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Effect of Curcumin on Interleukin-6 Expression and Malondialdehyde Levels in Liver Fibrosis

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Abstract

BACKGROUND: Chronic inflammation and excessive oxidative stress are the main mechanisms causing liver fibrosis. It releases anti-inflammatory cytokines, namely, interleukin 6 (IL-6), nitric oxide, and malondialdehyde (MDA). Curcumin acts as an anti-inflammatory, antioxidant, and antifibrotic.

AIM: This study is aimed to analyze differences in IL-6 expression and MDA levels in (Deutschland, Denken, and Yoken) DDY mice with liver fibrosis after common bile duct ligation between the curcumin-treated and control groups.

METHODS: This research is an experimental study with a post-test-only control group design. Seventy-five male DDY mice 20–30g were used in this study (n = 5). Mice were randomly divided into five groups, each consisting of 15 mice. The first group healthy control (HC) was the HC group given phosphate-buffered saline (PBS) solution and did not perform the CBDL. The second group negative control (NC) was a NC group given PBS solution and completed the CBDL. The third group positive control (PC) was a PC group given oral ursodeoxycholic acid (UDCA) and performed CBDL. The fourth group (P1) was assigned oral curcumin and performed the CBDL. The fifth group (P2) was given oral curcumin and UDCA and performed the CBDL. Seven, fourteen, and 21 days after ligation, liver samples were taken to examine IL-6 expression and MDA levels.

RESULTS: There was a significant difference between the NC and PC groups (p = 0.00), NC and P1 (p = 0.00), NC with P2 (p = 0.00), PC with P1 (p = 0.04), PC with P2 (p = 0.04), on day 21 between the NC and PC groups (p = 0.00), NC with P1 (p = 0.00), and PC with P2 (p = 0.00). Statistical analysis of the comparison of MDA levels on days 7 and 14 found no significant difference. On day 21, there was a substantial difference between the NC group and P1 (p = 0.02).

CONCLUSION: This study concluded that curcumin effectively reduced IL-6 expression and MDA levels in liver fibrosis.

Introduction

Mesenchymal cell populations in the liver may get activated and grow as a result of liver damage, which will alter the extracellular matrix as a part of the body's response to healing a lesion. Chronic injury leads proteins buildup resulting in scar tissue formation (fibrosis) [1]. This has become a significant health problem because it can affect the mortality rate of more than 1.5 million people globally. The final stages of death are generally cirrhosis and liver cancer [2].

Liver fibrosis results from excessive accumulation of extracellular matrix proteins, including collagen, in the extracellular space. Activated hepatic stellate cells (HSC) are the primary source of collagen products that can cause an imbalance between the formation and degradation of the extracellular matrix in the liver tissue. Chronic tissue injury, oxidative stress, inflammatory cytokines, and apoptotic signals can activate HSC, causing changes in transport function and membrane permeability of hepatocytes [1]. Interleukin-6 (IL-6) has been recognized as an essential pro-inflammatory cytokine whose expression is associated with organ inflammation. Serum and intrahepatic IL-6 levels were also significantly increased in patients with acute and chronic liver disease [3]. Malondialdehyde (MDA) is a marker used to measure liver damage. MDA results from lipid peroxidation caused by increased free radicals resulting from liver cell damage [4].

Liver fibrogenesis in humans is a clinical problem that often occurs worldwide, so several experimental studies of rodent models with acute and chronic liver failure have been carried out over the last few decades. Ligation of the common duct in rodents has been performed as an experimental procedure in research for many years. In this protocol, cannulation/obstruction and ligation cause hepatic fibrosis in rodents with morphological changes similar to those observed in humans [5].

Ursodeoxycholic acid (UDCA) is a hydrophilic dihydroxy bile acid [6]. UDCA works by replacing endogenous hepatotoxic bile acids that facilitate apoptosis, minimize their toxicity and cause a reduction

in oxidative stress and liver injury [7]. The hydrophilic nature of UDCA protects liver cells from damage due to bile accumulation [8].

The previous studies found that fibrotic inflammatory signals such as IL-6 decreased with curcumin and UDCA supplementation. These profibrotic markers are implicated in the development of fibrosis. These facts have prompted researchers to seek hepatic fibrosis preventive interventions. Curcumin is relatively safe in humans and bioavailable enough to produce beneficial systemic and hepatic effects. This study analyzed differences in IL-6 expression and MDA levels in mice with liver fibrosis after CBDL between the curcumin-treated and the control group.

Materials and Methods

Study design and animal

The study was experimental with a randomized "post-test only control group design." Samples were 75 Deutschland, Denken, and Yoken (DDY) mice with inclusion criteria: Male, body weight is about 20–30 g. Animal care and intervention were carried out in IRatco, Bogor, Indonesia. The process took 1 month on August 2021.

The mice were caged in Ventilated Cages Air model cages with temperatures between 22 and 25°C, humidity 55–67%, placed in population cages, per cage consisting of five mice. All mice were acclimatized to the laboratory conditions for 7 days before the actual study. The mice were terminated in days 7, 14, and 21 to evaluate the dynamic changes of pro inflammatory mediators and the effect of curcumin and its combination with UDCA on IL-6 and MDA on liver fibrosis.

The Ethical Committee clearance for experimental protocol from the Faculty of Medicine, University of Diponegoro, Kariadi Hospital Semarang, Indonesia (97/EC/H/FK-UNDIP/VIII/2021). The animals care criteria, prepared by the National Academy of Sciences and outlined in the Guide for the Care and Use of Laboratory Animals, were applied throughout the experiment.

Materials

Male DDY mice (10 weeks, 20–30 g) were purchased from the IRatco Laboratory, Bogor, Indonesia. Curcumin (curcuminoid content ≥94%), common chemicals, and protease inhibitors were obtained from Sigma Chemical Company (St Louis, MO). UDCA was purchased from Dexa Medica Company (Palembang, Indonesia). IL-6 assay kit was purchased from Abcam (CA, USA). Kit antibody IHC detection MDA assay kit was purchased from Elabscienc (Houston, Texas).

Induction of liver fibrosis and treatment

Seventy-five mice have randomly divided into five groups with 15 mice in each group: The first group was a healthy control group given 0.5 ml of phosphate buffer saline (PBS) solution/day for 7 days until the study sample was taken. The second group was the negative control (NC) group, given 0.5 ml of PBS solution/day for 7 days, and did the common bile duct ligation, and then the solution was given again during the study. The third group was the positive control (PC) group, given oral UDCA 80 mg/kg in 0.5 ml of saline/day for 7 days, performed CBDL, and the solution was given again during the study. The fourth treatment group (P1) was assigned oral curcumin 200 mg/kg in 0.5 ml saline/day for 7 days and performed CBDL; the solution was given again during the study. The fifth treatment group (P2) was assigned oral curcumin 200 mg/kg in 0.25 ml/day saline and oral UDCA 80 mg/kg in 0.25 ml/day saline for 7 days performed CBDL, then the solution was given back during the study. CBDL was done in 7th day after the treatment was initiated. Every group that received UDCA treatment was given UDCA straight after the acclimation period was complete (1st day after acclimation). After the CBDL procedure in the NC, PC, P1, and P2 groups, all mice were re-enclosed and received standard feed and drink as in the initial treatment.

Preparation of sample

Seven days after common bile duct ligation, five mice in each group were terminated. Liver tissue was processed to the paraffin blocks, cut approximately 4 microns, and stained with immunohistochemistry to evaluate the IL-6 expression. Quantification is done by program image analysis and color intensity inspection. MDA levels were examined and measured from liver tissue using the TBARs (thiobarbituric acid reactive substances) method, the tool used is spectrophotometry. This examination was also carried out on the 14th and 21st days.

Quantification of Stained Cells

To allow quantification of the whole slide, the complete area of the slide was counted using the KS300 versus 3.0 software from Carl Zeiss Vision GMBH, Jena, Germany. Stained cells were counted as follows: All sections were scanned at ×100 magnification and the total area and the stained area of each section were measured. Then the total stained area of the slide was measured by semi-automatic counting. The area of immunoreactive cells was expressed as the percentage of stained area out of the total area of the section.

Data management

As all data IL-6 and MDA were normally distributed, the data were analyzed using

one-way ANOVA and post-hoc LSD (Least Significant Difference) test, with significant consideration if $p < 0.05$. Correlation test between the level of the expression of IL-6 with MDA levels with the Pearson statistical test.

Results

All experimental animals were alive and able to follow this study during the study period, from the adaptation period to the 4th week. All mice were terminated on days 7th, 14th, and 21st after ligating the common bile duct. Liver fibrosis was positive in all samples performed by ligating the common bile duct. Liver fibrosis process was confirmed through the results of Il-6 and MDA expression in control group in days 7, 14, and 21 after treatment was initiated. The results show that IL-6 and MDA expression is significantly higher in control group that was treated using CBDL. The body weight was homogenous.

IL-6 expression 7 days

The mean ± SD of IL-6 on healthy group, NC group, control group, Group P1, and Group P2 were, respectively, 0 ± 0, 78,82 ± 5.06, 76.77 ± 2.26, 74.84 ± 0.41, and 74.92 ± 3.31 (ANOVA = 0.00). *Post hoc* LSD tests were also significantly different between healthy group versus NC group (p = 0.00), healthy group versus control group (p = 0.00), healthy group versus Group P1 (p = 0.00), and healthy group versus Group P2 (p = 0.00) (Figure 1).

IL-6 expression 14 days

The mean ± SD of IL-6 on healthy group, NC group, control group, Group P1, and Group P2 were, respectively, 0 ± 0, 72.98 ± 2.87, 45.97 ± 5.86, 53.59 ± 4.18, and 56.32 ± 5.68 (p value = 0.00). *Post hoc* LSD test also significantly different between healthy group versus NC group (p = 0.00), healthy group versus control group (p = 0.00), healthy group versus Group P1 (p = 0.00), healthy group versus Group P2 (p = 0.00), NC group versus control group (p = 0.00), NC group versus P1 group (p = 0.00), NC group

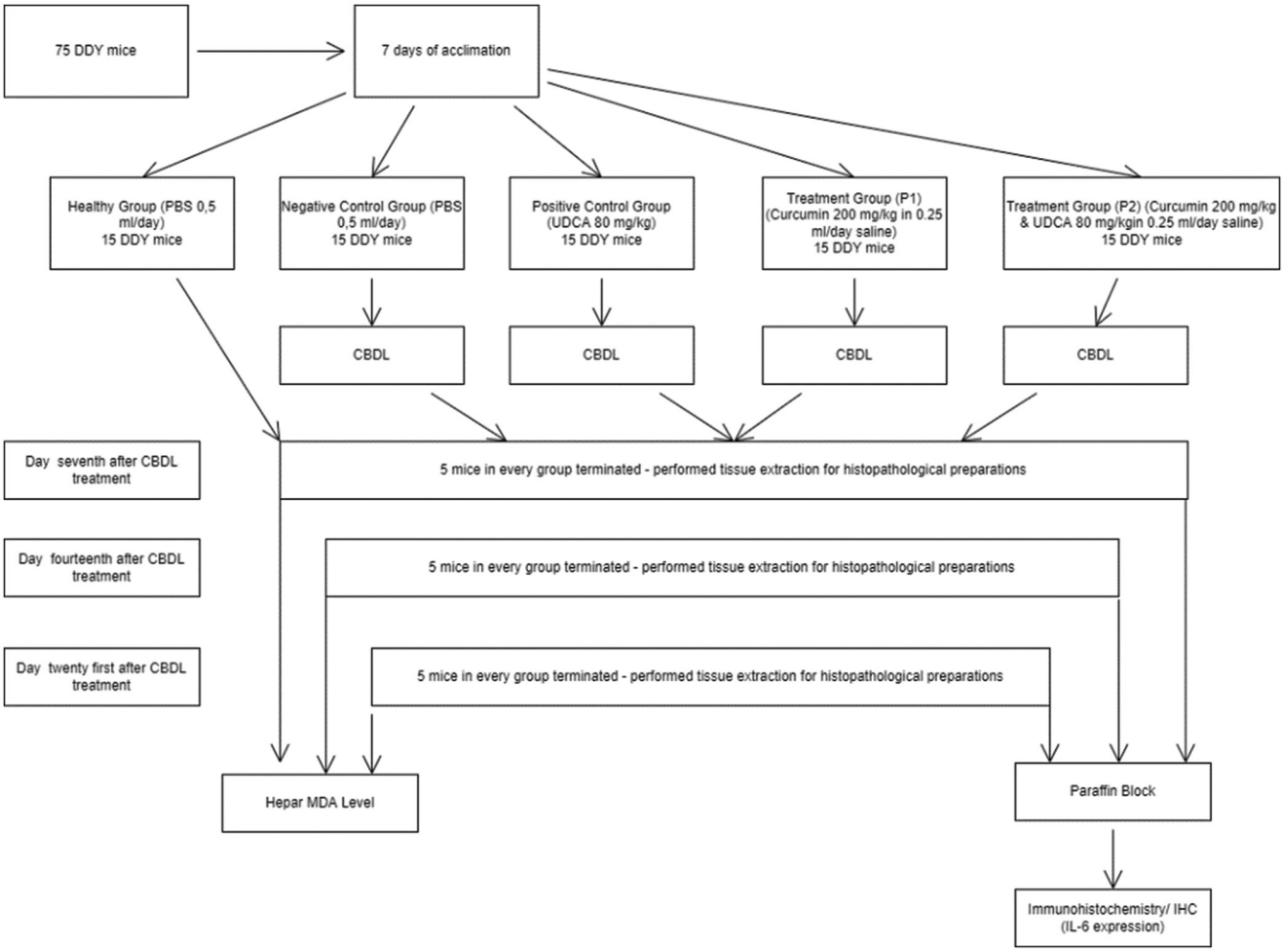

Figure 1: Research workflow

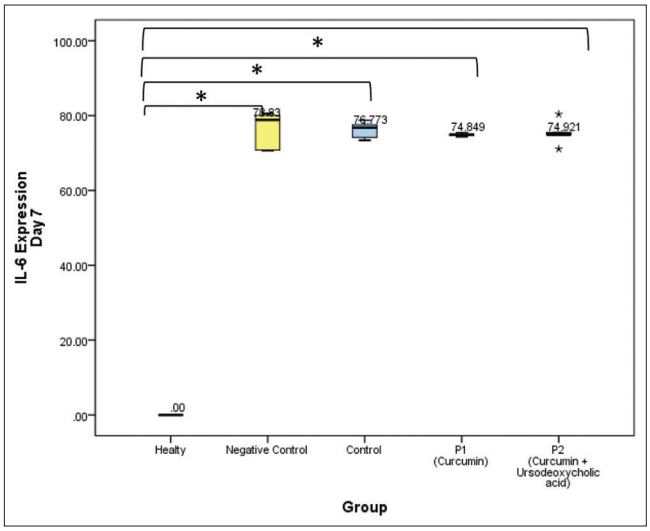

*Figure 2: IL-6 expression day 7 of healthy, negative, control, curcumin, and combination of curcumin and UDCA groups. One-way ANOVA p = 0.00. Post hoc LSD: * p < 0.05*

versus P2 group (p = 0.00), control group versus P1 group (p = 0.04), and control group versus P2 group (p = 0.04) (Figure 2).

IL-6 expression 21 days

The mean ± SD of IL-6 on healthy group, NC group, control group, Group P1, and Group P2 were 0 ± 0, 77.60 ± 2.76, 31.16 ± 11.43, 33.46 ± 10.44, and 50.18 ± 14.92 (ANOVA = 0.00). *Post hoc* LSD test also significantly different between healthy group versus NC group (p = 0.00), healthy group versus control group (p = 0.00), healthy group versus Group P1 (p = 0.00), healthy group versus Group P2 (p = 0.00), NC group versus control group (p = 0.00), NC group versus P1 group (p = 0.00), and NC group versus P2 group (p = 0.00) (Figure 3).

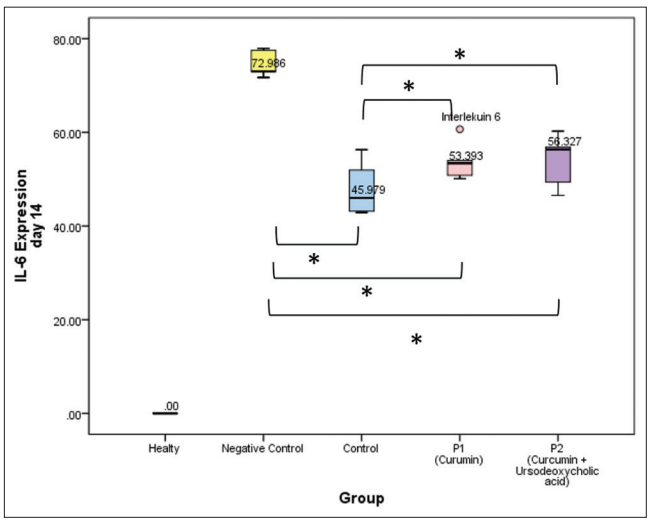

*Figure 3: IL-6 expression day 14 of healthy, negative, control, curcumin, and combination of curcumin and UDCA groups. One-way ANOVA p = 0.00. Post hoc LSD: * p < 0.05*

MDA Level 7 days

The mean ± SD of MDA levels in the healthy group, NC group, control group, Group P1, and Group P2 were 13.91 ± 1.43, 34.59 ± 7.19, 31.43 ± 3.92, 31.43 ± 3.56, and 29.28 ± 8.08 (ANOVA = 0.00). *Post hoc* LSD tests were also significantly different between healthy group versus NC group (p = 0.00), healthy group versus control group (p = 0.00), healthy group versus Group P1 (p = 0.00), and healthy group versus Group P2 (p = 0.00) (Figure 4).

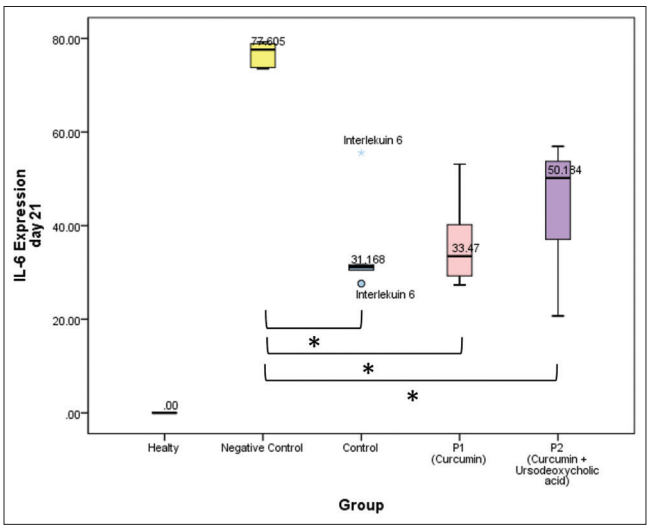

*Figure 4: IL-6 expression day 14 of healthy, negative, control, curcumin and combination of curcumin and UDCA groups. One-way ANOVA p = 0.00. Post hoc LSD: * p < 0.05*

MDA Level 14 days

The mean ± SD of MDA levels in the healthy group, NC group, control group, Group P1, and Group P2 were 12.96 ± 1.87, 29.54 ± 9.59, 28.80 ± 5.37, 26.10 ± 4.43, and 30.28 ± 3.47 (ANOVA = 0.00). *Post hoc* LSD tests were also significantly different between healthy group versus NC group (p = 0.00), healthy group versus control group (p = 0.00), healthy group versus Group P1 (p = 0.00), and healthy group versus Group P2 (p = 0.00) (Figure 5).

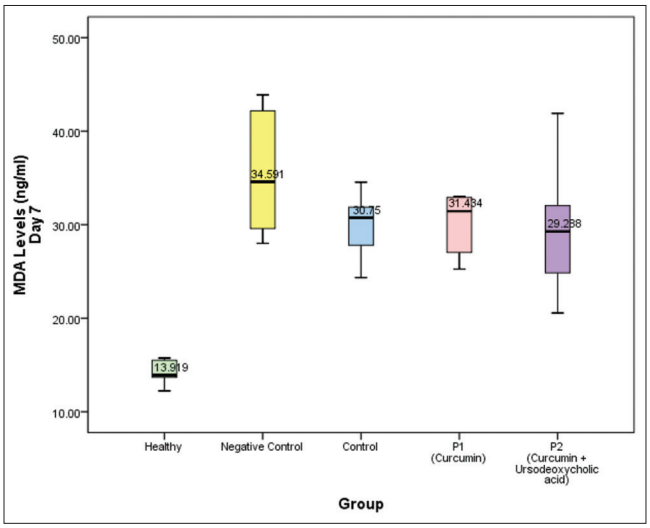

*Figure 5: MDA Levels Day 7 of healthy, negative, control, curcumin, and combination of curcumin and UDCA groups. One way ANOVA p = 0.00. Post hoc LSD: * p < 0.05*

MDA Level 21 days

The mean ± SD of MDA levels in the healthy group, NC group, control group, Group P1, and Group P2 were 11.93 ± 2.81, 41.23 ± 10.60, 27.70 ± 4.22, 21.02 ± 4.42, and 28,02 ± 3.73 (ANOVA = 0.00). *Post hoc* LSD test was also significantly different between healthy group versus NC group (p = 0.00), healthy group versus control group (p = 0.00), healthy group versus Group P1 (p = 0.00), healthy group versus Group P2 (p = 0.00), and NC group versus P1 group (p = 0.02) (Figure 6).

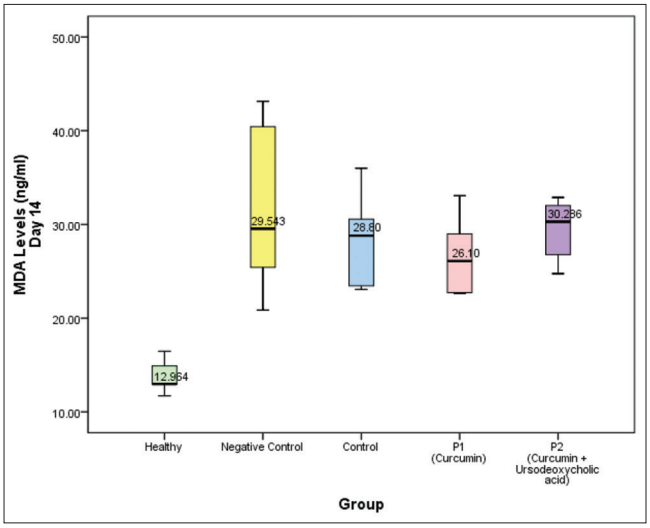

*Figure 6: MDA Levels Day 14 of healthy, negative, control, curcumin, and combination of curcumin and UDCA groups. One-way ANOVA p = 0.00. Post hoc LSD: * p < 0.05*

IL-6 immunohistochemistry

The immunohistochemistry reaction and digital quantification evaluate the inflammatory process of liver fibrosis by analyzing the interleukins profile (Figure 7). An increase in the expression of IL-6 was noted during the fibrosis process. We found that in the curcumin group, IL-6 and fibrotic liver significantly decreased 21 days after the CBDL compared to the control groups (Figure 8).

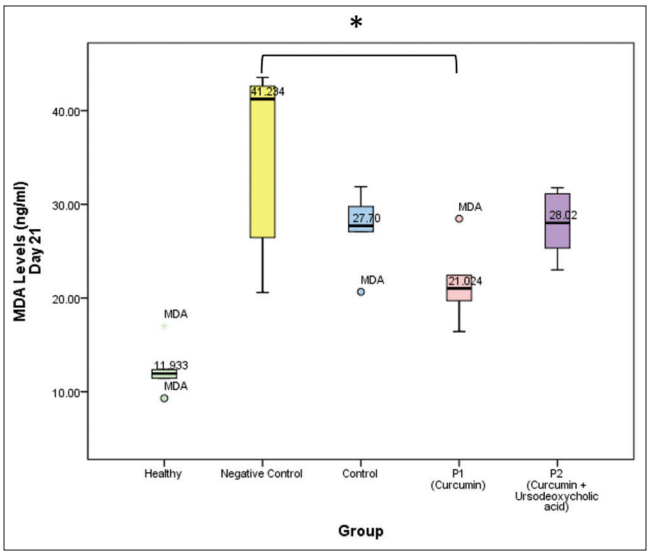

*Figure 7: MDA Levels Day 21 of healthy, negative, control, curcumin, and combination of curcumin and UDCA groups. One-way ANOVA p = 0.00. Post hoc LSD: * p < 0.05*

Correlation between IL-6 expression and MDA levels

The correlation test between IL-6 expression and MDA levels showed a strong correlation (r = 0.6–0.8) between IL-6 expression and MDA levels with p < 0.05 (Table 1).

Table 1: Correlation between Interleukin-6 expression and malondialdehyde levels

Time	n (Mice)	Variable	MDA levels (%)	
			r (correlation coefficient)	p
Day 7	25	IL-6 expression (%)	0.78	0.00
Day 14	25		0.78	0.00
Day 21	25		0.75	0.00

*p<0.05 (significant), Pearson correlation test. MDA: Malondialdehyde, IL-6: Interleukin 6.

Discussion

IL-6 is a pro-inflammatory cytokine, where an overproduction of this cytokine indicates inflammation. Inflammation is the initial stage of a series of fibrosis. When IL-6 increases, this will impact acute-phase protein (APP) synthesis and HSC activation. Liver fibrosis is caused by increased collagen production mediated by APP and the cytokine IL-6 [9].

This study showed a significant difference in the expression of IL-6 in the NC and P1 groups. The P1 group given curcumin 200 mg/kg/day was the leading group considered in this study. From the results of this study, it can be concluded that curcumin 200 mg/kg/day affects IL-6 expression with a tendency for lower values than NC on days 7, 14, and 21. And IL-6 faces a trend for lower values than PC in the 7th and 14th-day groups [10]. Curcumin has an antifibrotic role, which is through the mechanism of reducing the expression of pro-inflammatory mediators such as TNF-α (Tumor Necrosis Factor-alpha), IL-6, and MCP-1 (Monocyte chemoattractant protein-1) through downregulation of high mobility group box-1 protein, TLR4 (transmembrane protein), and TLR2 expression in mice with liver fibrosis [11]. According to research by Azam *et al*. (2018), curcumin has an antioxidant and anti-inflammatory function, inhibiting oxidative stress, apoptosis, and NF-kB (nuclear factor kappa-light-chain-enhancer of activated B cells) activation. NF-kB will stick to the deoxyribonucleic acid (DNA) wall, forming an NF-kB DNA-Binding complex. The NF-kB DNA-binding complex then regulates the release of cytokines, including TNF-α and IL-6. Curcumin inhibits NF-kB DNA-binding by releasing inflammatory cytokines, thereby inhibiting the inflammatory response

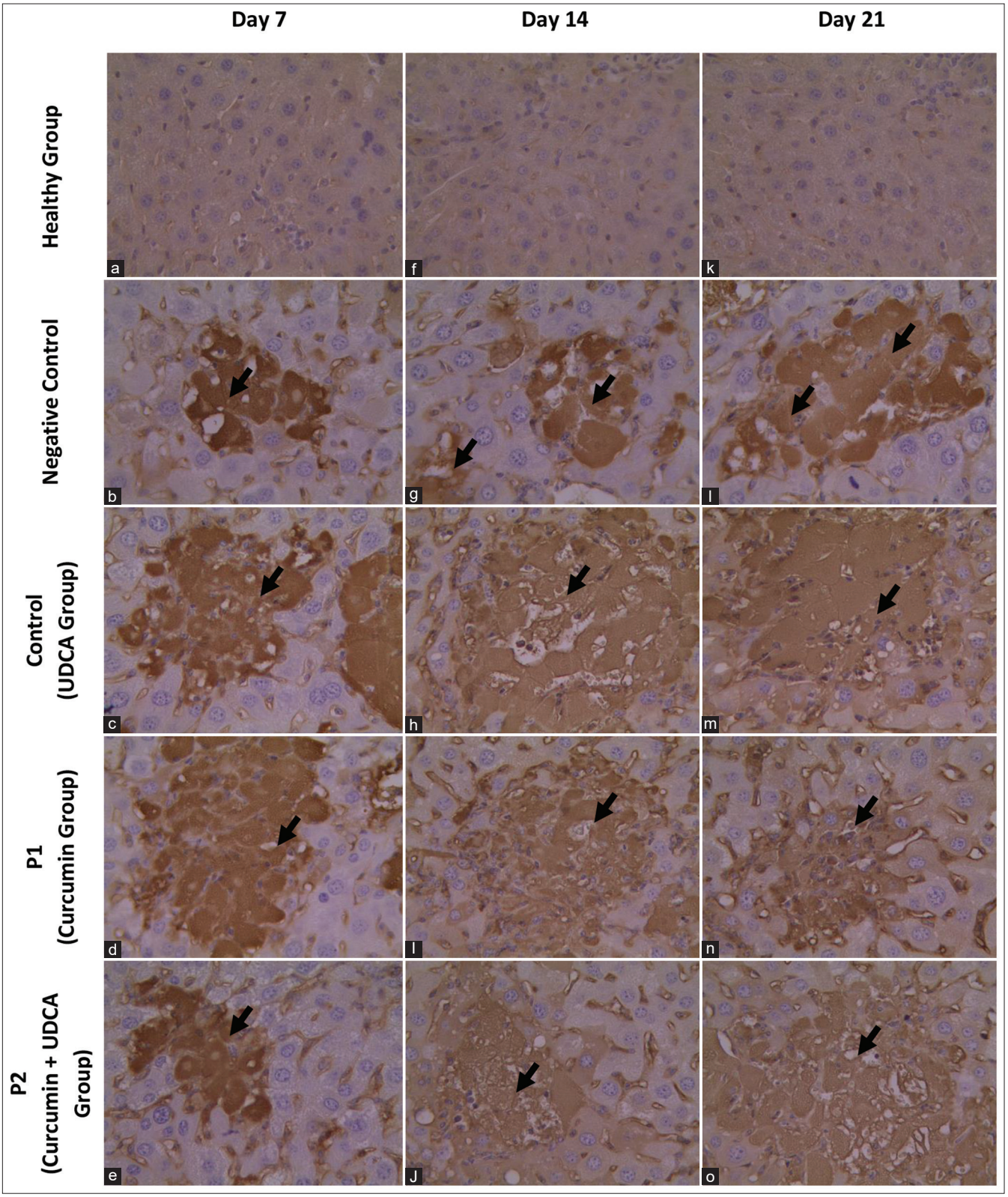

Figure 8: IL-6 Immunoexpression Immunohistochemistry reaction in the liver fibrosis after CBDL (7, 14, and 21 days) staining with ×100. Healthy Groups A, F, and K (samples obtained from mice did not CBDL procedure). The NC group exhibited high reactivity to IL-6 (B, G, and L). UDCA groups showed reduced reactivity for IL-6 (C, H, and M). Curcumin groups exhibited reduced reactivity for IL-6 (D, I, and N). Curcumin + UDCA groups showed reduced reactivity for IL-6 (E, J, and O)

cascade [12]. The anti-inflammatory effect of curcumin is expected to be a therapy in accelerating the healing process of liver fibrosis and providing good outcomes.

The results of this study were that the administration of UDCA or the combination of UDCA and curcumin compared to the control group had a decreasing effect on IL-6 with $p < 0.05$. UDCA has

an anti-apoptotic effect because UDCA can activate the anti-apoptotic pathway and reduce the results of the injury on hepatocyte cells. According to Wan et al. (2018), UDCA can reduce pro-inflammatory cytokine expression within 24 h after administration [13]. Among these are TNF-α, IL-1, and IL-6.

Administration of a combination of curcumin 200 mg/kg/day and UDCA 80 mg/kg/day also exerted an antioxidant effect and anti-inflammatory response, according to a study by Gheibi *et al*. (2019). A survey by Farashbandi *et al*. (2018) reported that a combination of curcumin 100 mg/kg/day and UDCA 20 mg/kg/day has a therapeutic effect on alcohol-induced acute liver injury by inhibiting oxidative stress and tissue inflammation [14], [15].

This study showed that the expression of IL-6 in the NC group was significantly different compared to the P1 and P2 groups ($p < 0.05$) on days 14 and 21. Administration of UDCA and its combination with curcumin in this study did not provide a significant difference in the expression of IL- 6 when compared with curcumin alone. This is slightly different from the survey conducted by Gheibi *et al*. Where the results of the combination of curcumin and UDCA at the same dose in this study gave better results (judging by SGOT, SGPT, and Caspase III) than curcumin alone or UDCA alone in mice with NAFLD (non-alcoholic fatty liver disease). This may differ due to the different hepatotoxicity. In contrast, the current study looked at hepatotoxicity due to obstruction of the secondary common bile duct, whereas the study by Gheibi *et al*. looked at alcohol-induced hepatotoxicity [16].

Free radicals can cause oxidative stress. Oxidative stress occurs due to an imbalance between oxidants and antioxidants, which can cause cell damage and is reported to play an essential role in the process of liver damage [17]. Increased production of reactive oxygen species (ROS) causes cell damage to the lipid portion of the membrane, NCown as lipid peroxidation reaction. Increased ROS in cell membranes increases the formation of MDA [18]. MDA is a product of lipid peroxidation by free radicals in the body, which is one of the indicators to determine oxidative stress in the body and is a biomarker of oxidative stress.

This study showed significant differences in MDA levels in the NC and P1 groups. The P1 group given curcumin 200 mg/kg/day was the leading group considered in this study. From the results of this study, it can be concluded that curcumin 200 mg/kg/day affects MDA levels with a tendency for lower values than NC on days 7, 14, and 21. In this study, the mean expression of IL-6 on day 21 was higher. Lower compared to the 7^{th} and 14^{th}-day groups.

Likewise, Azam *et al*. (2018) found that curcumin can inhibit lipid peroxidation initiated by free radicals. Curcumin therapy can reduce MDA levels by donating a hydrogen atom (H) from the phenolic hydroxyl (OH) group when reacting with free radicals (R*) [19].

Research conducted by Li *et al*. revealed that curcumin could reduce oxidative stress and inhibit the transcription of genes associated with oxidative stress and inflammatory responses. It was also reported that using curcumin as a pre-treatment can cause a decrease in lipid peroxidation in hepatocytes and decrease free radical-induced DNA damage [20].

This study showed that NC with P1 compared to the control group had a significant decreasing effect on MDA levels with $p < 0.05$. MDA levels in the current study even found substantial differences between NC and P2, P1 and P2. The visible trend is the lower MDA levels in the P1 group than in the PC or P2 groups.

Farashbandi *et al*. reported that UDCA could inhibit ROS formation in Kupffer cells to reduce oxidative stress in liver cells. UDCA also increased superoxide dismutase and catalase levels and decreased MDA and nitric oxide (NO) levels in CCLI4-induced mice by creating a protective effect on liver tissue [15]. The results in this study are also in line with the survey by Gheibi *et al*. They reported a significant decrease in MDA values with the combined administration of UDCA and curcumin [16]. Salman *et al*. also found a protective and therapeutic effect of the combination of curcumin and UDCA is mediated by reducing oxidative stress and tissue inflammation [21].

This study showed that the MDA levels in the NC group were significantly different from those in the P1 group ($p < 0.05$) on day 21. In this study, the administration of UDCA and its combination with curcumin did not significantly differ in MDA levels compared with the administration of curcumin alone. Research by Gheibi *et al*. (2019) with the same dose as this study showed that the combination of UDCA and curcumin gave significant results. However, it was not found that curcumin gave better results on SGOT, SGPT, p53, and Caspase III. This difference could be due to differences in the onset of fibrosis for different causes of hepatotoxicity (NAFLD, common bile duct ligation, or alcohol), differences in observation time, and different doses of curcumin or UDCA.

The correlation analysis of this study found a correlation between the expression of IL-6 and MDA levels with $p <0.05$. There was a strong correlation ($r = 0.6–0.8$) on days 7, 14, and 21. Suliman *et al*. (2020) reported that oxidative stress and inflammation are involved in the pathology of liver injury in animal and human models. Hepatocytes that ROS has injured will release the pro-inflammatory cytokine interleukin. This report is consistent with the finding that there are elevated p53, IL-6, IL-11, and MDA levels in acetaminophen-induced acute liver injury [22].

There were some limitations in this study. First, the limitation of this study is that we did not test on multiple inflammatory mediators. Future research would be better

to carry out several inflammatory mediators such as IL-10, IL-11, and NO. In addition, it would be better for further research to confirm a more varied dose of curcumin to NCow how much IL-6 expression and MDA levels are optimal to reduce hepatocyte cell damage.

Conclusion

Curcumin significantly decreases IL-6 expression and MDA levels compared to the control group in DDY mice induced by liver fibrosis with common bile duct ligation.

Data Availability

The materials and data presented in work are available from the authors on reasonable request.

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