ORIGINAL RESEARCH

Exposure to Goat Bile for 28-days Causes Hepatocyte Injury: a Histopathological Study

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ABSTRACT

Background: Bile consumption by Indonesians is believed to have therapeutic effects, especially goat bile. Goat bile is thought to contain harmful ingredients that can cause toxic effects on the liver. However, the 28-days oral toxicity study of goat bile has not been performed. **Objective**: To analyze the hepatotoxic effect of subchronic administration of goat bile on the liver of mice (M*us musculus*). **Material and Method**: This was an experimental research with a post-test-only control group design. The samples used were 32 Balb/C mice (*Mus musculus*), which were grouped into 4 groups. The samples were administered with goat bile orally (3.2, 6.4, or 12.8 mL/kg/day) for 28 days. The liver was taken for histopathological examination and the hepatocytes injury score was performed. The scoring results were analyzed using Kruskal-Wallis, Mann-Whitney, and Spearman correlation tests ($p<0.05$). **Result**: Goat bile administration was associated with hepatocyte injury ($p= 0.004$). Groups with goat bile administration of 6.4 and 12.8 mL/kg/day had significant differences with the control group ($p= .015$ and $.029$ respectively) and the 3.2 mL/kg/day administered group ($p= 0.006$ and 0.009 respectively). Moreover, the increased administration of goat bile had a positive correlation with the level of hepatocyte injury ($p= 0.004$ and $r_s= 0.504$) **Conclusion**: Goat bile administration for 28 days had a significant toxic effect on the liver of mice at a dose of 6.4 mL/kg/day.

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BACKGROUND

The phenomenon of consuming animal bile in Indonesia is quite popular, such as cobra, goat, and chicken bile. Generally, the bile is processed into dishes or drinks, but some Indonesians, especially

Javanese, believe that eating raw bile from those three animals will be more effective to bring benefits such as increased fertility and immunity (Sudardi, 2011).

Bile is generally composed of protein, fat, carbohydrates, vitamins, minerals, and other dissolved components. The main components of bile are bile acids and bile pigments. In mammals, there are 2 main bile acids produced by hepatocytes, namely cholic acid (CA) and chenodeoxycholic acid (CDCA) and there are also secondary bile acids which are the metabolites of bacterial flora in the digestive tract, namely lithocholic acid (LCA), ursodeoxycholic acid (UDCA), and deoxycholic acid (DCA) (Reshetnyak, 2013). This secondary bile acid is extracted and widely used as medicine. UDCA is one of the bile acids that has been mass-produced in the form of active compounds for the treatment of gallstones, primary biliary cirrhosis, and cholestasis (Tonin & Arends, 2018).

UDCA is very useful, however, it turns out that LCA is very toxic when consumed. LCA in mice was used as a mouse model for hepatobiliary damage. Accumulation of LCA causes blockage of the biliary system that resembles biliary cholestasis (Woolbright, et al., 2014). Then, a report of toxic effects occurred in Saudi Arabia with toxicity problems to patients' hearts and kidneys after consuming goat bile as a treatment for diabetes mellitus (Centers for Disease Control and Prevention (CDC), 1996). In addition, it is important to know that bile also plays a role in the process of xenobiotic excretion of drugs and heavy metals.

Recent research by (Arwati, et al., 2020) shows that in an acute toxicity study scenario, mild diarrhea was observed after being administered with goat bile within 2 days, which indicates slight intestinal toxicity. However, the use of bile as long-term traditional treatment is possible to produce toxic effects. Nevertheless, research on sub-chronic toxicity studies on liver cell damage, especially goat bile, is lacking. A toxicity study needs to be performed before the activity test to determine the limit of the dose range and duration of administration.

OBJECTIVE

The objective of this study was to analyze the hepatotoxic effect of sub-chronic administration of goat bile on the liver of mice (*Mus musculus*).

MATERIAL AND METHOD

This research was conducted at the laboratory of the Department of Anatomy, Histology and Pharmacology, Faculty of Medicine, Universitas Airlangga, Surabaya, Indonesia. This research had been declared ethical with certificate number 44/EC/KEPK/FKUA/2018 and the experiment began in February-August 2018.

The sample used was 32 BALB/c mice (*Mus musculus*) aged 6-8 weeks with 20-25 g body weight. The mice were placed in separated cage for 7 days before the experiment, allowing the mice to adapt to their new environment. After acclimatization, 32 mice were divided into four groups, namely 1 control group and 3 treatment groups. The control group received distilled water only, while the other 3 treatment groups were administered with goat bile consumption at different doses. Each group consists of 8 mice, 4 male mice and 4 female mice. Every 2 mice of the same sex and the same dose were placed in 1 cage.

This study used goat bile (*Capra aegagrus hircus*) from Surabaya Slaughterhouse on Pegirian Street, Surabaya, Indonesia. Bile fluid was aspirated with a sterile syringe into the beaker glass as much as needed every day. The remaining bile would not be used the next day. Then, the clean gall bladder that had not been opened, was stored in the refrigerator, for a maximum period of 2 days, to prevent contamination of other materials, prevent bacterial colonization, and slow the reaction of changes in substances in the bile and gallbladder decay. Goat bile was administered orally, and the dose was adjusted to the body weight of the mice. There were 3 treatment groups: low dose (dose 1; 3.2 mL/kg/day), medium dose (dose 2; 6.4 mL/kg/day), and high dose (dose 3; 12.8 mL/kg/day). Each group received the treatment orally once in every hour, at 10 AM, daily for 28 days. After the treatment was done, the mice were terminated, and the livers were collected for histological examination and the hepatocytes injury scoring was performed. The hepatocyte injury was evaluated by calculating the level of hepatocyte injury based on the degree of change in liver cell histological structure according to the modification of Manja Roenigk's Histopathological Scoring by reading each liver tissue slide in five visual fields with 400x magnification (Prasetiawan, Sabri & Ilyas, 2012). The higher score was given

to the most injured hepatocyte and vice versa. The scoring used in this experiment illustrated in Table 1 and Figure 1. The Scoring results were analyzed using Kruskal-Wallis, Mann-Whitney, and Spearman correlation test ($p<0.05$).

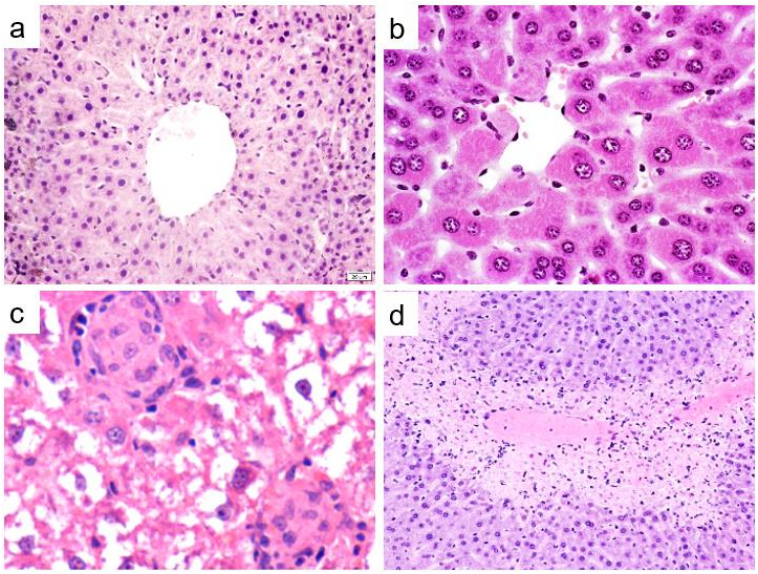

Figure 1. Scoring standard A. Score 1: Normal; B. Score 2: Cloudy swelling; C. Score 3: Ballooning degeneration; D. Score 4: Necrosis. Magnification 400x (Mescher, 2016; Kumar, et al 2018).

Table 1. The scoring system in the research modified from Prasetyawan, Sabri & Ilyas (2012)

Score	Description
1	Normal. The cells are arranged radially, oval shape, hepatocyte plates and the boundaries between cells are visible, and sinusoids are almost evenly sized.
2	Cloudy swelling. The hepatocytes are not arranged radially, the cell size becomes more diverse, the cell nucleus is enlarged, the boundaries between cells become blurred, and sinusoids appear irregularly. In addition, vacuoles appear in the cytoplasm, which results in cells becoming larger and hollow.
3	Ballooning degeneration. Hepatocytes become 2-3 times larger than normal cells, visible vacuoles in the cytoplasm that contain water, with the nucleus in the middle. The cytoplasm looks cloudy, with the formation of a spider-like nest. The boundaries between cells and sinusoids cannot be identified.
4	Necrosis. Karyorrhexis with the nucleus that is hypnotic, or partially undergoes fragmentation, or karyolysis, which is characterized by core chromatin becoming pale.

RESULT

In the control group, most samples showed cloudy swelling (Score 2) (75%) (Figure 2). Then, at the 1st treatment group, with the dose of 3.2 mL/kg of goat bile, most of the samples showed normal hepatocytes (57%) (Figure 3). Whereas in the 2nd treatment group, with a dose of 6.4 mL/kg of bile goat, the result was cloudy swelling (50%) (Score 2) and hydropic degeneration (50%) (Score 3) (Figure 4), and in the 3rd treatment group, with a dose of 12.8 mL/kg of goat bile, most samples showed cloudy swelling (Score 2) (63%) (Figure 5). Complete data is shown in Table 2.

Table 2. Results of scoring of hepatocyte injury

Groups	Score 1		Score 2		Score 3		Total samples (n)
	Frequency (f)	Percentage (%)	Frequency (f)	Percentage (%)	Frequency (f)	Percentage (%)	
Control	2	25%	6	75%	0	0%	8
3.2 ml/kg	4	57%	3	43%	0	0%	7
6.4 ml/kg	0	0%	4	50%	4	50%	8
12.8 ml/kg	0	0%	5	63%	3	38%	8

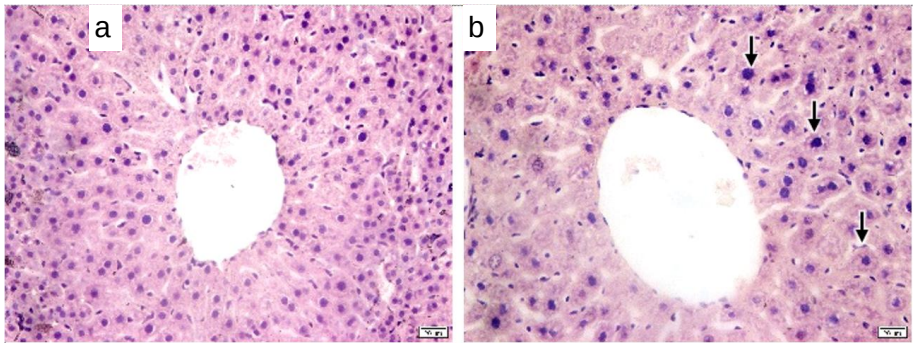

Figure 2. Control group. Central vein surrounded by hepatocytes with A: normal hepatocytes (Score 1) (Magnification 400x), and B: cloudy swelling (Score 2), such as cell and core enlargement (black arrow) (Magnification 400x).

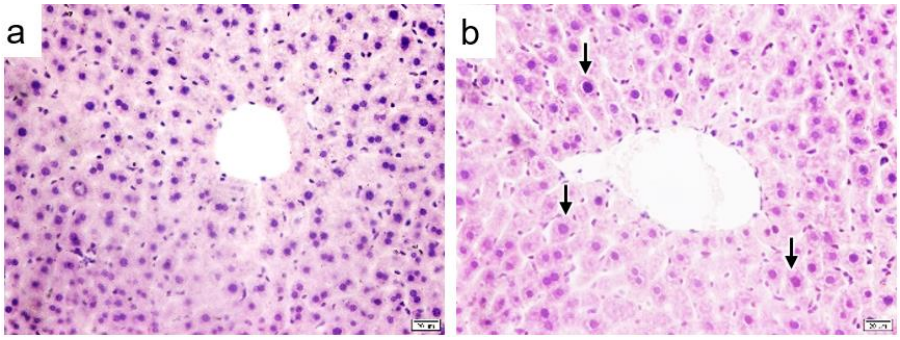

Figure 3. Group of 3.2 ml/kg. Central vein surrounded by hepatocytes with A: normal hepatocytes (Score 1) (Magnification 400x), and B: cloudy swelling (Score 2), such as cell and core enlargement (black arrow) (Magnification 400x).

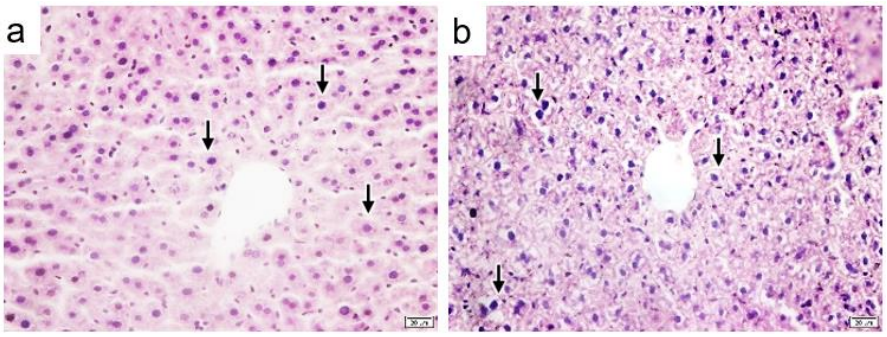

Figure 4. Group of 6.4 ml/kg: Central vein surrounded by hepatocytes with A: cloudy swelling (Score 2), such as cell and core enlargement (black arrow) (Magnification 400x), and B: hydropic degeneration (Score 3), such as an enlarged cell, with a cytoplasm filled with water (black arrow) (Magnification 400x).

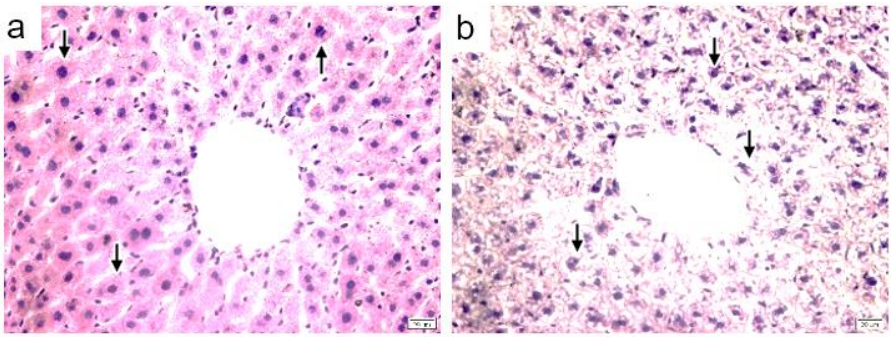

Figure 5. Group of 12.8 ml/kg Central vein surrounded by hepatocytes with A: cloudy swelling (Score 2), such as cell and core enlargement (black arrow) (Magnification 400x), and B: hydropic degeneration (Score 3), such as an enlarged cell, with a cytoplasm filled with water (black arrow) (Magnification 400x).

The comparative test of hepatocyte injury scores for the four groups was conducted using Kruskal Wallis non-parametric statistics test using the modus value of the hepatocytes injury scores. The result of Kruskal Wallis test is presented in Table 3.

Table 3. Results of The Kruskal-Wallis Test of hepatocyte injury scores

Group	Mean Rank	*p*-value	Notes
Control (n=8)	12.50	0.004	Statistically significant different (p<0.05)
3.2 ml/kg (n=7)	8.64		
6.4 ml/kg (n=8)	21.75		
12.8 ml/kg (n=8)	20.19		

This study also analyzed the comparison of hepatocyte injury scores of each group using the modus value of the hepatocytes injury scores. The number of samples for each group was relatively small (<20) so it was decided to use a non-parametric statistical test of Mann Whitney U Test as presented in Table 4.

Table 4. Results of the comparative test of hepatocyte injury scores

Group	Compared with group	*p*-value
Control	3.2 ml/kg	0.221
	6.4 ml/kg	0.015*
	12.8 ml/kg	0.029*
3.2 ml/kg	6.4 ml/kg	0.006*
	12.8 ml/kg	0.009*
6.4 ml/kg	12.8 ml/kg	0.626

*: Statistically significant different (p<0.05)

This study also analyzed the correlation of increased administration of goat bile with the level of hepatocyte injury using Spearman Correlation Test. The result was that the increased administration of goat bile had a positive correlation with the level of hepatocyte injury (p= 0.004 and r_s= 0.504).

DISCUSSION

Goat Bile Toxicity

Research on sub-chronic toxicity study of goat bile has not been well established, because the consumption of goat bile is not as intensive as the consumption of bile from other animals, such as snakes, bears, or fish. Nonetheless, because goat bile is CDCA-based bile acid, several studies correspond to the results of our study. After CDCA administration, a histopathologic proliferation of bile canalicular cells, inflammation in the periportal region, focal necrosis and cloudy swelling in the centrilobular region were obtained (Song, et al., 2011). In this study, the toxic effect was seen to be significant starting with the administration dose of 6.4 mL/kg and the results did not differ significantly from the increase in dose to 12.8 mL/kg. However, the Spearman correlation test showed a positive correlation between increasing doses and the level of hepatocyte damage.

In addition to the toxic effects that arose on histopathological examination in this study, CDCA and LCA in several other studies have also been shown to have toxic effects. Giving CDCA or LCA could increase COX-2 expression (Song, et al., 2011; Ridlon & Bajaj, 2015), erbB2 gene and Epidermal Growth Factor Receptor (EGFR) (Kitamura et al., 2015), which were the main mechanisms in the carcinogenesis process. It also affected the expression of the metalloproteinase mRNA matrix (MMP7), which is one of the proteins that plays a role in cancer metastasis and the inflammatory process of cancer (Raufman, et al., 2015). In cancers with higher MMP7 concentrations, cancer cells were found with higher metastatic and aggressive ability.

Mechanistic studies of bile acid hepatotoxicity

The mechanism of liver toxicity due to bile acid has been explained by several theories. Based on its physical properties, bile acid is an amphipathic compound that has a hydrophilic group (hydroxyl group) and a hydrophobic group (steroid group) (Hofmann & Hagey, 2014). The amount of the two groups influences the physical properties of bile acid to be more hydrophilic or hydrophobic. Therefore, bile acids have the following sequence based on their hydrophobicity, UDCA<CA<CDA<DCA<LCA (Ashby, et al., 2018). Furthermore, with the physical properties of bile acids that are hydrophobic, the possibility of retention and accumulation in the hepatobiliary system is quite high. In some studies, using mice with cholestasis, the accumulation of bile in the liver caused degeneration of hepatocyte parenchyma with features of cell swelling, pleomorphism and abnormal crest formation in mitochondrial hepatocytes. In addition, the accumulation of bile acids in the liver tissue could increase the intracellular ROS (reactive oxygen species). The increasing ROS in cells will change the intracellular structure into radicals and causes apoptosis (Perez & Briz, 2009).

Bile acid at certain concentrations induces cell death which may occur through apoptosis. Toxic bile salts, such as glycine-conjugated CDCA, enhance the expression of Fas receptor, TRAIL – R2, and TNF – R1 which are cell death receptors. After receptor activation, Fas and TRAIL - R2 form a death-

inducing signalling complex (DISC), which activates the caspase 8 protease. This results in recruitment of death-inducing signalling complex and activation of caspase 8, which initiates an apoptosis signalling cascade that is amplified via mitochondrial dysfunction (Ashby, et al., 2018).

Bile acids have also been reported to induce an innate immune response. Damages to hepatocytes due to toxic bile acids could trigger innate immunity by activating Toll-like receptor 9 (Tlr 9), one of the receptors that plays a role in the immune system (Cai, et al., 2017). After innate immunity is activated, the cell will respond to the release of inflammatory chemoattractants, such as Ccl2 and Cxcl 2, and carry out neutrophil chemotaxis. This neutrophil accumulation causes necrosis in hepatocytes (Li, et al., 2017).

Some limitations of this study could have influenced the results. Among those limitations were the measurements of food and drink consumption, daily weight measurements, and the measurements of urine and faecal production that were not carried out every day, while determining the subchronic toxic effects was not only based on the results of haematological examination, urinalysis, and organ histopathology, but also based on the process of these toxic effects occurrence which was not assesed (Organisation for Economic Co-operation and Development, 2008). In addition, the size of the sample was too small, so the dose for No Observed Adverse Effect Level (NOAEL) and No Observed Effect Level (NOEL) had not been obtained.

CONCLUSION

The administration of goat bile (*Capra aegagrus hircus*) for 28 days in mice (*Mus musculus*) had a toxic effect on the liver of the mice in the form of cloudy swelling and hydropic degeneration starting from a dose of 6.4 mL/kg/day.

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Conflict of interest
All authors have no conflict of interest.

Ethics consideration
This research has been declared ethical with an ethics-worthy certificate number 44/EC/KEPK/FKUA/2018.

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Author contribution
All authors have contributed to all process in this research, including preparation, data collection and analysis, drafting and approval for publication of this manuscript.

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