International Journal of Public Health Science (IJPHS)
Vol. 15, No. 1, March 2026, pp. 190~196
ISSN: 2252-8806, DOI: 10.11591/ijphs.v15i1.27001

Hepatotoxicity effect of alcoholic beverages on histology and IL-6 gene detection in *Rattus norvegicus* using polymerase chain reaction

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Article Info

Article history:

Received Jan 27, 2026
Revised Feb 10, 2026
Accepted Feb 16, 2026

Keywords:

Alcohol
Hepatotoxicity
Histology
IL-6
PCR
Rattus norvegicus

ABSTRACT

The liver is an organ that has an important role in metabolic and detoxification processes. Alcohol consumption will cause metabolic disorders in the liver and can cause changes in histological structures. Interleukin-6 has a profound role in liver pathology and plays an important role in the body's defense mechanism, and contributes to the manifestation of tissue damage. This study was an experimental study using 20 *Rattus norvegicus* treated with 25% alcohol, and a normal treatment only given distilled water. Histological observations were carried out on each preparation in 5 fields of view. The hepatotoxicity effect of alcohol was observed based on the presence of cell degeneration and necrosis in histological preparations stained with hematoxylin eosin. IL-6 detection with polymerase chain reaction was analyzed qualitatively using electrophoresis. Based on the statistical test results, the sig. (2 tailed) of 0.00 < 0.05 so that it can be seen that there is a significant difference in histological results between the normal control group without treatment and the group induced by alcohol. The administration of alcoholic beverages with 25% ethanol content in vivo for 21 days causes necrosis and bleeding around the hepatocyte cells. IL-6 overview appeared to be stronger in the group with alcohol-induced drinking, it could indicate that giving alcohol causes a higher inflammatory response. This study provides scientific evidence that alcohol consumption significantly damages the liver. Further research can be conducted to increase the sample size and quantitatively assess IL-6 expression.

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1. INTRODUCTION

Alcoholic beverages are one type of addictive substance whose abuse has a serious impact on public health and social problems. Addiction to consuming alcoholic beverages has become a serious health problem today. One type of alcoholic drink that is often found in Indonesia is the traditional ciu liquor [1]. Ciu is made from fermented liquid waste from the sugar-making process, namely fermented cane molasses, and undergoes a distillation process. Ciu is included in group C because it has an alcohol content of 25-40% [2]. Alcohol is widely known to cause disease manifestations in the body [3].

The organ that has a role in neutralizing toxins, metabolism, and other important roles, and is sensitive to disorders is the liver [4]. Excessive alcohol consumption will cause metabolic disorders in the

liver. Fatty liver, hepatitis, and cirrhosis are three main manifestations of liver disease caused by alcohol. More than 60% of the population in western countries have thats, and as many as 40% to 50% of deaths are caused by alcohol-related cirrhosis [5]. In 2015, the death rate from cirrhosis of the liver increased to 11th in the world with 15.8 cases per 100,000 population [6]. The prevalence of chronic liver disease in Indonesia reaches 20 million people, [7] of which 20-40% develop liver cirrhosis [8], [9].

Interleukin-6 is a pleiotropic cytokine that plays various biological activities and plays an important role in the body's immune response in the liver. In experimental animals associated with hepatotoxicity, IL-6 plays a role in hepatocytes to stimulate cell regeneration and repair. Interleukin-6 has a role in the pathology of liver disease and is very complex. IL-6 was originally thought to be a hepatoprotector in hepatic steatosis. IL-6 is able to reduce liver steatosis, able to reduce oxidative stress, and prevent mitochondrial dysfunction [10]. IL-6 contributes to the manifestation of tissue damage. Previous research has shown that elevated levels of the inflammatory cytokines IL-6, IL-1b, and TNFα are associated with a high-fat diet [11], [12]. IL-6 has also received particular attention as a neuroimmune regulator of the effects of alcohol. The study found significantly higher IL-6 levels in individuals with AUD compared to healthy controls, while other cytokines showed no significant changes [13].

Alcohol induction can have a toxic effect on the liver [14] and will also affect the expression of IL-6, so this research aims to see the toxic influence of ciu alcohol based on histopathological examination of the liver and also the expression of IL-6. This study aims to look at alcohol hepatotoxicity based on histology features and IL-6 profile as a pro-inflammatory cytokine marker. This research needs to be conducted to study the basis of alcohol toxicity on histological features and its relationship to the inflammatory cytokine IL-6.

2. METHOD

2.1. Experimental animal treatment

This research was an experimental study with 20 test animals divided into 2 treatment groups using a simple random sampling technique. The group that was given ciu, the traditional alcoholic drink 3.3 ml/ 200 g BB *Rattus norvegicus,* and the group that was only given distilled water. The treatment was carried out within 21 days. The preparation is carried out according to [15], [16]. The first step is washing the tissue with NaCl. This washing aims to remove blood, after that it is put into a tissue cassette, then fixed with 10% NBF for 10 hours before histology preparation.

2.2. Histology preparations

The procedure for making preparations refers to [17]. Preparation of histological tissue preparations can be carried out in several stages, including fixation, dehydration, clearing, embedding, blocking, and staining. The macros cut was put into cassette tissue and then fixed with 10% NBF for 1½ hours, then 10% NBF for 1½ hours. The next stage is dehydration. Dehydration was performed by immersing the tissue in 50% alcohol for 1½ hours, 70% alcohol for 1½ hours, 95% alcohol for ½ hour, 100% alcohol for 2 hours, and 100% alcohol for 2 hours. The process of removing the water-drawing agent and replacing it with chemicals, in this process, used a solution of xylol I for 1 hour and xylol II for 2 hours.

The embedding process was carried out to replace the clarification material with liquid paraffin so that the tissue was easily cut using a microtome. The tissue was placed in liquid paraffin I for 2 hours and liquid paraffin II for 4 hours. The procedure is continued with blocking. The process of cutting tissue into blocks using a microtome at a thickness of 3-5 mm and capturing it with a glass object in a floating bath (55-60 °C). The tissue then stained with hematoxylin-eosin dye [18].

Hematoxylin is used to stain cells pink, which is acidic, and eosin can be used to colour cytoplasmic proteins to be purplish blue and is alkaline. Before staining, it is necessary to carry out the deparaffinization process by immersing the tissue in xylol, then after that it is put into absolute alcohol, 90% alcohol, and 70% alcohol. After that, soak the hematoxylin paint for 5-10 minutes. The tissue is soaked in eosin paint for 2-5 minutes and washed with alcohol. The tissue is put in xylol for further mounting of the tissue with enthelan. The tissue is then read using a light microscope at up to 400x magnification.

2.3. IL-6 detection

Gene testing begins with DNA isolation from serum samples using the DNA Extraction and Isolation Kit from Geneaid (GS100). IL-6 genotyping was determined by amplifying DNA fragments with specific primers F: 5'-GGAGTCACACACTCCACCT3' and R: 5'-CTGATTGGAAACCTTATTAG-3'. PCR reaction mixture (25 µL final volume). The temperature conditions for the IL-6 gene PCR reaction were as follows: Initial denaturation at 95 °C, following 56 °C for 1 minute, 72 °C for 1 minute, and 72 °C 10 minutes for extension. The PCR product obtained from the PCR process with the adjusted PCR conditions was then electrophoresed using an agarose gel.

2.4. Data analysis

This study used a simple random sampling technique with histological observations carried out on all preparations in a blinded manner. The parameters observed in this study were the macroscopic assessment of the liver, including the texture, colour, and size of the liver in a descriptive way. Microscopic observation with 400x magnification in 5 fields of view calculated the average damage value by observing the preparations.

- Scale 1: normal cell, the cell nucleus is round and blue, and the cytoplasm is red.
- Scale 2: degeneration, the cell appears swollen, pale, and clear, containing a lot of water (hydropic degeneration). The intercellular spaces appear swollen, the cytoplasm is rough, and there are granules (parenchymatous degeneration).
- Scale 3: Necrotic, the dense nucleus appears dark (pyknosis), the nucleus appears broken (karyorrhexis), and the nucleus appears lost due to chromatin hydrolysis (karyolysis).

The data obtained were analysed using by T-test to find the different histopathological pictures of the liver of white rats (*Rattus norvegicus*) induced by ciu, traditional alcohol.

3. RESULTS AND DISCUSSION

The hepatotoxicity effect of alcohol was observed based on microscopic observations of livers that had been treated in vivo. Assessment of the histopathological picture of the liver due to alcohol exposure can be seen in Table 1. Observations were made using 5 visual fields. Histological analysis was performed to determine the effects of alcohol.

Table 1. The results of the microscopic observation of the liver of white rats

Treatment	Histological object	Field of view				
		1	2	3	4	5
Control group without treatment	1	1	1	1	1	1
	2	1	1	1	1	1
	3	1	1	1	1	1
	4	1	1	1	1	1
	5	1	1	1	1	1
Alcohol induction group	1	3	2	2	3	2
	2	2	2	3	3	2
	3	2	3	3	3	3
	4	2	3	3	3	3
	5	3	2	2	3	3

Description: results of microscopic observation of the liver of *Rattus norvegicus:*
1 = Normal; 2 = Degeneration; 3 = Necrosis

Microscopic observation of the liver of white rats was analysed based on the presence or absence of normal cells, epithelial cell swelling (parenchymatous and hydropic degeneration), and epithelial cell damage or necrosis [19]. The results of microscopic observations can be seen in Table 1. Based on the statistical test results, the sig. (2 tailed) of $0.000 < 0.05$, so that it can be seen that there is a significant difference in histological results between the normal control group, without microscopic observation of the liver of white rats, which was analyzed based on the presence or absence of normal cells, epithelial cell swelling (parenchymatous and hydropic degeneration), and epithelial cell damage or necrosis.

Based on the results of the study, it can be seen that the ciu alcohol induced in *Rattus norvegicus* has a histopathological effect on the liver. In the normal control without treatment, which can be seen in Figure 1, the liver cells are well distributed, and the arrangement of the nucleus and cytoplasm is clearly stained. Figure 2 shows no visible degeneration or necrosis, indicating that the hepatocytes are not swollen. This is because the group without treatment was only given distilled water, which is not toxic to the liver. Aquades is clear, tasteless, and distilled water, so the percentage of possible contamination by toxic compounds and microorganisms is very small. Water that enters the body will be immediately absorbed by the digestive system and distributed throughout the body by blood vessels. The absence of hepatic alterations in the untreated group may be attributed to the administration of distilled water, which is considered biologically inert and non-hepatotoxic under normal experimental conditions [20].

Figure 2 is the histological structure of the liver that has been induced by alcohol. Based on observations, it can be seen in the picture that there is cell damage in the form of degenerated cells and necrotic cells. In alcohol-induced livers, it can be seen that the histological structure contains many blood vessels around the hepatocyte cells. Necrosis and degeneration are also found in some parts. Degenerated

cells can still recover or return to normal if exposure to the toxin is stopped [21]. Parenchymatous degeneration is characterized by swelling of the cytoplasm and granular cytoplasm. This is because the cell is unable to eliminate water, so it accumulates inside the cell, and the cell organelles also absorb water and swell, causing the cytoplasm to appear granular [22]. The level of damage to parenchymatous degenerative cells was more common in the group induced by ciu alcohol due to the induction of the toxic substance of traditional alcoholic beverages ciu which was able to damage the livers of white rats.

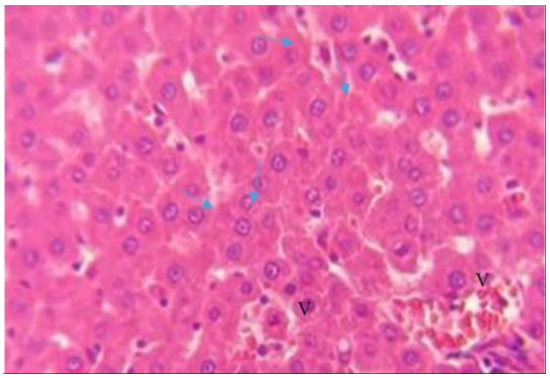

Figure 1. Histopathological picture of the liver of white rats in the control group without treatment, hematoxylin eosin staining with 400x magnification. Description: blue arrow = normal cell and V = vein

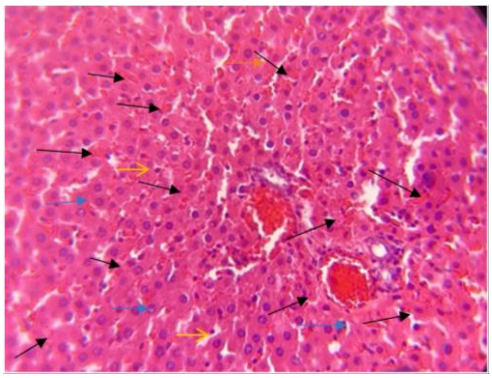

Figure 2. Histopathological picture of the liver induced by kissing alcohol with Hematoxylin-Eosin staining and 400x magnification. Description: blue arrows: degeneration, yellow arrows: necrosis, and black arrows: lots of blood vessels around hepatocytes

Necrosis is a morphological change as a result of progressive degradation by enzymes in cells that experience lethal injury characterized by changes and nuclear destruction [23]. The formation of necrosis can be seen from the formation of pyknosis, which is characterized by shrinkage of the cell nucleus, compaction and dark colour. Necrosis are irreversible cell changes due to damage to the plasma membrane or lysosomal membrane, as well as loss of DNA or mitochondria. Dysfunction of the cell membrane and mitochondria is the main factor causing irreversible cell damage [24]. The physical effects experienced from consuming alcoholic beverages include damage to the liver and pancreatic [25].

Another parameter that can be used to observe liver function after alcohol induction is IL-6 detection. According to [26], A low annealing temperature during polymerase chain reaction (PCR) may reduce primer specificity, allowing primers to hybridize to partially complementary or non-target DNA sequences, thereby increasing the likelihood of non-specific amplification products. According to [27], proper adjustment of this parameter enhances amplification yield while minimizing non-specific products.

Interleukin-6 is a pleiotropic cytokine that is secreted from body tissues during the acute and chronic phases of infection [27]. IL-6 expression can be visualized in the electrophoresis results, which are read using Gel Doc. IL-6 expression can be observed in Figure 3. Based on Figure 3, it can be seen that in the group with alcohol-induced kissing, it appears that IL-6 is expressed more strongly on electrophoretic visualization compared to the control group without any treatment. Interleukin-6 is a phosphorylated glycoprotein containing 185 amino acids, including pleiotropic cytokines involved in inflammation, bone metabolism, C-reactive protein synthesis, and carcinogenesis. These cytokines and receptors are a class of polypeptides that mediate the inflammatory process. These polypeptides are divided into pro- and anti-inflammatory cytokines. In nonspecific immunity, interleukin-6 stimulates hepatocytes to produce acute phase protein (APP) and cerebrospinal fluid (CSF), which stimulate progenitors in the bone marrow to produce neutrophils. The interleukin 6 gene is at the 7th chromosome position 21 (7p21). Interleukin-6 is a multifunctional cytokine with an important role in various cell biological activities [28]. In addition, interleukin 6 plays a role in the normal immune response to foreign antigens [29]. Furthermore, alcohol drinking has also been shown to alter IL-6 expression in various tissues across humans and animals [30]-[33]. According to Wijayanti and Sayekti [34], conventional PCR can be seen qualitatively but quantitatively it cannot be analyzed. This research can be expanded by examining gene expression quantitatively and add treatment variations for further analysis of the relationship between histological structure, gene, and protein level of IL-6.

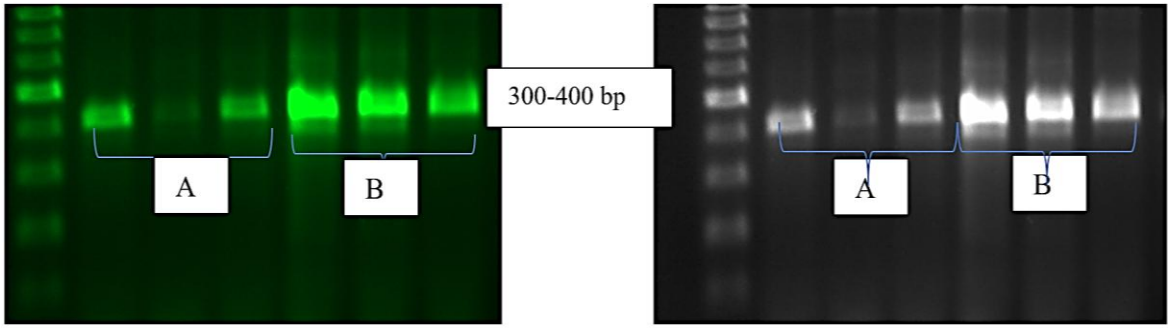

Figure 3. IL-6 overview qualitatively using electrophoresis. Description: A: normal control without treatment and B: alcohol-induced group

4. CONCLUSION

Based on the research results it can be seen that ciu alcoholic drink has a toxic effect on the histopathological picture of the liver in the form of cell degeneration and necrosis, and lots of bleeding around the hepatocyte cells. Based on the statistical test results, the sig. (2 tailed) of 0.000 < 0.05, so that it can be seen that there is a significant difference in histological results between the normal control group without treatment and the group induced by kissing alcohol. The IL-6 gene was detected more clearly in the group with the induction of alcohol; this indicates that giving alcohol causes a higher inflammatory response. Further research can be conducted to increase the sample size and quantitatively assess IL-6 expression.

ACKNOWLEDGMENTS

The author thanks the Study Program Medical Laboratory Technology, STIKES Nasional.

FUNDING INFORMATION

This research was funded by LPPM STIKES Nasional, Surakarta, Jawa Tengah, Indonesia.

AUTHOR CONTRIBUTIONS STATEMENT

This journal uses the Contributor Roles Taxonomy (CRediT) to recognize individual author contributions, reduce authorship disputes, and facilitate collaboration.

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C : **C**onceptualization
M : **M**ethodology
So : **So**ftware
Va : **Va**lidation
Fo : **Fo**rmal analysis
I : **I**nvestigation
R : **R**esources
D : **D**ata Curation
O : Writing - **O**riginal Draft
E : Writing - Review & **E**diting
Vi : **Vi**sualization
Su : **Su**pervision
P : **P**roject administration
Fu : **Fu**nding acquisition

CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest.

INFORMED CONSENT

This research did not use human subjects. This research has passed ethical review.

ETHICAL APPROVAL

This study was approved by the Health Research Ethics Committee of UMP, Indonesia (Ethical Clearance No. KEPK/UMP/34/VI/2023). Ethical approval confirmed that the research protocol met all institutional and international ethical standards.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author, [FDJS], upon reasonable request.

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