THE EFFECT OF STORAGE TIME AND VARIATIONS OF GLYCEROL CONCENTRATION IN FROZEN PACKED RED CELL ON ERYTHROCYTEINDICES VALUES

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Abstract, Background: Storage of Packed Red Cells depends on the anticoagulant used. Generally Packed Red Cell can last 35 days to 42 days. Storage of Packed Red Cells can be extended by a freezing process called cryopreservation. Cryopreservation is the process of preserving biological samples that are susceptible to damage and maintained at low temperatures for a long time to maintain cell structure. PRC stored using the cryopreservation method was carried out by adding one of the cryopreservation agents, namely glycerol. Glycerol used to protect red blood cells during clotting and thawing. The qualityof red blood cells can be seen from several blood test parameters, one of which is the erythrocyte indices. Erythrocyte indices is a value that describes the characteristics of erythrocytes in size, content and concentration. The erythrocyte indices consists of: MCV (Mean Cospucular Volume), MCH (Mean Cospucular Haemoglobin), MCHC (Mean Cospucular Hemoglobin Concentration). The aim of this studywas to determine the effect of storage time in frozen packed red cells on the erythrocyte indices value and to determine the effect of glycerol concentration in frozen packed red cells on the erythrocyte indicesvalue.

Method: The type of research is experiment research with a quasy experiment research design. In thisstudy, glycerol was added to Packed Red Cells with varying concentrations of 35%, 40%, and 45%, then the samples were stored frozen at -20°C for 35 days, 42 days, and 49 days. Then deglycerolization was carried out, then tested using the sample to see the erythrocyte indices. The research data were statistically analyzed using the SPSS version 22 application.

Result: The results of the statistical test of the concentration difference in the significant of MCV value,namely sig. 0.000 ($p<0.05$) and MCHC sig. 0.000 ($p<0.05$). And for MCH there is no difference, namelysig. 0.185 ($p<0.05$).

Conclusion: Based on the results of statistical tests, it can be concluded that there is an effect of storage time on frozen packed red cells on the erythrocyte indices value. There is an effect of 35%, 40%, and 45% glycerol concentrations on the MCV and MCHC values. However, there was no effect of MCH valueon glycerol concentrations of 35%, 40%, and 45%.

Keywords: Storage, Cryopreservation, Packed Red Cell, Glycerol, Erythrocyte Indices

Background

Cryopreservation is the process of preserving biological samples that are susceptible to damage and maintained by cooling at low temperatures for a long time to maintain cell structure. This preservation is also useful for emergency transfusion services, military transfusions, and storage of rare blood because it can store PRC for years.[1]

Freezing PRC is recommended by the AABB at -65°C or colder. Storage of red blood cells at low temperatures below 0°C aims to suppress erythrocyte metabolism so that it is possible to maintain them in frozen conditions for longer. Erythrocyte metabolism is severely inhibited at -20°C and almost stops at -70°C to -140°C.[2,3,4]

Cryopreservation is carried out by adding a cryopreservation agent (cryoprotectant) to red blood cells. There are several types of cryoprotectants used for cryopreservation of red blood cells, including hydroxyethyl starch, glycerol. Glycerol is the standard ingredient used for cryopreservation because of its cost and safety. The process of adding glycerol to PRC (glycerolization), namely by adding glycerol with a certain concentration in Packed Red Cells and then

freezing. Glycerol is used to protect red blood cells during clotting and thawing. Glycerol can pass through the cell membrane and enter the cytoplasm, then it will create a hyperosmotic environment to prevent the release of fluid from the cell. Glycerol must be removed first (deglycerolization) before blood is transfused.[1,5,6]

The concentrations that can be used in the glycerolization process for PRC preservation are 40% for high concentrations at -80°C and 20% for low concentrations at -190°C. Some Blood Banks that freeze PRC use high concentrations. Freezing of PRC using low concentrations of glycerol is rarely used because the freezing temperature required is very low and can only be achieved using liquid nitrogen which is considered impractical, and tends to cause hemolysis.[6,7]

The aim of this study was to determine the effect of storage time in frozen packed red cells on the erythrocyte indices value and to determine the effect of glycerol concentration in frozen packed red cells on the erythrocyte indices value.

Method

The type of research is a quasi-experimental. In this study, Packed Red Cell was added with glycerol with varying concentrations of 35%, 40%, 45%, then the PRC was stored frozen at -20°C for 35 days, 42 days, and 49 days. After that, deglycerolization was carried out, then the sample was tested to see the erythrocyte indices. Packed Red Cells that were not added with glycerol (0%) and not frozen were used as controls (0 days).

Samples were obtained from PMI Bandung City. This study was held at Laboratory of Hematology. Department of Medical Laboratory Technology Poltekkes Ministry of Health Bandung on March to June 2022.

The data in this study is used primary data obtained from the measurement of the erythrocyte indices in Packed Red Cells added with glycerol then frozen and deglycerolized with variations in concentration to variations in storage time, then measured using a hematology analyzer.

The tools used in this research are Hematology Analyzer, Freezer -20°C, Laminar Airflow, centrifuge, timer, vial tube, beaker, analytical balance, stir bar, tube, tube rack, micropipette, blue tip, measuring pipette. The materials used in this study were Packed Red Cell, glycerol 85%, deionized water, NaCl (12%, 1.6%, 0.9%).

Sample collection

In this study PRC with SAGM (Saline-Adenine-Glucose-Mannitol) was used.

Freezing

The first step in this process is to freeze the PRC, a process known as glycerolization. During this step, a glycerol solution is introduced into the container with the PRC. Glycerol was added to the PRC dropwise for small PRC aliquots according to the predetermined concentration. After that, freezing sample at -20°C storage.

Thawing and deglycerolization

Thaw samples at room temperature. The next step is deglycerolization, which is the process of removing the glycerol solution from the PRC by gradually lowering the hypertonic intracellular environment of the PRC using salt solutions of decreasing concentrations (12%, 1,6%, 0,9% NaCl solutions).

While mixing continuously, add a volume of 12 percent NaCl solution equal to one-half the volume of the PRC mixture in a dropwise manner; for example, if thawing an aliquot of 1 mL PRC mixture, add 500 µL of 12 percent NaCl solution. Gently mix the contents and incubate for 3 minutes at room temperature. Fill the test tube to the halfway mark with 1.6 percent NaCl solution. Then fill almost to the top with blood bank saline. Centrifuge the samples at 3000 rpm for 30 seconds. Aspirate the supernatant. Wash the RBCs with blood bank saline until no hemolysis is seen in the supernatant.[10] Then the sample is measured using a hematology analyzer.

Data Analysis

Based on the results of the measurement of the erythrocyte index value, statistical data processing was carried out with univariate data with descriptive and bivariate tests with the General Linear Model Repeated Measures Two Way test on the SPSS version 22 application.

Result

The research data on the effect of storage time and variations in the concentration of glycerol in frozen packed red cells on the erythrocyte indices value were carried out 5 times repetition. The mean of data that has been collected, as shown in the table below.

Table 1. Results of Erythrocyte Indices Measurement

Treatment Variables	Storage Time	Mean		
		MCV (fL)	MCH (pg/sel)	MCHC (g/dL)
Control without Glycerol (0%)	0 Hari	81,52	24,00	29,44
Concentration 35%	35 Hari	81,74	23,98	29,28
	42 Hari	82,38	24,14	29,32
	49 Hari	83,48	23,96	28,68
Concentration 40%	35 Hari	81,78	24,04	29,36
	42 Hari	82,14	24,20	29,46
	49 Hari	82,96	24,04	28,96
Concentration 45%	35 Hari	81,78	24,08	29,26
	42 Hari	82,02	24,10	29,38
	49 Hari	82,86	24,08	29,02

The table 1.1 above shows the mean of MCV values based on variations in glycerol concentrations of 35%, 40%, and 45% stored for 35 days, 42 days, and 49 days.

Mean Corpuscular Volume

Table 2. The Results statistical Analysis of MCV

	Sig.	Result	Conclusion
Storage Time	0,000	p<0,05	There is difference
Concentration	0,000	p<0,05	There is difference

Based on table 2 for the length of storage, the Sig value is obtained. 0.000 <0.05, it was concluded that there were differences in MCV values in frozen PRC stored for 35 days, 42 days, and 49 days. For the concentration obtained the value of sig. 0.000 <0.05, it can be concluded that there are differences in MCV values with variations in glycerol concentrations of 35%, 40%, and 45% in frozen PRC. To see the location of the difference in each storage time and its concentration can be seen through the results of the Within-Subjects Contrast test in table 3.

Table 3. The Results statistical Analysis of MCV

	Storage Time	Sig.	Result	Conclusion
Storage Time	35 days vs 0 day	0,013	p<0,05	There is difference
	42 days vs 0 day	0,000	p<0,05	There is difference
	49 days vs 0 day	0,000	p<0,05	There is difference
Concentration	35% vs control	0,000	p<0,05	There is difference
	40% vs control	0,000	p<0,05	There is difference
	45% vs control	0,000	p<0,05	There is difference

Table 3. above shows that there is a difference in the MCV value stored for 35 days with 0 days (sig. 0.013 <0.005), there is a difference in the MCV value between 42 days of storage and 0 days, and there is also a difference in MCV value between 49 storage times. day with 0 day. Furthermore, it can be seen for concentrations, where the table above shows that there are differences in MCV values with glycerol concentrations of 35%, 40%, and 45% compared to the control because the significance value obtained is sig. 0.000 < 0.05. An illustration to show that there are differences in shelf life and glycerol concentration is presented in Figure 1.

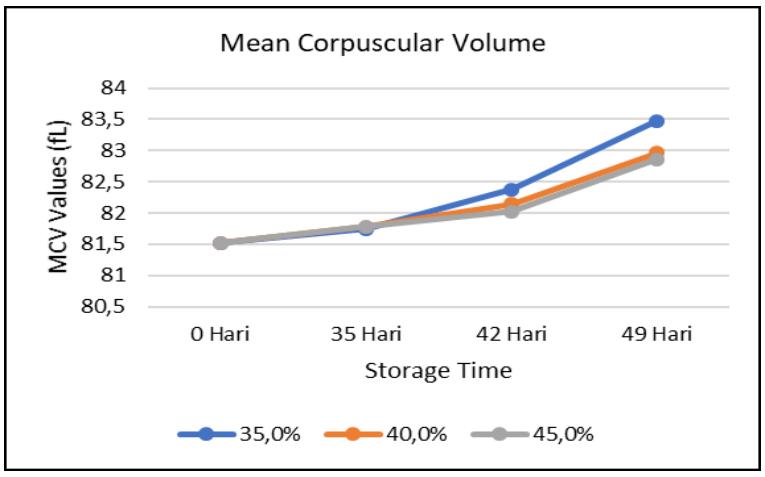

Figure 1. Graphic Plots of MCV Measurement

Based on Figure 1, It can be seen that the mean value of MCV in each concentration tends to increase with the length of time the PRC is stored. However, the mean value closest to the control was a concentration of 45%.

Mean Corpuscular Haemoglobin

Table 4. The Results statistical Analysis of MCH

	Sig.	Result	Conclusion
Storage Time	0,035	p<0,05	Ada perbedaan
Concentration	0,185	p<0,05	Ada perbedaan

Based on table 4, it can be seen for the length of storage the significance results obtained are Sig. 0.035 > 0.05, it can be concluded that there is a difference in the MCH value with the shelf life of 35 days, 42 days, and 49 days in frozen PRC. For the concentration obtained the value of sig. 0.185 > 0.05, it can be concluded that there is no difference in MCH values with variations in glycerol concentrations of 35%, 40%, and 45% in frozen PRC. To see the location of the difference in each storage time and concentration, it can be seen through the results of the Within-Subjects Contrast test in table 5.

Table 5. The Results statistical Analysis of MCH

	Storage Time	Sig.	Result	Conclusion
Storage Time	35 days vs 0 day	0,504	p>0,05	There is no difference
	42 days vs 0 day	0,004	p<0,05	There is difference
	49 days vs 0 day	0,602	p>0,05	There is no difference
Concentration	35% vs control	0,503	p>0,05	There is no difference
	40% vs control	0,085	p>0,05	There is no difference
	45% vs control	0,118	p>0,05	There is no difference

Based on table 5, it can be seen that for storage time there is no difference in the MCH value stored for 35 days compared to 0 days because the significance value obtained is sig. 0.504 > 0.05, but there is a difference in the MCH value stored for 42 days compared to 0 days (sig. 0.004 <0.05), and there is no difference in the MCH value stored for 49 days compared to 0 days (sig value 0.602 > 0.05). Furthermore, for the concentration, the table above shows the value of sig. obtained > 0.05, it can be concluded that there is no difference in the MCH value between the glycerol concentrations of 35%, 40%, and 45% compared to the control. An illustration to show that there are differences in shelf life and glycerol concentration is presented in Figure 2.

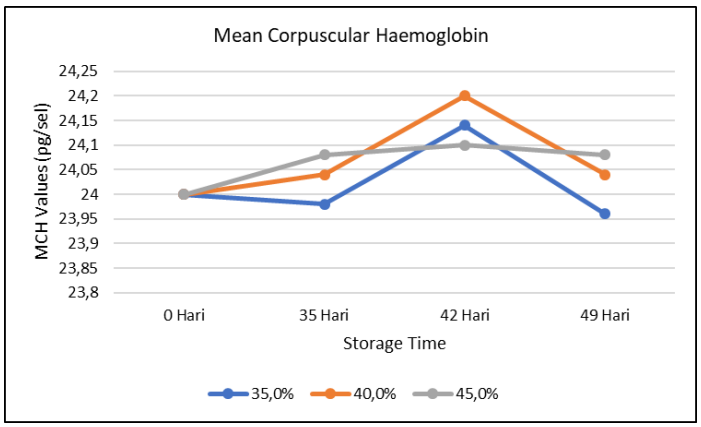

Figure 2. Graphic Plots of MCH Measurement

Based on Figure 2, It can be seen that the mean value of MCH at 42 days of storage shows a difference due to the increased MCH yield. However, judging from the concentration the most stable and close to control is a concentration of 45%.

Mean Corpuscular Haemoglobin Concentration

Table 6. The Results statistical Analysis of MCHC

	Sig.	Result	Conclusion
Storage Time	0,00	p<0,05	There is difference
Concentration	0,00	p>0,05	There is difference

Based on table 6, it can be seen that the significance results obtained are 0.000 <0.05, it can be concluded that there are differences in MCHC values in frozen PRC stored for 35 days, 42 days, and 49 days. For the concentration of sig. The obtained value is 0.000 <0.05, it can be concluded that there are differences in MCHC values with variations in glycerol concentrations of 35%, 40%, and 45% in frozen PRC. To see the location of the difference in each storage time and its concentration can be seen through the results of the Within-Subjects Contrast test in table 7.

Table 7. The Results statistical Analysis of MCHC

	Storage Time	Sig.	Result	Conclusion
Storage Time	35 days vs 0 day	0,080	p>0,05	There is no difference
	42 days vs 0 day	0,279	p>0,05	There is no difference
	49 days vs 0 day	0,000	p<0,05	There is difference
Concentration	35% vs control	0,000	p<0,05	There is difference
	40% vs control	0,003	p>0,05	There is difference
	45% vs kontrol	0,089	p>0,05	There is no difference

From table 7. can be seen the value of sig. obtained 0.080 > 0.05, it can be concluded that there is no difference in the value of MCHC stored for 35 days and 42 days compared to 0 days. And there is a difference in the value of MCHC stored for 49 days compared to 0 days because of the sig value. obtained 0.000 <0.05. Furthermore, for the concentration, there is a difference in the MCHC value between the 35% concentration and the control (sig. 0.000 <0.05), there is a difference in the MCHC value between the 40% concentration and the control (sig. 0.003 <0.05) and there is no difference in the value. MCHC between 45% concentration and control (sig. 0.089 > 0.05). An illustration to show that there is a difference in shelf life and glycerol concentration is presented in Figure 3.

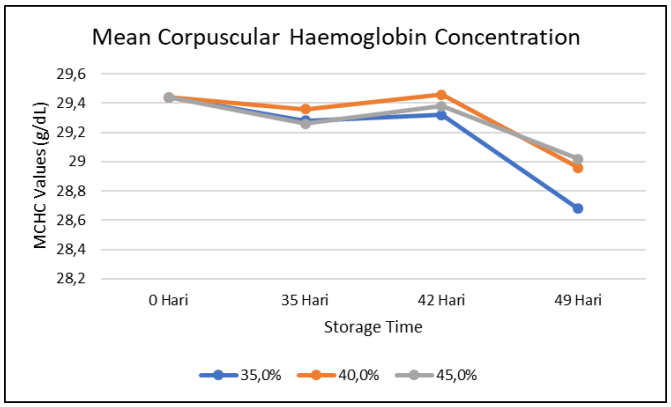

Figure 3. Graphic Plots of MCHC Measurement

Based on Figure 2, It can be seen that the mean value of MCHC in each concentration tends to decrease with the length of time the PRC is stored. However, the mean values closest to the control were concentrations of 40% on day 42 and 45% on day 49.

Discussion

Preservation of red blood cells by cryopreservation causes physiological changes, because during the freezing process it will form ice crystals in the extracellular which causes changes in intracellular osmotic stress. To prevent damage to cells, cryoprotective or protective agents such as glycerol are needed[9]

Glycerol is used to protect red blood cells from damage, by resisting cellular dehydration and maintaining osmotic stress. Glycerol also depresses the system's freezing point, which may help mitigate the formation of extracellular.[10,11]

However, glycerol protects red blood cells, after the liquefaction and washing process it will produce osmotic volume changes that can damage cells. The glycerol washing process in red blood cells was carried out using graded NaCl solution (12%, 1.6%, 0.9%). The use of a hypertonic saline solution serves to reduce the glycerol concentration, reduce cell volume, increase cell density and provide ample latitude for the hypotonic stress associated with glycerol removal. The use of hypertonic solutions can cause osmotic swelling of cells, so the solution must be added slowly with sufficient time for mixing and osmotic balance. With the addition of a solution with a decreased concentration can overcome the swelling of red blood cells.[12,3]

From the measurement results in figure 1, it was found that the results of the Mean Corpuscular Volume (MCV) at storage times of 35 days, 42 days, and 49 days with glycerol concentrations of 35%, 40%,and 45% tended to increase. From the results of statistical data in table 3 processing using the General Linear Model Repeated Measures Two Way with variations in shelf life of 35 days, 42 days, and 49 days it is known that there are significant differences, and also with variations in glycerol concentrations, namely 35%, 40%, and 45 % concluded that there is a difference.

These results are in line with research conducted by Palotta et al., 2012, which stated that the shape of red blood cells changed significantly after glycerolization, the MCV value increased, and persisted when frozen at -80°C and returned to normal after the thawing process. and washing, but the results of the MCV value were still higher than the control.[9]

Changes in the shape of red blood cells occur associated with increased permeability to ions and increased osmotic fragility. The permeability of red blood cell membranes to glycerol depends on the presence of aquaporin channels. When glycerol penetrates cells, on the one hand it protects erythrocytes from many clotting damage, while on the other it appears to trigger and alter protein-protein interactions in the cytosol and cytoskeleton. Glycerol is a polar molecule, at high concentrations, known to change the ionic strength and dielectric constant of aqueous solutions. The penetrating effect of glycerol in red blood cells can result in harmful changes in cell structure.

The concentration of glycerol used for cryopreservation also affects red blood cells. The concentration of solutes and cell dehydration that is too high has a damaging effect on the lipid-protein complex of the cell membrane and can weaken the cell and then cause swelling of the cell to the point of cell rupture.[13]

Furthermore, on the measurement of Mean Corpuscular Haemoglobin (MCH) in figure 2, it was found that the MCH values obtained at shelf life of 35 days, 42 days, and 49 days with glycerol concentrations of 35%, 40%, and 45% tended to be stable. From the results of statistical data in table 5 processing with variations in shelf life of 35 days, 42 days, and 49 days it is known that there are significant differences, this is explained in more detail in table 3 for 42 days of storage there are differences, but in save 35 days and 49 days there is no difference. Then with variations in the concentration of glycerol, namely 35%, 40%, and 45%, it was concluded that there was no difference.

Based on the literature, frozen storage of red blood cells does not completely affect red blood cells. Frozen storage of red blood cells also does not cause the loss of Adenosine 5'-triphosphate (ATP) and 2,3-Diphosphoglycerate (DPG).[9]

According to the results of research conducted by Rogers et al., 2018, said that the MCV value in the glycerolization process has increased, changes in the shape of red blood cells do not affect the MCH value, but affect the MCHC value which has decreased.[14]

From the measurement results in figure 3, it was found that the results of the Mean Corpuscular Haemoglobin Concentration (MCHC) at storage times of 35 days, 42 days, and 49 days with glycerol concentrations of 35%, 40%, and 45% tended to decrease. From the results of statistical data in table 7 processing with variations in shelf life of 35 days, 42 days, and 49 days, it is known that there is a significant difference, this is explained in more detail in table 4 on the shelf life of 35 days and 42 days there is no difference , but at 49 days of storage there is a difference. Then with variations in the concentration of glycerol, namely 35%, 40% there is a difference, but at a concentration of 45% there is no difference.

The results of the decreasing MCHC value are in line with research conducted by Palotta et al., 2012. That there is a decrease in the MCHC value after the thawing and washing process. MCHC measures the concentration of hemoglobin in red blood cells, the larger the cell size, the lower the concentration. The decrease in MCHC values is related to hemoglobin and hematocrit values, not all red blood cells present at the beginning before glycerolization survive until the end of storage.[9]

Conclusion

Based on the results of statistical tests, it can be concluded that there is an effect of storage time on frozen packed red cells on the erythrocyte indices value. There is an effect of 35%, 40%, and 45% glycerol concentrations on the MCV and MCHC values. However, there was no effect of MCH value on glycerol concentrations of 35%, 40%, and 45%.

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