that contribute to the gut barrier being breached.(4)

Recent developments in the pathophysiology of intestinal failure in cholestasis have demonstrated that disturbance of the immunologic, biological, mechanical, and biochemical barriers is just one of several factors that contribute to the breaching of gut barrier. In cases of cholestasis, more advance medical therapy is being developed, especially to halt the cellular deterioration. For cholestasis, ursodeoxycholic acid (UDCA) is the first-line treatment (7) and considered as gold standard of primary biliary cholangitis (PBC) treatment (8). UDCA is a physiological hydrophilic bile acid that functions as a choleric, immunomodulator, and cytoprotective agent. (9) UDCA promotes epithelial cell migration after cellular injury and protects the intestinal barrier.(10) Optimal dosage of UDCA ranging from 13-15 mg/kg BW/day to give significant results while not exhibiting significant side effects after two years of usage.(11) However, 35-40% of patients with PBC do not respond to UDCA treatment and have a poor outcome (12), thus adding another treatment regimen would aid to a better outcome. Combination of UDCA dan S-adenosyl-L-methionine (SAME) has been used in treating hepatocyte injury during cholestasis, and combining both regimens gives better clinical outcomes.(13) SAME is an essential protein of methylation in glutathione synthesis.(14) UDCA also increases the glutathione level in the HepG2 cell line.(15)

One of the endogenous antioxidants in the liver is glutathione. Because it is a cofactor for the enzyme glutathione peroxidase, glutathione eliminates free radicals. (16-18) The antioxidant defence system is disrupted by decreased glutathione reductase and glutathione peroxidase activity under conditions of cholestasis.(19) High glutathione levels can prevent cellular damage caused by oxidative

stress due to reactive oxygen species (ROS), which have an essential role in the pathogenesis of cholestasis.(20,21)

Previous studies have not elucidated the effect of UDCA-glutathione combination to reduce inflammatory response. Therefore, this study was conducted to evaluate tumor necrosis factor (TNF)- α expression and mucosal villus height of terminal ileal after UDCA-glutathione administration in cholestatic rats model.

Methods

Animal Treatment

Twenty-eight Sprague Dawley male rats aged 6-8 weeks and weighed 100-200 g were obtained from Institut Biosains, Malang, housed in a controlled environment and provided with standard rodent chow and water ad libitum. Cholestasis was induced by ligating the common bile duct. Before surgery, the rats were given 18 mg cefotaxime (Indofarma, Jakarta, Indonesia) intramuscularly for prophylaxis antibiotics. Then the rats were given 0.5 mL ketamine hydrochloride (Dexa Medica, Cikarang, Indonesia) intramuscularly as anaesthesia. Subsequently, 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). Postoperative analgesia 7 mg Ibuprofen (Pharos, Semarang, Indonesia) orally every 8 hours for three consecutive days was given to relieve pain.

UDCA (Dexa Medica) was given in 10, 20, or 30 mg dose as human dose for UCDA was 8-25 mg/kg and converted to a rat dose of 10-30 mg. While the glutathione (Sigma Aldrich, St. Louis, MO, USA) was given in 10, 15, or 20 mg dose as the human dose for glutathione was 600-1200 mg per day and converted to a rat dose of 10-20 mg. Twenty-eight rats were randomly divided into four groups, each consist of seven rats who had previously undergone bile duct ligation to induce cholestasis: group C was given 20 mg UDCA supplementation; group T1 was given 10 mg UDCA and 10 mg glutathione supplementation; group T2 was given 20 mg UDCA and 15 mg glutathione supplementation; and group T3 was given 30 mg UDCA and 20 mg glutathione supplementation. UDCA was given once daily with an oral gastric tube (7 gauge feeding tube) inserted daily and taken off after supplementation. Meanwhile, glutathione was administered intramuscularly daily. Each treatment was given for 21 days.

Rats were sacrificed by a lethal dose of ketamine after 22 days of cholestasis-induction. The experiments

were conducted following the institutional guidelines, and the protocol was approved by the Health Research Ethics Committee of the Faculty of Medicine, Universitas Diponegoro (Protocol Numbers: 32/EC/H/FK-UNDIP/IV/2022).

Sample Handling Process

Terminal ileal samples were obtained from 5 cm of the ileocecal junction and were fixed in 10% neutral buffered formalin for 3 hours. Cell dehydration process was done by using different concentration of alcohol (70, 80, 90, and 100%) for 1 hour in each alcohol concentration, respectively. Then, the tissue underwent a clearing process in xylol for 3 hours. The tissue was inserted in liquid paraffin for 4 hours and embedded into cassette using Histostar (Thermo Fischer, Waltham, MA, USA). Paraffin block was cut into 2-5 mm block size.

Immunohistochemistry Examination

The ileal samples were deparaffinized and incubated overnight at 4°C with the following primary antibodies: Mouse Anti-TNF Alpha Monoclonal Antibody (Bioenzy, Shanghai, China). Slides were counterstained with hematoxylin and eosin (HE), dehydrated, cleared, and mounted. Ileal samples labelled with TNF- α immunohistochemistry would appear in brown colour. The evaluation of cell staining was performed around crypt of ileal tissue. Using ImageJ (National Institutes of Health, Bethesda, MD, USA), the percentage of TNF- α labelled brown was calculated in four areas in 1000x magnification of samples.

Histopathological Examination

Samples were dehydrated in graded ethanol before being embedded in paraffin after being fixed for a week at room temperature in 10% (wt/vol) PBS-buffered formaldehyde. After that, 50 μ m sections were deparaffinized with xylene and stained with hematoxylin-eosin (HE) (Elabscience Biotechnology, Wuhan, China) for light microscopic examination. Histopathological examinations were performed by a pathologist blinded to the study design, and

photographs were taken with an Olympus CX23 microscope (Olympus, Tokyo, Japan). Ileal villus height was measured at 400x magnification in a minimum of 20 well-preserved villus in each randomly selected sample from each tissue block with ImageJ. Measurement was conducted by one certified pathologist who was blinded to this study design. The pathologist examined each sample in 4 different area using an objective tool from ImageJ software to reduce bias.

Statistical Analysis

Scores of TNF- α expression and villus height were presented as mean \pm SD and compared by Kruskal-Wallis variance analysis. Differences between groups were analysed with the Mann-Whitney U test. The correlation was tested by the Spearman correlation test. Statistical analyses were performed with the Statistical Package for the Social Sciences (SPSS), version 26.0 for Windows (IBM Corporation, Armonk, NY, USA). A $p < 0.05$ was considered to be statistically significant.

Results

TNF- α Level

Non-parametric Kruskal-Wallis test showed there was a significant difference in TNF- α level in all rats ($p=0.023$) (Table 1). Post-Hoc Mann-Whitney U test showed that there was a significant reduction in TNF- α expression in group T2 ($p=0.047$) and group T3 ($p=0.004$) compared to group C, and there was no significant difference ($p > 0.05$) between the treatment groups (Table 2). Figure 1 showed TNF- α immunohistochemical staining results. The mean expression of TNF- α expression in group T3 was 2.1 ± 1.4 . Furthermore, it can be concluded that the optimum dose of UDCA-glutathione combination was 30 mg UDCA + 20 mg glutathione, as given to group T3.

Ileal Villus Height

The intestinal morphology samples were examined with HE stains under light microscopic examination (Figure 2).

Table 1. Mean TNF- α expression and mean ileal villus height.

Groups	Mean TNF- α Expression (%)	Mean Ileal Villus Height (μ m)
UDCA only (Group C)	11.4 \pm 1.6	177 \pm 11.1
10 mg UDCA + 10 mg Glutathione (Group T1)	7.7 \pm 3.5	225 \pm 13.7*
20 mg UDCA + 15 mg Glutathione (Group T2)	3.9 \pm 2.1*	237 \pm 13.2*
30 mg UDCA + 20 mg Glutathione (Group T3)	2.1 \pm 1.4*	241 \pm 12*

* $p < 0.05$ is considered as significant compared to Group C, tested with Kruskal Wallis test.

Table 2. Mean difference analysis of TNF- α expression between groups.

Groups	UDCA Only (Group C)	10 mg UDCA + 10 mg Glutathione (Group T1)	20 mg UDCA + 15 mg Glutathione (Group T2)	30 mg UDCA + 20 mg Glutathione (Group T3)
UDCA only (Group C)	-	0.084	0.047*	0.004*
10 mg UDCA + 10 mg Glutathione (Group T1)	0.084	-	0.337	0.141
20 mg UDCA + 15 mg Glutathione (Group T2)	0.047*	0.337	-	0.564
30 mg UDCA + 20 mg Glutathione (Group T3)	0.004*	0.141	0.564	-

* $p < 0.05$ is considered as significant, tested Post Hoc Mann-Whitney U test.

Non-parametric Kruskal-Wallis test showed there was a significant difference in TNF- α level in all rats ($p=0.013$) (Table 1). Post Hoc Mann-Whitney test showed that there was a significant difference in ileal villus height ($p < 0.05$) in group T1 ($p=0.018$), group T2 ($p=0.013$), and group T3 ($p=0.006$) compared to the UDCA only group (Table 3).

Using the Spearman correlation test, TNF- α expression was found to be significantly correlated with ileum villus height ($p=0.023$).

Discussion

Recent developments in the pathophysiology of intestinal failure in cholestasis have demonstrated that disturbance of the immunologic, biological, mechanical, and biochemical barriers is just one of several factors that contribute to the gut barrier being breached.(4) Obstructive jaundice decreases the number of T lymphocytes in the gut by inhibiting the ability of Kupffer cells to remove debris and the activity of natural killer cells. Inflammatory bowel illness and primary sclerosing cholangitis (PSC) are intimately related, emphasizing the significance of the gut-hepatic axis. Ligation of the common bile duct impairs intestinal function

and alters intestinal mucosal structure by decreasing villus height and mucosal crypt density terminal ileum.(22)

The data seen in the UDCA-only group were considerably worse than the mean mucosal height of the UDCA-glutathione group. This is most likely caused by the additive antioxidant properties of glutathione and UDCA. In a mouse model of colonic inflammation, UDCA prevents intestinal inflammation *in vivo* by preventing epithelium apoptosis and enhancing barrier function.(23) UDCA also acts as a choleric, hepatocyte cell protector, anti-apoptotic, and antioxidant.(24)

As a result, glutathione frequently targets ROS in uncatalyzed and catalysed processes. Unbalances in the expression of glutathione and related enzymes are implicated in a range of situations because ROS has a definite role in cell signalling events as well as in human illnesses.(25) Glutathione has a crucial cytoprotective role, based on its mechanism of action as an antioxidant.(26) Research on the use of antioxidants in cholestasis was previously conducted with pomegranate in rats undergoing ligation of the common bile duct. In this study, the mean villus height in the treatment group was higher than in the control group ($p=0.010$). The thickness of the mucosa of the ileal samples increased in the group that received honey and immune nutrients compared

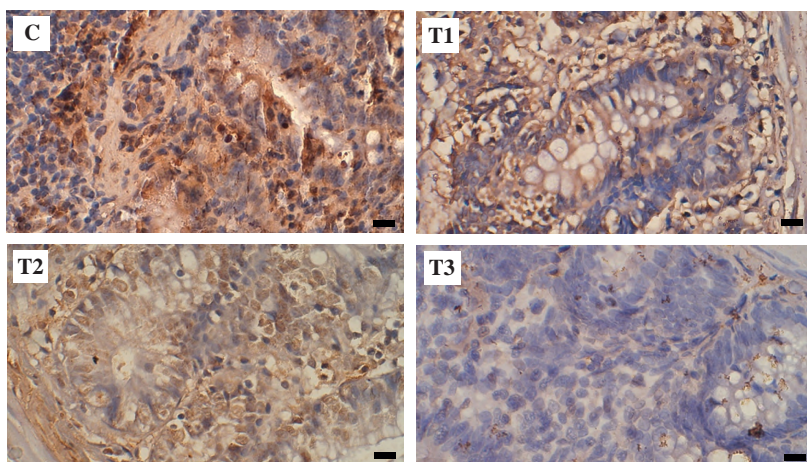


Figure 1. TNF- α immunohistochemical staining of terminal ileum in each group at 400x magnification. C: rats given 20 mg UDCA supplementation; T1: rats given 10 mg UDCA and 10 mg glutathione supplementation; T2: rats given 20 mg UDCA and 15 mg glutathione supplementation; T3: rats given 30 mg UDCA and 20 mg glutathione supplementation. Black bar: 100 μ m.

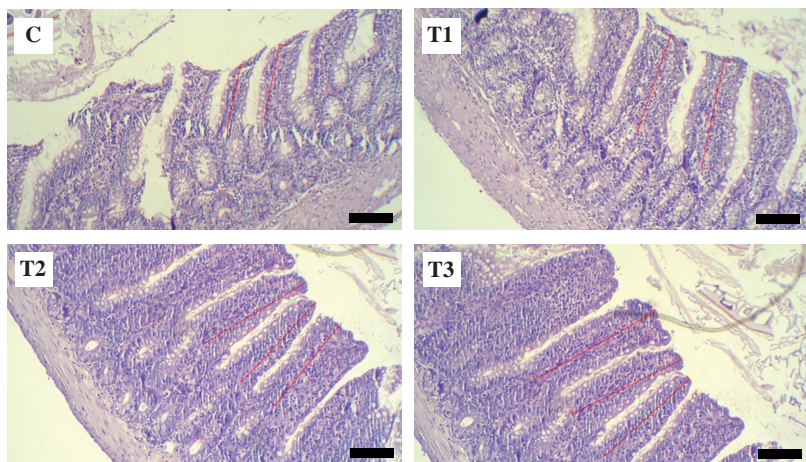


Figure 2. Terminal ileum with HE staining in each group at 1000x magnification. C: rats given 20 mg UDCA supplementation; T1: rats given 10 mg UDCA and 10 mg glutathione supplementation; T2: rats given 20 mg UDCA and 15 mg glutathione supplementation; T3: rats given 30 mg UDCA and 20 mg glutathione supplementation. Black bar: 100 μm .

to the control group. When compared to the other groups, the groups who received immunonutrients and honey had considerably lower alanine aminotransferase (ALT) levels. Mean ileum mucosal thickness has significantly increased in both the honey and immunonutrient group and the control group.(24) Obstructive jaundice induced intestinal oxidative stress, which play a key role in intestinal barrier failure and the development of septic complications. Inhibition of proliferation and promotion of apoptotic death of enterocyte was the main cause of intestinal mucosal atrophy in obstructive jaundice as resulted from, possibly, ROS.(4)

In our study, there was a significant decrease in TNF- α expression in cholestatic mice in the UDCA and glutathione combination group compared to the UDCA-only group. UDCA has the ability in reducing inflammation and apoptosis during cholestatic conditions. Intravenous administration of UDCA on endotoxemia with obstructive jaundice could provide protective effect by promoting biliary lipopolysaccharide (LPS) excretion via hepatocytes. However, UDCA did not affect serum TNF- α levels and Kupffer cells.(27) Administration of UDCA alone was inadequate in reducing inflammation, especially TNF- α

levels, so additional therapy regimens were needed.(28) TNF- α and interleukin (IL)-1 proinflammatory cytokine levels, nitric oxide (NO) concentration, and oxidative stress indicators malondialdehyde (MDA) and 3-NT were all elevated. The therapy with glutathione lowered each of those. In rats, glutathione supplementation prevented obstructive jaundice-induced vascular hypo responsiveness and attenuated overexpressed ONOO(-) from the interaction of excessive NO with O $^{2-}$.(29) The protective effect of glutathione on liver injury may be mediated by regulation of the excessive inflammatory response and its cascade, according to the considerable reduction in TNF- α and IL-1 production after glutathione supplementation.(21)

Patients with inflammatory diseases of the stomach have significantly higher TNF- α levels.(30,31) It is widely known that intestinal tight junction permeability is increased by TNF- α both *in vivo* and *in vitro*, allowing for greater intestinal penetration of luminal antigens.(30) Anti-TNF medication causes the intestinal barrier to retighten, and normalizing intestinal permeability is linked to sustained clinical remission.(32) TNF- α regulates the proliferation and death of ileal mucosal epithelial cells through NFB activation and apoptotic pathways. TNF- α plays an essential

Table 2. Mean difference analysis of ileal villus height between groups.

Groups	UDCA Only (Group C)	10 mg UDCA + 10 mg Glutathione (Group T1)	20 mg UDCA + 15 mg Glutathione (Group T2)	30 mg UDCA + 20 mg Glutathione (Group T3)
UDCA only (Group C)	-	0.018*	0.013*	0.006*
10 mg UDCA + 10 mg Glutathione (Group T1)	0.018*	-	0.338	0.337
20 mg UDCA + 15 mg Glutathione (Group T2)	0.013*	0.338	-	0.949
30 mg UDCA + 20 mg Glutathione (Group T3)	0.006*	0.337	0.949	-

* $p < 0.05$ is considered as significant, tested Post Hoc Mann-Whitney U test.

role in the pathogenesis of inflammatory bowel disease (IBD), infection, and intestinal wound healing.(33)

To maintain epithelial function, intestinal stem cells in the crypt divide to produce progenitor cells or daughter cells that develop along the crypt-villus axis into secretory and absorptive lineage cells. A constant replacement of differentiated cells placed within the crypt base with the migration of these cells down the crypt-villus axis allows intestinal epithelial cell layers to maintain a balance between cell proliferation at the base of the crypts and cell death at the villus tips.(34) The secretory Paneth cells were the only cells that did not move towards the villus ends and remained in the Lieberkühn crypts and persisted for more than three weeks.(35) Increasing TNF- α would increase the release of epithelial cells at the tips of intestinal villi and interfere with the work of tight junctions and intestinal permeability. (36) The interaction of TNF- α with the TNF receptor causes apoptosis in intestinal mucosal cells, and a decrease in villus height in high-grade burns is associated with severe inflammation.(37)

This study gave an insight of future therapeutic pathway in management of cholestasis through combination of glutathione and UDCA. However, further studies are needed to evaluate the effect of glutathione and UDCA combination on other inflammatory mediators and the dynamic changes in a time series study. The UDCA-glutathione combination might be used to prevent the harmful effects of cholestasis in clinical settings.

Conclusion

The combination of UDCA and glutathione improved ileum mucosal villus height by downregulating TNF- α expression in cholestasis.

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Authors Contribution

JANH was involved in the planning of the study, collected the data, performed the analysis, and wrote the manuscript;

YWP, CHNP, US, NS gave critical revision of the draft for important intellectual content and finally approved the manuscript. All authors read and approved the final manuscript.

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