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TINJAUAN PEWARNAAN HEMAKTOSILIN-EOSIN DAN *PERIODIC ACID-SCHIFF* TERHADAP KERUSAKAN HATI MENCIT YANG DIINDUKSI ALOKSAN

A Review of Hematoxylin-Eosin and Periodic Acid Schiff staining to assess alloxan-induced liver injury in mice

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Abstrak

Hati merupakan organ yang memiliki banyak fungsi termasuk metabolisme glukosa untuk menyediakan energi bagi jaringan. Hepatosit adalah sel fungsional utama hati, yang juga mengatur pelepasan glukosa hati melalui protein transpor glukosa-2. Kerusakan hepatosit daoat disebabkan oleh zat beracun dan penyakit seperti diabetes melitus. Aloksan merupakan senyawa organik yang umum digunakan dalam penelitian diabetes melitus sebagai agen diabetogenik. Aloksan menyebabkan diabetes melalui penghambatan selektif sekresi insulin yang distimulasi glukosa dan induksi pembentukan spesies oksigen reaktif (ROS) yang memicu nekrosis sel beta pankreas. Aloksan juga berpengaruh terhadap kondisi histologi hati termasuk terhadap struktur hepatoseluler dan kandungan glikogen. Pewarnaan Hematoksilin Eosin dan *Periodic Acid Schiff* digunakan untuk mengevaluasi kondisi. Namun, keduanya perlu ditinjau untuk mengevaluasi kemampuan penegakan diagnosisnya. Mencit dibagi menjadi kelompok kontrol dan uji, setiap kelompok terdiri dari 5 ekor mencit. Kelompok uji diinduksi dengan aloksan dosis 25, 50 dan 100mg/KgBB pada hari kedua kedatangan. Hati mencit kemudian diambil pada hari ketujuh, selanjutnya dilakukan pengolahan jaringan untuk mendapatkan 20 blok hati mencit. Dua potongan jaringan dalam parafin setebal 5 µm dari setiap kelompok diambil dan diwarnai masing-masing dengan hematoxylin-eosin, dan *Periodic Acid Schiff*. Slide hati mencit diperiksa secara mikroskopis untuk melihat derajat kerusakan dan konsentrasi glikogen. Selanjutnya digunakan aplikasi pencitraan digital ImageJ untuk evaluasi lebih lanjut. Penelitian ini menemukan bahwa secara mikroskopis dan uji beda ANOVA kedua metode pewarnaan HE dan PAS mampu menghasilkan perbedaan yang signifikan antara kelompok mencit yang diinduksi aloksan dengan berbagai dosis dan kontrol.

Kata Kunci:
Aloksan, diabetes melitus, Hemaktosilin Eosin, Periodic Acid Schiff, kerusakan hati.

Keywords:
Alloxan, diabetes mellitus, Hematoxylin-Eosin, Periodic Acid Schiff, liver injury.

Abstract

The liver is an organ that serves several functions in the body, including glucose metabolism to provide energy to other tissues.. Hepatocytes are the primary functional cells of the liver, which also regulates liver glucose release via glucose transport protein-2. Hepatocytes injury could occured by toxic substance and disease such as diabetes melitus. Alloxan as an organic compound that is commonly used in diabetes research as a diabetogenic agent. Alloxan causes diabetes through selective inhibition of glucose-stimulated insulin secretion and induced formation of reactive oxygen species (ROS) that promotes pancreatic beta cell necrosis. Alloxan also has an effect on the liver's histological condition including the hepatocellular structure and glycogen content. HE and PAS used for evaluating this condition. However, both should be reviewed to evaluate their abilities. Mice were divide in control and test group, which each group consist of 5 mice. Test group were intervein-induced with 25, 50 and 100mg/KgBB alloxan on the second day of arrival. The mice's livers were then taken on the seventh day, then tissue processing is carried out to get 20 blocks of mice's livers. Two 5-µm-thick paraffin-embedded sections from each group were taken and stained with hematoxylin-eosin, and periodic acid Schiff, respectively. Mice's liver slides are examined microscopically for the degree of injury and glycogen concentration for further evaluation using ImageJ digital imaging application. This study was found that microscopical and ANOVA test of both staining methods were succesfully able to produce significant differences between control and various dose alloxan-induced groups of mice

INTRODUCTION

The liver is an organ that serves several functions in the body, such as digestion, detoxification, elimination of substances from the body, and also regulates glucose metabolism (Rubin and Strayer, 2012). The liver is made up of thousands of hepatic lobules, which are distinguished by a prominent central vein and a slightly pale central portion of the lobule when compared to the lobule edges. Hepatocytes are the primary cells of the liver parenchyma. These are large polygonal cells with basophilic nuclei and eosinophilic cytoplasm (USMLE, 2023). The nucleus is round or oval with a regular surface and varies in size; some cells have two nuclei. Each nucleus has one or more nuclei and is vesicular in shape, with well-defined and scattered chromatin granules (Scudamore, 2014).

Hepatocytes are the primary functional cells of the liver, responsible for a wide range of functions including the production of bile, a number of plasma proteins, and non-essential amino acids, breakdown or detoxification of metabolic waste products, drugs, and toxins; fat, carbs, and protein metabolism; glucose, vitamin, and iron storage (USMLE, 2023).

The liver is important in glucose homeostasis because it regulates glycogenesis, glycogenolysis, glycolysis, and gluconeogenesis, all of which are required to provide energy to other tissues. Glucose is stored as glycogen and/or converted into fatty acids or amino acids in the postprandial (fed) state. This process is also known as glycogenesis. Pancreatic cells secrete insulin when fed. Insulin stimulates glycogen synthase and the expression of glucokinase (GK/hexokinase-4) in the liver. Glycogen synthase is a key enzyme that aids in the extension of glycogen chains. Glucose enters hepatocytes via glucose transport protein-2 (GLUT2), which also regulates liver glucose release (Rubin & Strayer, 2012; Sarikaya, Schierz & Sarikaya, 2021).

Liver toxicity, also known as hepatotoxicity, is an inflammation of the liver caused by a toxic substance. Alcohol, chemicals, and herbal and nutritional supplements can all cause liver toxicity. Alcohol, chemicals, and herbal and nutritional supplements can all cause liver toxicity. Some medications, which doctors may refer to as drug-induced liver injury, can also cause this condition (DILI). Medical treatment can sometimes reverse the damage done to the hepatocytes, depending on its cause and severity. However, if not detected and treated promptly, liver toxicity can result in an irreversible buildup of tissue injury, resulting in permanent liver damage or acute liver failure(Trull and Ewumi, 2022). The liver injury also can occur by diabetes mellitus (DM) (Coman *et al.*, 2021).

DM is widely regarded as a major public health issue today, and the World Health Organization predicts that it will be the seventh leading cause of death by 2030 (WHO, 2016). DM is a chronic metabolic condition marked by high blood sugar levels. The liver is also associated with DM because it has a vital function in glucose homeostasis (Park *et al.*, 2022).

Hyperglycemia results in oxidative stress, which can damage liver tissue. Following that, disruptions in protein, carbohydrate, and lipid metabolism increased oxidative stress and triggered the inflammatory cascade. Diabetes is caused by both oxidative stress and inflammatory responses. Diabetes complications are divided into two categories: macrovascular and microvascular. Microvascular complications include hepapathy, neuropathy, retinopathy, and nephropathy, while macrovascular complications include peripheral artery disease, stroke, and coronary artery disease (Aslam, 2022).

Alloxan (5,5-dihydroxyl pyrimidine-2,4,6-trione) is an organic compound that is commonly used in diabetes research as a diabetogenic agent. It is a hydrophilic b-cell toxic glucose analog. Alloxan-induced diabetes is a type of insulin-dependent diabetes caused by the administration or injection of alloxan into animals. Alloxan's diabetogenicity is highlighted by its selective cellular uptake by pancreatic beta cells and subsequent accumulation in these cells. Alloxan causes diabetes through a mechanism that entails partial degradation of pancreatic islet beta (b) cells and a subsequent reduction in the quality and quantity of insulin produced by these cells. The model employs two distinct pathological effects: selective inhibition of glucose-stimulated insulin secretion and induced formation of reactive oxygen

species (ROS) that promotes pancreatic beta cell necrosis (Ighodaro, Adeosun & Akinloye, 2017).

Alloxan also has an effect on the liver's histological condition including the hepatocellular structure and glycogen content . As a result, we require a method for accurately assessing liver damage. HE and PAS are two commonly used methods. However, the ability of these two methods in evaluating liver damage caused by alloxan or DM should be reviewed.

METHODOLOGY

MATERIAL

The population of this study is mice's liver (*Mus musculus*) provided by *Laboratorium Sitohistoteknologi* STIKES Jenderal Achmad Yani that divide into four study groups. Inclusion criteria are healthy male mice weighing 25-30 grams without any intervention prior to use. Exclusion criteria are male dropout mice during the experiment.

The study group included:

- 5 mice for the control group
- 5 mice for each intervein alloxan induce dose: 25mg/ KgBB, 50mg/KgBB, and 100mg/KgBB.

Organ preservation: neutral buffered formaldehyde 10%

HE staining: xylol, alcohol 70%, alcohol 80%, alcohol 90%, alcohol 100%, Mayer's Hematoxylin Solution, alcohol acid1% (1% HCl, Alcohol 70%), lithium carbonate 0,5%, eosin 1%, Canada balsam (Bancroft & Gamble, 2013).

PAS staining: xylol, alcohol 70%, alcohol 80%, alcohol 90%, alcohol 100%, periodic acid 1%, Schiff reagent, Mayer's Hematoxylin Solution (Bancroft & Gamble, 2013).

METHOD

A total number of 15 mice were intervein-induced on the second day of arrival with various alloxan doses. The mice's livers were then taken on the seventh day (Pourghasem, Nasiri, & Shafi, 2014). Then tissue processing is carried out to get 20 blocks of mice's livers (Ahmad, 2009; Bancroft & Gamble, 2013).

Two 5-µm-thick paraffin-embedded sections were taken and stained with hematoxylin-eosin, and periodic acid Schiff, respectively.

HE staining: HE staining: Sections were deparafinized in xylol and hydrated to water with ethyl alcohol in descending order. Staining was performed using hematoxylin which were then counterstained with 0.5% lithium carbonate and 1% aqueous eosin for nucleus and cytoplasm, then examined under a light microscope examination.

PAS staining: Sections were deparafinized in xylol and hydrated to water through descending series of ethyl alcohol. The section was oxidized with 1% Periodic Acid before being washed in water. Schiff reagen was used for staining, and hematoxylin was used as a counterstain.

All stained sections were dehydrated through ascending series of ethanol (100%, 90%, 70% respectively) purified in xylol and finally mounted with Canada balsam.

Detailed procedures provide in Bancroft & Gamble, 2013.

Mice's liver slides are examined microscopically for the degree of injury and glycogen concentration for further evaluation using *ImageJ* digital imaging application.

RESULT AND DISCUSSION

Alloxan injury is characterized by cellular swelling and pyknotic in the cell nucleus, with an inability to Glycogen levels are likely to increase, which appears to be caused by a decrease in hepatic glucokinase activity, which results in changes in the cellular morphology of the liver parenchyma and inhibits gluconeogenesis. HE and PAS staining can be used to detect this damage (Srikanta & et al, 2104).

In the present study, HE and PAS staining methods show differences between control and alloxan induce mice in various doses. However, both staining methods applied different evaluation techniques.

HE stains evaluated the degree of injury based on the cell morphology. Normal hepatocyte cells show a clear nucleus, cytoplasm and scattered granular chromatin. On the other hand, the degree of injury of abnormal/damaged cells was evaluated in three degrees of cell damage: parenchymal degeneration (PD), hydropic degeneration (HD), and necrosis.

Mice's liver slides from each test group and control had been cut with a thickness of 5µm and stained with HE staining

were observed microscopically with 400x magnification at 5 random points (Fig.1).

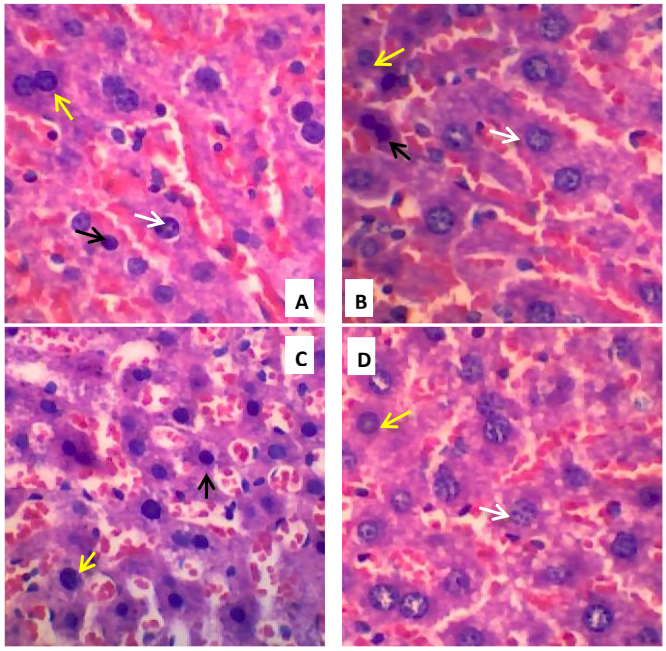

Figure 1. Mice's liver slide using HE stain between alloxan dose group at 400x magnification showing normal cell (white), parenchymal degeneration (yellow), and picnotic necrosis (black). Alloxan doses: 25mg/KgBB (A), 50mg/KgBB (B), and 100mg/KgBB (C), and Control (D)).

The microscopic observation shows normal, PD, HD, and necrosis cell within every group. Microscopic results were then counted for the number of normal, PD, HD, and necrosis cells. The results of these calculations are included in the degree of injury. The results of the normality test showed that the data are normally distributed. The mean and standard deviation distributions are used to describe the degree of injury (Table 1).

Table 1 shows the lowest degree of injury in the control group with a mean and SD of 1.09 ± 0.06. This value indicates that there are two PD cells in the 20-cell field of view. As a result, the control group falls into the normal category. The degree of injury was less than 1.5 for the 25 and 50 mg/kgBB treatment groups. This value indicates that there are 1–2 PD cells and 2–3 necrotic cells in 20 cells in the field of view. This indicates that the 25 and 50 mg/kgBB dosing groups are still in the normal category.

In contrast, the 100 mg/kg BB groups showed mean values and an SD of 2.49 ± 0.34. This value indicates that there are 8-10 PD, 1-2 HD, and 6 necrotic cells in 20 cells in the field of view. This value also indicates that the 100mL/kgBB group progresses through the PD category relative leading to the HD category. The standard deviation for this group is also indicating that the distribution of data in this group is wider than in the control group.

Table 1. Mice liver degree of injury with HE staining. Alloxan doses: 25mg/KgBB (A), 50mg/KgBB (B), 100mg/KgBB (C) and Control (D).

Group	Mean ± SD
D	1.09 ± 0.06
A	1,25 ± 0.08
B	1,42 ± 0.19
C	2,49 ± 0.34

The average mice hepatocellular degree of injury is proportional to the concentration of alloxan-induced dosage, as shown in Table 1. The extent of degree of injury increases with alloxan dose.

Unlike HE staining, PAS staining evaluated cell damage based on glycogen concentration within liver cells. Glycogen shows a pink to purple color within the liver slide. Mice's liver side stained with PAS staining were microscopically observed with 400x magnification at 5 random points (Fig.2). The pictures were taken for digital imaging using *ImageJ*.

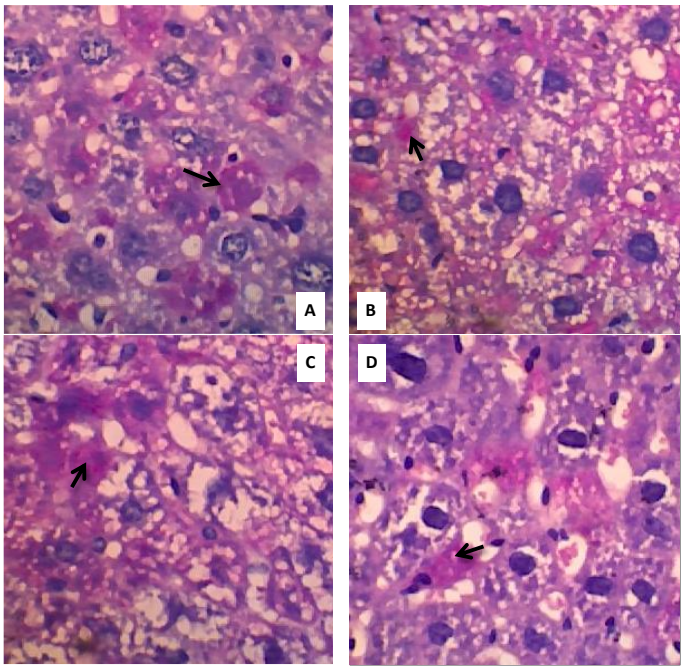

Figure 2. Mice's liver slide using PAS stain between alloxan dose group at 400x magnification showing glycogen within liver cells (black). Alloxan doses: 25mg/KgBB (A), 50mg/KgBB (B), 100mg/KgBB (C) and Control (D).

Microscopic observation shows glycogen concentration in the control group is more scattered in all fields of view than in the test group. The higher the concentration administered to the test group, the less scattered it appeared.

After the images are acquired, digital image processing is performed using ImageJ to obtain the average color intensity of glycogen contained in liver tissue. The normality test results yielded sig values >0.05. Hence the data distributions used are mean and SD (Table II).

Table II. The color intensity of mice's liver glycogen with PAS staining. Alloxan doses: 25mg/KgBB (A), 50mg/KgBB (B), 100mg/KgBB (C) and Control (D).

Group	Mean ± SD
D	90,48 ± 5,81
A	86,18 ± 7,10
B	80,06 ± 2,08
C	75,82 ± 3,42

Glycogen depletion occurs with increasing alloxan dose in mice (Table II). The highest glycogen levels were in the control group with a mean and SD of 90.48 ± 5.81 and the lowest glycogen levels were in the 100 mg/kg body weight dose group with a mean and SD of 75.82 ± 3.42. This value is obtained in color intensity units output by the *ImageJ* digital image processor. This value indicates that the higher the color intensity value, the more enriched the glycogen content contained in the cell/tissue.

ANOVA test on both methods showed Sig values <0.001, suggesting that both HE and PAS staining may show distinct differences within control and test groups. HE staining showed a significant difference in the degree of injury between the control and alloxan-induced groups. PAS staining also successfully demonstrated significant differences in liver glycogen levels between control and alloxan-induced groups.

Alloxan is a substance that can induce reactive oxygen species (ROS) activity by forming superoxide (O_2), hydrogen peroxide (H_2O_2), and hydroxyl (-OH) free radicals (Lenzen & Panten, 1998). These ROS products induce pancreatic beta cell death, cause hyperglycemia, and also damage liver tissue (Lenzen & Mirzaie-Petri, 1996; Lenzen, Drinkgern & Tiedge, 1996). Liver damage caused by alloxan can attack the cells and chemicals found in the liver. Hepatocellular injury by alloxan has similar effect with cell damage caused by diabetes melitus (Khan, et al., 2006; Srikanta, et al, 2104). Various staining method, including HE and PAS staining, can be used to identify the damage to the cells.

The HE stain is considered routine in the histopathology laboratory. HE staining used for identifying microorganisms or specific structures like nuclei and cytoplasm or molecules in cells or in tissue sections (Pujar *et al.*, 2015). Hence, HE staining could show a difference in degree of injury. This present study is consistent with Zhang *et al.*, 2018, who found that HE can reveal differences between healthy and alloxan-damaged hepatocytes. HE demonstrated that healthy control mice had normal hepatocytes with well-preserved cytoplasm, nuclei, and central veins in the diabetic group, and a loss of hepatic architecture with lymphocyte inflammation and focal necrosis of hepatocytes. Naseer A.S and Khan M.R also stated that the HE staining method could show changes in liver cells induced by alloxan in the form of central vein blockage, dilation of sinusoids, steatosis, and necrosis (Naseer & Khan, 2014; Zhang *et al.*, 2018)

The present study also accord with Sharma & Rana, 2020 that state HE staining could demonstrates a change in nuclear size from densely packed nuclei to large enucleated areas resulting from the distortion of the hexagonal shape of hepatocytes and the formation of pyknotic nuclei in alloxan-induced mice. This shows that the degree of injury by various Alloxan doses can be evaluated with HE staining (Sharma & Rana, 2020).

HE staining show that the lack of insulin resulted in significant structural changes in the liver of mice with diabetes that was caused by alloxan. The main changes included necrosis of hepatocytes, infiltration of blood cells, clumping of nuclei in numerous locations, and degradation of fibrous connective tissue. The livers of diabetic mice exhibited immediate morphological changes, including hepatocyte enlargement, bile duct hyperplasia, and a rise of intracytoplasmic acidophilus pellets (Sharma & Rana, 2020).

Suputri *et al.*, (2020) who used HE staining to review Alloxan-induced diabetic mice hepatocellular injury after onion extract treatment state that onin (*Allium ascalonicum*) can improve the histopathological feature of rats liver. Data from this study show that when onion extract treatment group compared to the group that was only given CMC, there was a decrease in the mean value of liver cell degeneration that used HE staining.

Hence, it can be deduced from the studies mentioned above that HE has been shown to be a useful staining approach for assessing the level of injury within the hepatocelullar.

The H&E stain is widely used in the industry and is relatively simple to perform. Pathologists use a variety of other histological stains, in addition to H&E, with varying properties, to better highlight various tissue components. The periodic acid-Schiff (PAS) method can be used to examine the basement membrane and carbohydrate content more thoroughly (Noori-Mughahi, et al, 2014; de Haan *et al.*, 2021).

PAS staining was used to check for the presence and abnormalities of carbohydrates like glycogen. As a strong oxidizing agent, periodic acid acts on the 1.2 glycol linkage of carbohydrates in tissue sections to produce aldehyde, which is then colored with Schiff's reagent to form a scattered red to magenta particles in the cytoplasm (Noori-Mughahi & et al, 2014; Swapna Shedge *et al.*, 2020).

PAS staining was discovered to demonstrate a significant decrease in glycogen between control and test group. The higher the dose, the lower the liver glycogen in the mice group. This is presumably because the mechanism of alloxan-induced liver damage causes structural changes in liver parenchyma cells and a failure to increase glycogen levels, which is believed to occur due to decreased glucokinase activity in the liver and inhibits glycogenesis (Srikanta, et al, 2104).

This founding was accorded with Zhao *et al.*, (2021) who demonstrate that PAS staining could presence glycogen accumulation in cytoplasm of liver cells in diabetic mice. PAS staining also show difference glycogen presence. PAS staining in this test can show a significant improvement in glycogen after carvacrol treatment in type 2 diabetic mice. This improvement is evidenced by a more even glycogen distribution and a decrease in glycogen accumulation in the treatment group the compared to control group.

The present study also in line with the research conducted by Pérez-García *et al.*, (2021). Pérez-García used PAS staining to demonstrate the difference of glycogen depots in liver between fasted and non-fasted wild-type and PAS kinase (PASK) deficient mice. Under non-fasted conditions, the majority of PAS-positive cells were found, indicating a high glycogen content in their cytoplasm in both WT and PASK-deficient livers. Pas staining was found to show significant differences in liver glycogen accumulation in this study. As a result, this study supports previous findings that PAS staining can be used to assess liver damage caused by alloxan or diabetes melitus.

CONCLUSION

The present study was found that HE and PAS staining methods were able to produce significant differences between control and various dose alloxan-induced groups of mice. Nonetheless, the sensitivity of the two methods should be compared to determine which is preferable in examining liver injury caused by alloxan or diabetes mellitus.

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