The Effect of Freezer Storage on Damage to the Microscopic Structure of Rat Liver with Hematoxylin Eosin and Masson Trichrome Staining

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The liver is the largest organ in the body which has many functions including detoxification, protein synthesis, and the production of chemicals needed for digestion. In organ research, the liver is an organ that is widely studied, so it can be used many times for research. So that the liver can be used again, the liver can be stored in preservation at -1960C or in a freezer at -800C. This study aims to assess the microscopic appearance of liver structure before and after storage in a -800C freezer equipped with DNAshield. Storage is carried out for one year as stored biological material (SBM). Fresh livers were made into histology preparations into paraffin blocks, and livers that had been stored in the freezer were also made into paraffin blocks. Both were stained with hematoxylin eosin and Masson trichrome. After that, observations were made with an Olympus CX 33 microscope and with the help of the Image J application, the research results were read. From the analysis using the ANOVA test, the total hepatocyte cell variable data was obtained at (0.117), meaning there was no significant

difference between the two groups. Meanwhile, significant data on the variable hepatocyte intake (0.18), the variable hepatocyte cells damaged (0.001) and the variable percentage damage (0.000), were below the p value of 0.05. This means that there are significant differences in the three variables between the two groups. The percentage of hepatocyte cell damage was (7.19+0.98), below organ damage of 20%. This means that rat liver stored in the freezer for one year is still suitable for reuse as research material during the storage process in the freezer in stable conditions.

Keywords: fresh liver, SBM liver, hematoxylin eosin, Masson trichrome, percentage of hepatocyte cell damage.

1. Introduction

Hepatocytes are the main parenchymal cells that make up the liver. They are polyhedral in shape and arranged in a collection of hepatic plates around the central vein. The nucleus is round and large, and some are double or multinucleated, making them highly differentiated cells[1]. Hepatocyte cells are cells that have high activity, so they are easily damaged, but also have the property of easily regenerating by means of mitosis to replace damaged cells. Hepatocyte cell damage is characterized by cell swelling and atrophy of the hepatocyte cells due to inhibited cell mitosis. Changes in cell structure in the form of cell swelling are referred to as degenerative changes, degenerative damage is reversible, which means it can be repaired if the cause is immediately removed [2].

In cryopreservation, cells or tissue are cooled to subzero temperatures in an attempt to stop biological activity. This is followed by subsequent heating to physiological temperature. The cryopreservation process is likely to cause cell damage during common biopreservation protocols through: (i) osmotic shock; (ii) dehydration; and (iii) the formation of extracellular or intracellular ice crystals [3]. Static cold storage (cryostorage) technology has become the primary method for liver transplantation and transportation; however, ischemia and hypoxia during cold storage can cause liver cell energy depletion, calcium overload, acidosis, cell edema, and other injuries, cold storage can cause changes in liver structure and function[4]. However, according to Mazur, organ cryopreservation allows organ storage indefinitely and reduces ischemic damage [5].

In the liver cold storage model, there is an increase in HIF-1α expression, which causes a decrease in AQP8 expression and ultimately inhibits liver cell secretion, makes bile lipids more concentrated, and accelerates the formation of cholesterol gallstones [6]. After cold storage, hepatocyte cytoplasmic edema occurs, the liver plate arrangement is irregular, the original shape is lost, part of the nucleus dissolves and ruptures, and the hepatic sinus fissure becomes more serious. Along with extending the cold storage time. Hepatocyte cells gradually show swelling, increase in cell volume, and sinus widening. Cold storage of liver grafts can alter cell volume homeostasis, leading to functional impairment [7]. The reperfusion process activates inflammatory factors and can cause severe liver damage. The primary targets of cold storage and reperfusion injury are hepatic sinusoidal endothelial cells (SEC)[8].

So that the liver is not easily damaged, according to Verman, during cold storage it must be fully perfused with an organ storage solution and then placed in an environment filled with storage solution [9]. Nano Heated livers were viable, maintained normal tissue architecture, preserved vascular endothelium, and demonstrated hepatocyte and organ level function[10]. An important issue in the success of freezing protocols is the prevention of intracellular ice formation, which is considered a major cause of cell damage. Intracellular ice formation can be prevented by slowly freezing the tissue to allow the cells to lose water in response to increasing solute concentrations in the extracellular medium. In this way, cells become dehydrated with no or minimal formation of intracellular ice crystals. On the other hand, intracellular ice formation can be avoided when cells are frozen very quickly in the presence of high concentrations of cryoprotectant causing tissue vitrification [11].

To protect plant tissue from the negative effects of freezing, it is necessary to dehydrate the cells. The use of cryoprotectants can maintain the integrity of the membrane by increasing the osmotic potential of the medium so that the fluid in the cell flows out and dehydration occurs. Optimal dehydration conditions can be achieved by using a cryoprotectant solution of the appropriate type, concentration and soaking time. A good cryoprotectant to use is one that can protect tissue during freezing without being toxic to the tissue itself [12]. For nucleated cells (mammals), the most common cryopreservation procedure is based on dimethyl sulfoxide (DMSO) (usually 10%), which protects cells from dehydration, replaces intracellular water, and reduces osmotic shock[13]. In cryopreservation of isolated primary hepatocytes, slow freezing rates have been shown to produce a higher number of live cells compared to fast freezing rates [14].

Hematoxylin and eosin (H and E) staining, which is the most widely used histological stain, provides a general morphological view of the nucleus and excellent details of the cytoplasm. The H&E procedure stains the nucleus and cytoplasm with contrasting colors to easily differentiate cellular components[15]. Hematoxylin, once oxidized, is a positively charged dye that colors basophilic structures, such as nuclei and ribosomes, blue, while eosin is a negatively charged dye that colors most other cellular organelles pink. Through differences in staining intensity, HE staining is able to display various morphological structures in great detail, making it a standard staining combination used in routine pathology [16].

2. Methods and Materials

2.1 METHOD

This research has received a certificate of passing ethical review from the Faculty of Medicine, University of Indonesia (FKUI) with number: KET-846/UN2.F1/ETIK/ PPM.00.02/2022, approval date 15 August 2022. This research is an experimental study of paraffin liver blocks and frozen liver stored in a -80^{0}C freezer obtained from IMERI's Stem cell and Tissue Engineering (SCTE) cluster.

2.2 MATERIAL

The research materials came from paraffin blocks of rat liver and stored biological materials obtained from Dr. Rico et al. From fresh rat liver organs, 2 paraffin blocks were made from normal rats. The remaining organs were stored in a -80^{0}C freezer provided by DNAshield for

1 year, then paraffin blocks were made. Before staining with hematoxylin eosin and Masson trichrome, the sample was thawed by placing the sample in a 50 cc centrifuge tube into a 1000C glass beaker filled with 37^0C warm water for 15 - 30 minutes. Then all materials were stained with Hematoxylin Eosin and Masson Trichrome.

2.2.1 Hematoxylin Eosin (HE) Staining

The preparation was deparaffinized in xylol for 5 minutes. Then dehydrate in graded alcohol solutions starting from 100%, 95%, 90%, 80% and 70% for 5 minutes each. After that, the preparation was incubated in Harris' hematoxylin solution and rinsed with running water. Next, the preparation is placed in an alcohol-acid mixture, examining color differentiation under a microscope. After that, rinse with running water and dip in lithium carbonate until the preparation is bright blue. Then washed with running water and incubated in eosin solution. Then dehydrated again with 70%, 80%, 90%, 95% and 100% alcohol. After that, it was incubated in xylol. Finally, it is swallowed and covered with a cover glass [17].

2.2.2 Masson Trichrome (MT) staining

The preparation was deparaffinized in Xylene, then dehydrated in a solution of absolute alcohol and 95% alcohol. Washed with distilled water. placed in Weigert's hematoxylin solution and rinsed with water. After that, it was incubated in a resorcin-fuchsin solution. examine under a microscope until the collagen fibers are black. Excess dye solution was rinsed with 95% alcohol and rinsed again with running water. After that, it was given aniline blue. Next, put the preparation into a solution of 95% alcohol and absolute alcohol. Then put it in the xylol solution. Finally, it is swallowed and covered with a cover glass [17].

2.2.3 Assessment of Hematoxylin Eosin and Masson Trichrome results

After the preparations were stained with HE and MT, they were read using an Olympus CX 33 microscope and photographed using an Optilab Advance microscope digital camera with CMOS Color Image 5 MP, photographed with 400x magnification. Assessment of research parameters using ImageJ software released by Wayne Rasband using a Windows 11 64 bit computer system from Microsoft.

Parameters assessed by HE and MT staining were the number of living hepatocytes, the number of intake hepatocytes, the number of damaged hepatocytes and the percentage of hepatocyte cells damaged. To reduce bias in reading the preparation being examined, write the sample number on the preparation without looking at the group of mice that were treated.

The assessment of research parameters is divided into 3 zones, namely zone 1 (the area around the portal area), zone 2 (the area in the middle of zones 1 and 3 which are mostly hepatocytes and sinusoidal cells) and zone 3 (the area around the central vein). Each zone was photographed 5 times, so there were 15 counting areas. Everything is added up and divided by 15 to get the average value for each preparation. Meanwhile, 8 preparations were used, so the number of photos analyzed was 120 photos. After completing the cell count, confirmation was carried out with a histologist to confirm hepatocyte cells and the accuracy of cell counting.

The assessment of intake hepatocyte cells is the presence of intact nuclei, cytoplasm and cell membranes. Hepatocyte cells are damaged if the nucleus, cytoplasm and/or cell membrane

are incomplete or damaged. The total number of hepatocyte cells is the number of intake hepatocyte cells plus the number of damaged hepatocyte cells. The percentage of hepatocyte cell damage is assessed by the number of damaged hepatocyte cells divided by the total number of hepatocyte cells and multiplied by 100%.

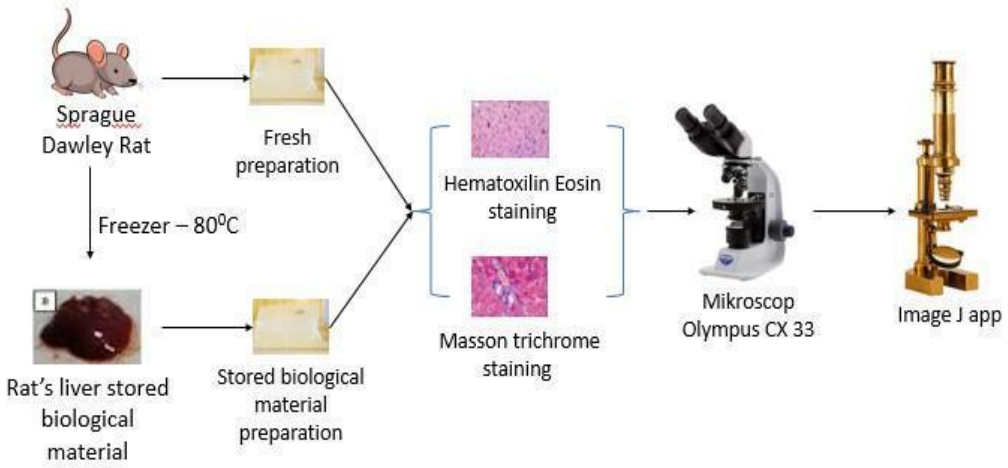

Figure 1.ProcedureStudy. Research materials were taken from fresh rat livers and stored rat livers frozen in freezer -80^{0}C, then HE and MT staining were carried out, examined with an Olympus microscope CX 33 and processed with the Image J application

2.3. Statistical analysis

Statistical analysis was carried out on a computer using the Statistical Program for Social Science (SPSS) software version 25 from IBM. Graphic creation using Prism 9 graphpad software from domatics. Data analysis begins with a descriptive test to determine the mean, minimum, maximum and standard deviation of each variable. Mean SD for normally distributed data or median min-max for non-normally distributed data. Then a normality test was carried out using the Shapiro-Wilk test and homogeneity using the Levene test. If the data is normal, continue with the Anova test and if the data is homogeneous, continue with the Bonferroni Post-Hoc test to compare between groups. If the data is not homogeneous, the Games-Howel test will be carried out to compare the data between groups. Data were considered statistically significant if the P value was <0.05[18].

3 Research Result

3.1 Histological examination with Hematoxylin Eosin and Masson trichrome staining

The assessment of the histology image was assisted by the ImageJ computer application and the following data was obtained:

Table 1. Results of hepatocyte cell counting using HE and MT staining

Group	Total Hepatocyte cells	Hepatocyte cells intake	Damage Hepatocyte cells	% damage of hepatocyte cells
Fresh Preparation N = 4	301 ± 16	292 ± 17	9 ± 1	3.05 ± 0.44
SBM Preparation N = 4	284 ± 8	264 ± 5	20 ± 3	7.19 ± 0.98

The results of examination of fresh preparations were obtained from paraffin blocks of fresh liver with HE and MT staining. The results of SBM preparations were obtained from SBM liver paraffin blocks with HE and MT staining. The total number of hepatocyte cells and intake were higher in the fresh preparation group compared to the SBM group, while the number of damaged hepatocyte cells and the percentage of damage were higher in the SBM group.

3.2 Microscopic images of HE and MT

Hepatocyte cell counting results obtained by HE and MT staining:

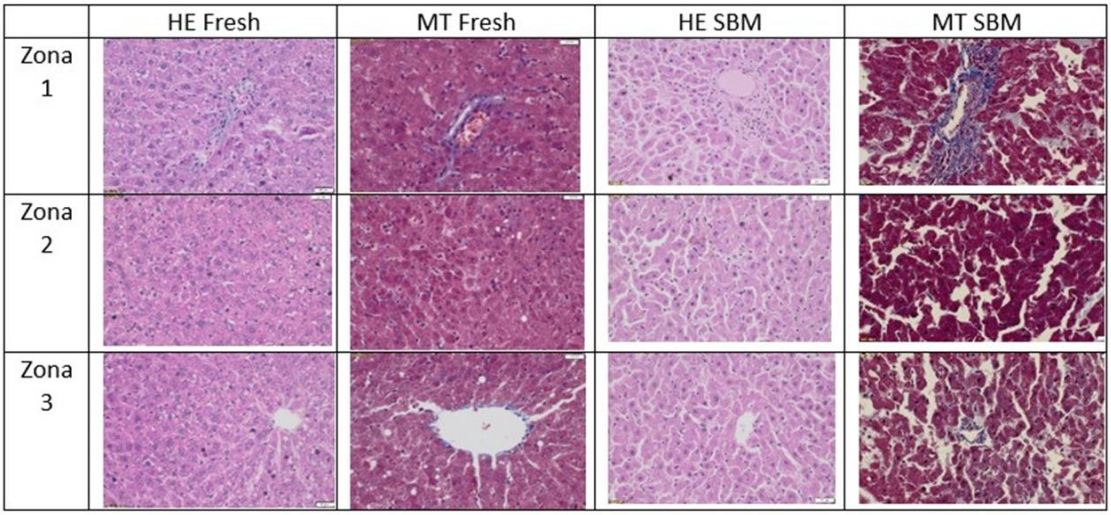

Figure 2. Microscopic images of fresh liver preparations and SBM liver preparations. Zone 1: area around the portal triangle, zone 2: area of hepatocyte cell plates and sinusoids, zone 3: area around the central vein. HE Fresh: HE staining on fresh liver, MT Fresh: MT staining on fresh liver, HE SBM: HE staining on stored biological material, MT SBM: MT staining on stored biological material.

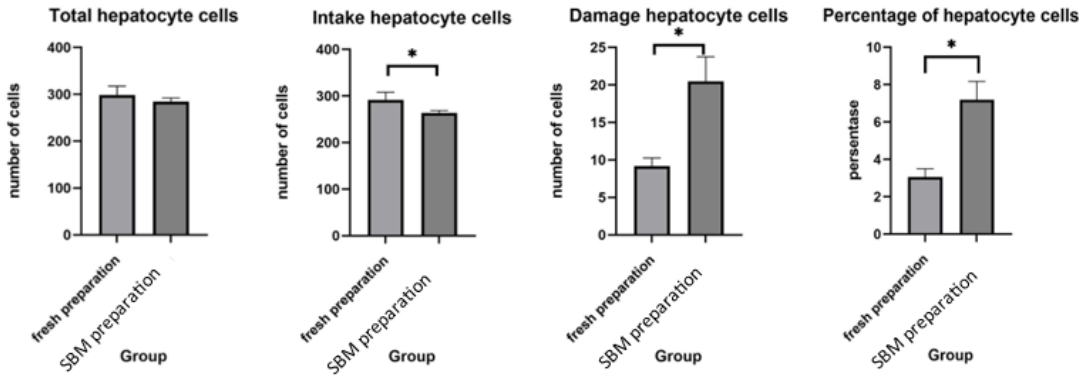

Figure 3. Comparison graph of examination results on fresh liver preparations compared to stored biological material (SBM) liver.

3.3 Data analysis

Analysis of descriptive data for each examination parameter, in the fresh preparation group, data on the total number of hepatocyte cells was (301 ± 16), the number of intake hepatocyte cells was (292 ± 17), the number of damaged hepatocyte cells was (9 ± 1), and the percentage of cell damage hepatocytes of (3.05 ± 0.44). Meanwhile, in the SBM preparation group, data on the total number of hepatocyte cells was (284 ± 8), the number of intake hepatocyte cells was (264 ± 5), the number of damaged hepatocyte cells was (20 ± 3), and the percentage of hepatocyte cell damage was (7.19 ± 0.98).

The normality test using the Shapiro Wilk test found that all significant data was greater than 0.05, so the research data was normal. The homogeneity test with the Levene test found that all significant values were greater than 0.05, so homogeneous research data was obtained. Because the data were normal and homogeneous, followed by an ANOVA test, the total hepatocyte variable was obtained with a significant value of 0.117, this shows that there was no significant data between the two groups. Meanwhile, the hepatocyte intake results had a significance value of 0.018, indicating significant data between the two groups, the damaged hepatocyte variable obtained significant data of 0.001, this indicates significant data. Finally, the damage percentage variable has a significant value of 0.000, indicating significant data. This means that there are three significant variables, namely hepatocyte intake, damaged hepatocytes and percentage of damage. This shows that there are significant differences between the fresh preparation group and the stored biological material preparation in these three variables.

4. Discussion

In this study, we summarized the variables into two, namely the fresh preparation group and the stored biological material (SBM) preparation group to facilitate data analysis. HE and MT staining for fresh preparations are combined into one into the fresh preparation group. Then HE and MT staining for stored biological material preparations were also combined into the SBM preparation group. So we get two large parts, namely the fresh preparation

variable and the stored biological material preparation variable.

In this study we obtained three significant variables from the four variables examined, these three variables are as follows, namely hepatocyte intake, damaged hepatocytes and percentage of hepatocyte damage. There is one variable that is not significant, namely the total hepatocyte variable. Data analysis shows that the significance value for intake hepatocytes is 0.018, damaged hepatocytes is 0.001 and the percentage of damage is 0.000. This value is below the p value of 0.05, meaning that there is a significant difference between the three variables in the fresh preparation group compared to the stored biological material preparation.

The total number of hepatocyte cells is the total number of hepatocyte cells in one examination field of view, consisting of intake hepatocyte cells and damaged hepatocyte cells. In the stored biological material preparation data, the total hepatocyte variable was (284±8), lower than the fresh preparation data of (301±16), this shows that there was a reduction in the total number of hepatocyte cells in the SBM group compared to the fresh preparation group. This situation makes it possible for the sinusoids to widen between the hepatocyte cells in the SBM preparation so that the hepatocyte cells can come out in one field of view. As a result, the number of hepatocytes in the SBM group was less than in the fresh preparation group. In data analysis using the ANOVA test, the significance value for the total hepatocyte variable was 0.117, greater than the p value of 0.05, so the data was not significant. So there was no significant difference in the total hepatocyte variable in the two research groups.

Intake hepatocyte cells are intact hepatocytes with the criteria for intact cell nucleus, cytoplasm and cell membrane. The number of intake hepatocyte cells in the SBM group was (264±5) less than in the fresh preparation group (292±17), this is possible due to some damage to the hepatocyte cells, so that the intake hepatocyte cells were fewer. In data analysis using the ANOVA test, significant data was obtained at 0.018, below the p value of 0.05, so significant data was obtained. So there are significant differences in hepatocyte cell intake variables between the two research groups.

Damaged hepatocyte cells are hepatocyte cells that experience damage to the cell nucleus, cytoplasm and/or cell membrane. The data shows that the number of damaged hepatocytes in the SBM group was (20±3) greater than the fresh group data of (9±1), this means greater damage to hepatocyte cells in the SBM group compared to the fresh group. Data analysis using the ANOVA test showed a significant value of 0.001, smaller than the p value of 0.05. This means that there is a significant difference in the damaged hepatocyte variable in the fresh group and the SBM group.

The percentage of damage is calculated by the number of damaged hepatocytes divided by the total number of hepatocytes multiplied by 100%. Data obtained on the percentage of damage in the SBM group was (7.19±0.98) greater than the fresh group of (3.05±0.44). This occurred because hepatocyte cell damage in the SBM group was higher than in the fresh group. Data analysis using the ANOVA test obtained a significant value of 0.000, smaller than the p value of 0.05. This means that there is a significant difference in the variable percentage of hepatocyte cell damage between the two groups. In accordance with research by Ishine N et al, who showed that in cryopreservation by freezing at temperatures below

zero degrees or in hypothermic preservation, the lobule structure and intercellular connections of hepatocytes are well preserved, the hepatocytes themselves are well preserved, and their nuclei do not appear pyknotic [19]. Fujii et al s research, with HE or PAS staining, the surface area of the tissue is compressed but only a few deeper tissue areas can retain it naturally morphologically, and the PAS reaction products are well preserved in both tissue areas [20].

5. Conclusion

In this study, we made two groups, namely a fresh preparation group stained with hematoxylin & eosin (HE) and Masson trichrome (MT), as well as a stored biological material group stained with HE and MT. There were significant differences between the two groups of fresh preparations and stored biological material preparations in the three variables, namely intake hepatocyte cells, damaged hepatocyte cells, and percentage of damage. The percentage of hepatocyte cell damage was (7.19 ± 0.98), including minor damage. Small damage is possible due to a cryoprotectant substance in the form of a DNA shield which is given to samples of stored biological material. Therefore, storing biological materials in a refrigerated freezer at -80^0C for one year is still feasible for further research, especially in terms of examining histology. In addition, during storage procedures with a -80^0C freezer, it is procedural and correct.

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