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Comparison of the effect of Sumbawa honey and sucrose as extracellular cryoprotectants on viability, morphology, and phenotype stability of CD34+ hematopoietic stem cells from umbilical cord blood

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ABSTRACT

Cryopreservation is a common practice for the long-term storage and maintenance of the quality of Hematopoietic Stem Cells (HSC) from Umbilical Cord Blood (UCB). Cryomedium containing 10% DMSO is the standard intracellular cryoprotectant agent (CPA) in HSC cryopreservation. However, DMSO is toxic to both cells and patients during transplantation. Therefore, the concentration of DMSO needs to be reduced by adding extracellular CPAs, such as sucrose or Sumbawa honey. The objective of this study was to compare the ability of Sumbawa honey and sucrose as extracellular CPAs to protect HSC CD34+ during cryopreservation. This in vitro study was designed using cryomedium consisting of 10% DMSO as a control, 5% DMSO + 5% Sumbawa honey, and 5% DMSO + 5% sucrose. The results showed that the cryoprotectant containing 5% DMSO + 5% Sumbawa honey had a positive effect and a significant difference ($p<0.05$) compared with 5% DMSO + 5% sucrose on the viability and morphology of HSC. However, the mean reduction in phenotype stability as indicated by the decrease in percentage CD34+ in the 10% DMSO (6.90 ± 8.60), 5% DMSO + 5% sucrose (10.60 ± 9.20), and 5% DMSO + 5% Sumbawa honey (8.60 ± 11.50) showed no significant difference ($p>0.05$). In conclusion, the combination of DMSO and Sumbawa honey was able to maintain the viability, morphology, and phenotype stability of HSC. Therefore, honey can be used as an alternative cryoprotectant for the cryopreservation of hematopoietic stem cells.

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1. Introduction

Hematopoietic stem cell transplantation (HSC) is a human cell-based cell therapy that has the potential to be used in regenerative therapies (Hunt, 2019). According to data collected by the Center for International Blood and Marrow Transplant Research (IBMTR) from 400 transplant centers, in 2006, HSC transplantation was used as a treatment for hematological malignancies. It was employed for acute myeloid leukemia by 33%, acute lymphoblastic leukemia by 16%, Hodgkin's lymphoma (HL) and non-Hodgkin's lymphoma (NHL) by 12%, and multiple myeloma (MM) by 3% (Hatzimichael and Tuthill, 2010). The clinical application of hematopoietic stem cells for the treatment of non-hematological conditions has also developed in recent years. These cells have been used to treat severe scleroderma, diabetes, metabolic disorders, and for gene therapy (Hornberger et al., 2019). HSC transplantation can be performed autologously and allogeneically by taking HSC from the peripheral blood, bone marrow, and umbilical cord blood (Hatzimichael and Tuthill, 2010). However, a major limitation of allogeneic HSC transplantation is the lack of donor-matched human leukocyte antigens (HLA), which limits graft acceptance and increases the risk of developing graft-versus-host disease (GVHD) (Hornberger et al., 2019).

One solution to overcome the shortage of donors is the development of cord blood banks, such as the Anthony Nolan Cord Blood Bank, located at Nottingham Trent University, England. In 2013, the umbilical cord blood bank stored stem cells from 50,000 cord blood centers using a cryopreservation procedure (Hatzimichael and Tuthill, 2010). The cryopreservation procedure has challenges, such as decreased cell viability after thawing and side effects in patients due to the use of cryoprotectants (Erol et al., 2021). Studying the optimization of cryopreservation protocols to uphold the quality of hematopoietic stem cells is crucial. This

effort aims to maintain the quality of HSCs stored for extended periods within cord blood banks (Rodrigues et al., 2008).

One of the most widely used cryoprotectants is 10% dimethylsulfoxide (DMSO) as an intracellular cryoprotectant agent in the cryopreservation of various cell types, including hematopoietic stem cells to maintain cell viability during cryopreservation (Hornberger et al., 2019; Rodrigues et al., 2008). However, DMSO is toxic to cells at room temperature and causes side effects when transplanted into patients, therefore DMSO concentrations need to be reduced (Fleming and Hubel, 2006). The addition of extracellular cryoprotectants in the form of saccharides, such as glucose, sucrose, and trehalose, is required to reduce the concentration of DMSO. Previous studies have shown that sucrose can be used in cryopreservation solutions to reduce DMSO concentration from 10% (v/v) to 5% (v/v). Similarly, trehalose can also reduce DMSO concentration from 10% (v/v) to 2.5% (v/v) (Rodrigues et al., 2008).

Besides sucrose and trehalose, honey is a natural ingredient that can also be used as an extracellular cryoprotectant. Honey was chosen as the extracellular cryoprotectant because it contains various types of saccharides (Sarmadi et al., 2019). Fructose and glucose are good osmotic buffers because they have lower viscosities than disaccharides (Alfoteisy et al., 2020). Honey is also known to have antibacterial and antioxidant properties because it contains vitamin C, phenolic compounds, and enzymes such as catalase and peroxidases (Meo et al., 2017). A study conducted by Sarmadi et al. (2019) on the cryopreservation of mouse embryos showed that natural honey has properties that are more stable to temperature changes than sucrose, thereby reducing the possibility of ice crystal formation.

Based on previous studies, sucrose has long been used as an extracellular cryoprotectant for cryopreservation of hematopoietic stem cells. Sumbawa honey also has potential as an alternative to sucrose for an extracellular cryoprotectant, because it has a more diverse sugar content and high antioxidant capacity. However, little is known about the effect of Sumbawa honey and sucrose as an extracellular cryoprotectants in HSC cryopreservation. This study aimed to determine the effect of the composition of cryoprotectants DMSO 10%, DMSO 5% + Sumbawa honey 5%, and DMSO 5% + sucrose 5% on the viability, morphology, and phenotypic stability of hematopoietic stem cells, as indicated by the percentage of postoperative CD34+ thawing after 48-72 h of cryopreservation.

2. Materials and methods

2.1. Materials

This research was conducted at the Stem Cell and Tissue Engineering (SCTE) Laboratory, Indonesia Medical Education and Research Institute (IMERI) FK UI, Central Jakarta, from October 2021 to July 2022. This study is an in vitro experimental study that compared two experimental groups: samples of HSC cryopreserved with DMSO 5% + Sumbawa Honey 5% and HSC cryopreserved with DMSO 5% + Sucrose 5% and one control group HSC cryopreserved with DMSO 10%. The chemical composition of Sumbawa Honey are shown in Table 1.

Table 1. Chemical composition of Sumbawa honey

Chemical composition	Concentration
Water (%)	28.2 4.92
Acidity (mL NaOH 1 N/kg)	160.42 56.31
Sucrose (%)	0.58 0.77
Fructose (%)	37,1 0.00
Glucose (%)	44.08 11.49
Hydroxymethylfurfural (mg/kg)	not detected
Ash	0.58 0.11

Source: Sari et al., 2013; Tanuwidjaya, 2014

The samples used in this study were hematopoietic stem cells isolated from fresh umbilical cord blood (100-120mL) resulting from caesarean section deliveries from patients at the National Central General Hospital (RSUPN) dr. Cipto Mangunkusumo. Sampling was appropriate based on the code of ethics (Protocol Number: 21-10-1076) reviewed by the Faculty of Medicine, University of Indonesia. The sample was obtained by a team of obstetrician at RSUPNCM with a collaborating researcher, dr. Gita Pratama Sp.OG(K) under research permit granted by RSUPNCM Innovate (Research Division) number 23223.

2.2. Methods

2.2.1. CD34+ cell isolation

The sample used in this study was umbilical cord blood which was collected by dr. Gita Pratama Sp.OG(K) from Caesarean section (SC) at RSUPN Dr. Cipto Mangunkusumo. Umbilical cord blood samples were collected from seven cesarean section. Isolation Mononuclear Cells (MNC) from fresh umbilical cord blood was initiated by collecting total nucleated cells (TNC) according to the method used by Antarianto et al. (2019). Fresh umbilical cord blood from the blood bag was diluted 1:1 in PBS and centrifuged at 1000 rpm for 10 min. The middle layer between the plasma and the pellet was centrifuged again at 3500 rpm for 15 min. The pellets of TNC were collected and slowly placed in a tube containing Ficoll-Paquein at a 1:1 ratio through the tube wall. The mixture was then centrifuged 400 × g for 10 min. MNC rings were collected slowly, then added at a ratio of 1:1 and centrifuged at 650 × g for 10 min. The pellets were resuspended in PBS up to 1 ml and the cells were counted. Subsequently, CD34+ hematopoietic stem cells were isolated using EasySep which refers to the protocol by Stemcell Technologies #17896 using EasySep and EasySep magnets Human Cord Blood CD34 Positive Selection Kit II (Stemcell technologies, Biopolis, Singapore).

2.2.2. Cryopreservation

Cryopreservation medium consisted of basal medium, medium diluent, and cryoprotectants. The isolated CD34+ hematopoietic stem cells were suspended in Roswell Park Memorial Institute (RPMI) 1640 basal medium and placed in a cryogenic tube. Subsequently, the cord blood serum was added to the cell suspension. Cryoprotectants consisted of three combinations of media, group 1 contains 10 % DMSO (control), group 2 contains 5% DMSO + 5% Sumbawa Honey, and group 3 contains 5% DMSO + 5% sucrose. A cryoprotectant combination from each group was then added to the cell suspension. The total volume of the cell suspension and cryopreservation medium in the cryogenic tube was 1 mL. Cryogenic tubes were placed in Mr. Frosty (Merck, Jakarta, Indonesia) and stored at -80°C for 48 h.

The cryopreservation procedure was carried out for 48 hours in order to mimic the cryopreservation time that was patented by Universitas Indonesia with Antarianto et al. (2020) (Indonesian awarded patent number S00201904436). Since this study supported the findings of the patent, the option of extending the term of cryopreservation or choosing another time-point was not taken into consideration.

2.2.3. Thawing and washing

The thawing and washing of cells were carried out according to the method used by Antarianto et al. (2019). After being cryopreserved for 48 hours, cells were thawed by warming the cryogenic tube in water at 37°C. The liquefied cells were then washed (washing) using a complete medium. Whole cell suspension in a cryogenic tube with a volume of 1 mL was placed in a 15 mL falcon tube containing 9 mL of complete medium. The

cell mixture and complete medium were centrifuged at 400 × g for 10 min. The supernatant was discarded, and the pellets were collected and resuspended in 1 mL complete medium.

2.2.4. Morphological observations and calculation of the number of living cells (viable)

The number of live cells was calculated before and after cryopreservation to determine the viability of hematopoietic stem cells after 48 h of cryopreservation. The cell suspension in 1 mL of complete medium was taken using a 10 L micropipette and placed on parafilm. Trypan blue was added into the cell suspension and resuspended by pipetting up and down. The mixture of cells and trypan blue (10 L) was added to Neubauer Improved Haemocytometer Counting Chambers and observed using an inverted microscope at 10x objective magnifications. The morphology of live cells in freshly isolated and post-cryopreserved samples was observed, and viable cell counts were determined by counting the unstained cells.

2.2.5. Flow cytometry analysis

Flow cytometry analysis was performed after the isolation of cord blood CD34+ cells according to the method used by Antarianto et al. (2019) to determine the purity of the isolated CD34+ cells by examining the percentage of cells expressing CD34+. Flow cytometry analysis was also performed to determine the percentage of cells that still expressed CD34+ after cryopreservation. CD34+ cells were isolated from fresh cord blood and cryopreserved by the same method. A cell suspension (100 L) was added to both stained and unstained tubes. Antibody fluorescein isothiocyanate-conjugated (FITC) anti-human CD34 was added to the stained tube to a volume of 3 L and resuspended by pipetting up and down. The tube was then covered with aluminium foil and incubated for 15 30 min in a refrigerator at 4 °C. After incubation, 100 L of PBS was added to both stained and unstained tubes. Then, final analysis was performed using BD FACS Aria III (BD Biosciences, New Jersey, US).

2.2.6. Progenitor cell observation

Giemsa staining was performed as described by Antarianto et al. (2019). The cell suspension (200 L) was pipetted and dripped on a glass slide, and dried using a hot plate at 50 °C for 30 min. Fixation was then carried out by immersing the glass slide in methanol for 30 min. The glass slide was then dripped with 5% Giemsa solution for 20-30 min. The slide was then rinsed with water and dried at room temperature. Progenitor cells were observed under a microscope and with Optilab at 400x magnification.

2.3. Data analysis

The data in this study were subjected to statistical analysis using SPSS 20. The analysis involved the examination of the percentage of hematopoietic stem cells that survived after cryopreservation and the percentage of CD34+ expression in hematopoietic stem cells before and after cryopreservation. To ensure the validity of the analysis, normality and homogeneity tests were conducted using the Shapiro-Wilk test and Levene's test, respectively. A p-value < 0.05 was considered indicative of a significant difference between the two groups."

3. Results and discussion

3.1. Results

3.1.1. Cell viability

There were no discernible variations in the quantity of viable cells between each treatment group's pre- and post-cryopreservation counts utilizing hemocytometer chambers (Fig. 1). These findings revealed that Sumbawa honey and sucrose, when used in combination with DMSO as extracellular cryoprotectants, were able to sustain the quantity of viable hematopoietic stem cells following cryopreservation. Additionally, the proportion of hematopoietic stem cells that survived cryopreservation in each therapy group was examined. In comparison to the DMSO group and the sucrose group, the Sumbawa honey group demonstrated a considerably higher percentage of surviving hematopoietic stem cells after cryopreservation (Fig. 2). Meanwhile, there is no significant difference between DMSO and sucrose in the percentage of viable hematopoietic stem cells after cryopreservation. Data on the average percentage of hematopoietic stem cells that were able to survive after cryopreservation are shown in Table 2. Table 2 shows that, in comparison to sucrose, Sumbawa honey can sustain more viable cells following cryopreservation.

Table 2. Percentage of viable cells and phenotype stability of CD34+ after cryopreservation

Group	Percentage of viable cells after cryopreservation (%)	Percentage of CD34+ after cryopreservation (%)
DMSO 10%	75.80 ± 8.54[a]	80.1 ± 22.7
DMSO 5% + Sumbawa honey 5%	86.16 ± 9.09[b]	78.4 ± 24.5
DMSO 5% + sucrose 5%	71.43 ± 3.55[c]	76.5 ± 25.1

The values are expressed as mean SD, n=5

(a) There is a significant difference between DMSO 10% and DMSO 5% + Sumbawa honey 5% group ($p<0.05$)

(b) There is a significant difference between DMSO 5% + Sumbawa honey 5% and DMSO 5% + sucrose 5% group ($p<0.05$)

(c) There is no statistically significant difference between DMSO 10% and DMSO 5% + sucrose 5% group ($p>0.05$)

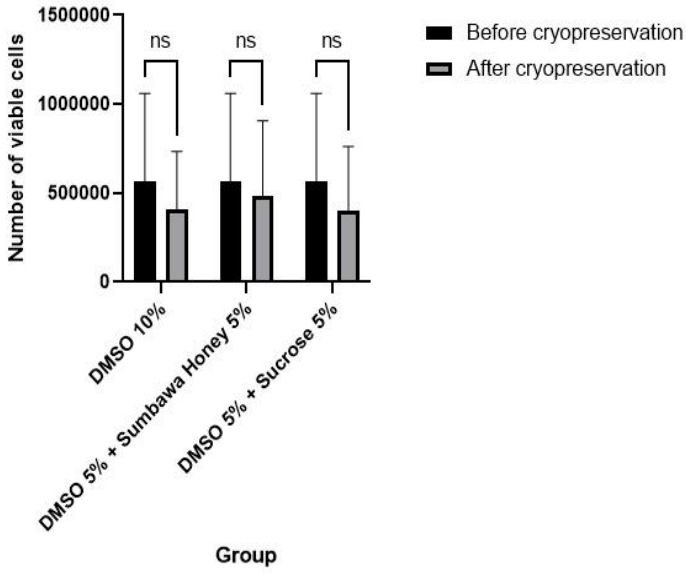


Fig. 1. Decreasing of the number of viable cells in fresh samples and after cryopreservation. There is no significant difference between before and after cryopreservation (ns, $p<0.05$)

3.1.2. Cell morphology

Each treatment group's cell morphology was evaluated following cryopreservation. Compared to DMSO and pre-cryopreserved cells (Fig. 3A and 3B, respectively), Sumbawa honey (Fig. 3C) did not exhibit any appreciable alterations in cell shape. Sucrose continued to exhibit some swollen cells at the same time (Fig. 3D). These findings suggested that Sumbawa honey tended to be more efficient at preserving cell morphological stability than sucrose (Fig. 3D).

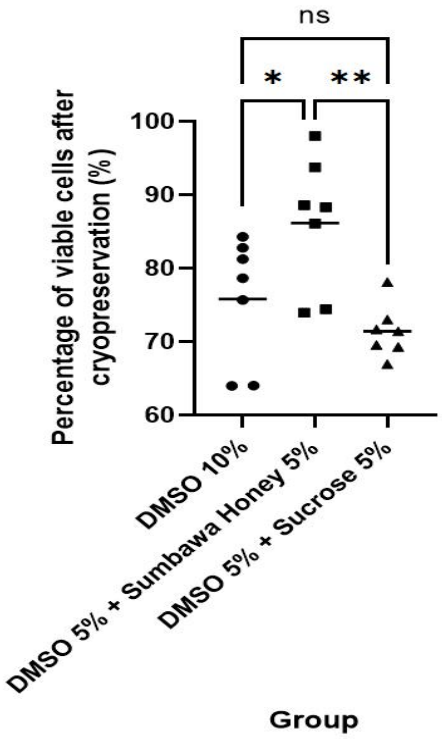


Fig. 2. Mean percentage of hematopoietic stem cells that are able to survive after cryopreservation. There is a significant difference between DMSO 10% and DMSO 5% + Sumbawa honey 5% group (*, p<0.05) and there is a significant difference between DMSO 5% + Sumbawa honey 5% and DMSO 5% + sucrose 5% group (*, p<0.05). However, there is no statistically significant difference between DMSO 10% and DMSO 5% + sucrose 5% group (ns, p>0.05)

Giemsa staining was used to further examine the changes in cellular structure. As demonstrated by the DMSO group's findings (Fig. 3F), Sumbawa honey did not exhibit any alterations in cellular structure. The arrows in Fig. 3H show that bigger, enlarged HSC were seen in the 5% DMSO + 5% sugar group. These results indicated that the 5% DMSO + 5% Sumbawa honey group can prevent cell swelling and maintain cell morphology as HSC before cryopreservation (Fig. 3E). These observation results of HSC in Giemsa staining were in accordance with observations of the morphology of living (viable) cells after cryopreservation.

Giemsa staining and microscopic examination revealed that the HSC was spherical, proportionately sized to that of a typical HSC, and had intact membranes. These findings indicated live cells and HSC features, which were supported by CD34 flow cytometry data (Fig. 4). It was determined that the complexity of cell morphology was relatively low. This was supported by the distribution of the cell population in the lower region, which exhibits low granularity. The distribution of the cell population, which tended to be in the left region, verified the size of the cells. These cells had a cell size of 6-10 m, which is typical of hematopoietic and progenitor stem cells that express CD34+ (Wagner et al. 1995). Cryopreservation was not followed by in vitro culture in this work, therefore more research is required.

3.1.3. Phenotype stability

The decrease in the percentage of CD34+ cells that occurred between the fresh sample and the sample after cryopreservation in group 1, group 2, and group 3 was relatively small (Fig. 5A). This shows that the three cryoprotectant combination treatments maintained the CD34+ percentage after cryopreservation. The percentage of CD34+ after cryopreservation was 80.1 22.7% (group 1), 78.4 24.5% (group 2), 76.5 25.1% (group 3) (Fig. 5B). Statistical tests showed that group 1, group 2, and group 3 had a mean percentage of CD34+ cells after cryopreservation, which was not significant (p>0.05; p=0.872).

Although the addition of honey or sucrose to the cryopreservation medium did not have a significant effect on maintaining stemness, based on the percentage of CD34+ cells, the addition of 5% Sumbawa honey together with 5% DMSO in hematopoietic stem cell cryopreservation medium resulted in a lower level of decreased CD34+ expression compared to the addition of 5% sucrose along with 5% DMSO. This result shows that the addition of honey was more effective than sucrose in maintaining the CD34+ percentage of hematopoietic stem cells during frozen storage.

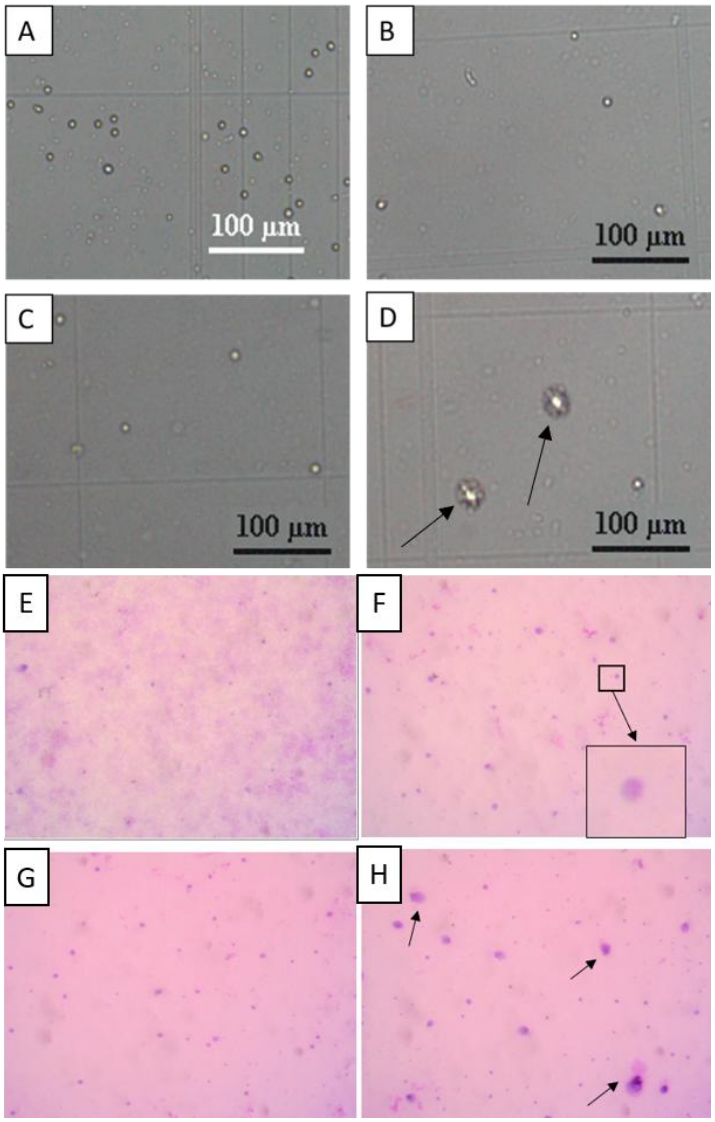


Fig. 3. Hematopoietic stem cells before cryopreservation (A); Hematopoietic stem cells after cryopreservation (10% DMSO) (B); Hematopoietic stem cells after cryopreservation (10% DMSO + 10%

Sumbawa honey) (C); Hematopoietic stem cells swelling after cryopreservation (5% DMSO + 5% sucrose) (D); Giemsa staining of HSC before cryopreservation (E); Giemsa staining of HSC after cryopreservation (10% DMSO) (F); Giemsa staining of HSC after cryopreservation (10% DMSO + 10% sumbawa honey) (G); Giemsa staining of swelled HSC after cryopreservation (5% DMSO + 5% sucrose) (H)

3.2. Discussion

This study showed that the addition of 5% Sumbawa honey together with 5% DMSO had a better effect on maintaining the cell number, morphology, and stemness during 48 h of cryopreservation at -80°C than the addition of 5% sucrose. This is because Sumbawa honey has advantages over sucrose, and it contains more sugar and antioxidants.

Honey contains various sugars in the form of monosaccharides and disaccharides. Sugar molecules are known to stabilize cell membranes through hydrogen bonds between sugars and lipid groups on cell membranes. This protective effect is correlated with an increase in the number of hydrogen bonds between the lipid head groups and sugar molecules. Disaccharide groups are able to bridge three or more lipid molecules, whereas monosaccharides, such as glucose, are able to bridge two lipid molecules to stabilize cell membranes (Svalgaard et al., 2016; Pereira et al., 2006).

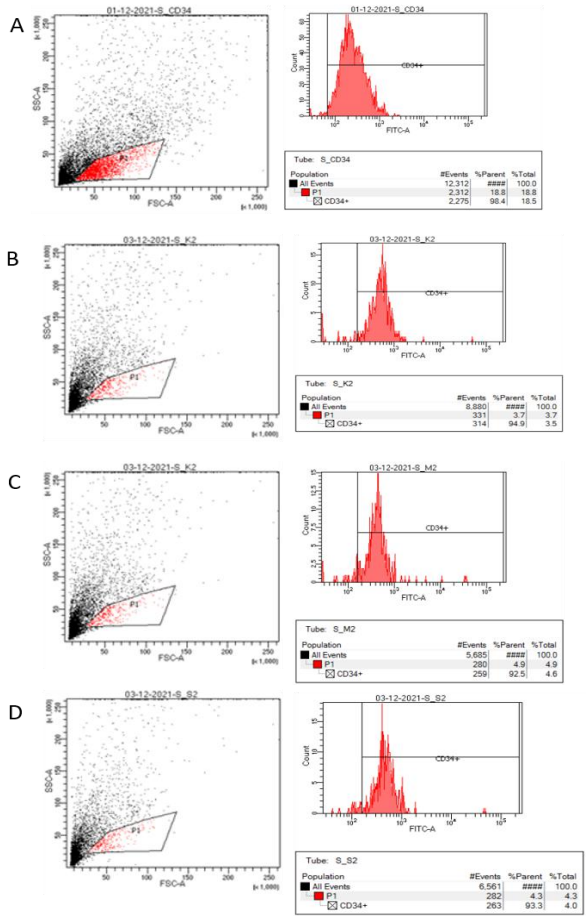
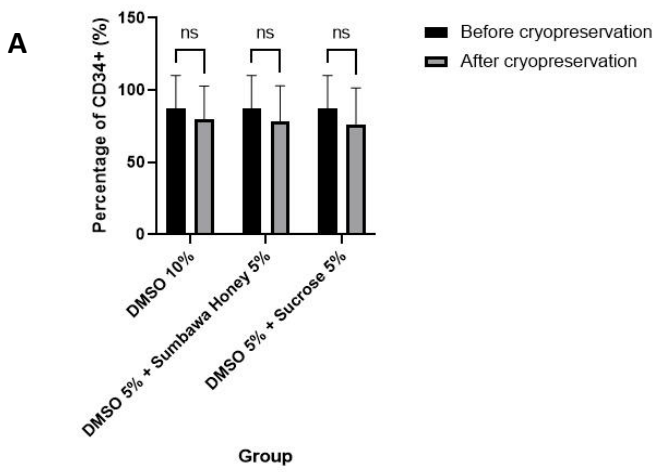


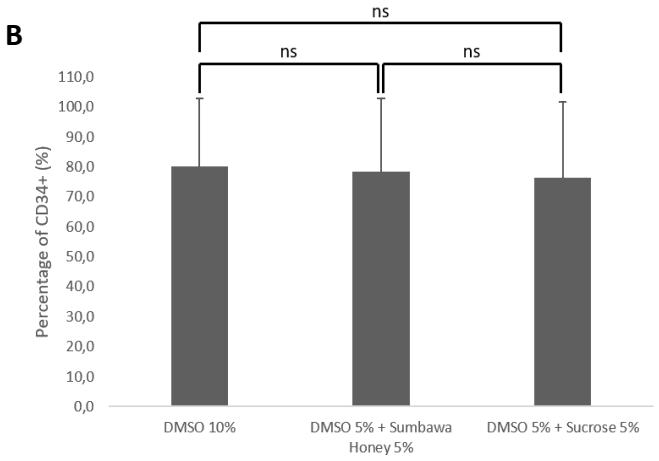
Fig. 4. Flow Cytometry analysis of fresh hematopoietic stem cells before and after cryopreservation. Population and histogram of CD34+ expression in (a) HSC before cryopreservation, (b) Hematopoietic stem cells after cryopreservation with DMSO 10%, (c) Hematopoietic stem cells after cryopreservation with 5% DMSO 5% sumbawa honey, and (d) Hematopoietic stem cells after cryopreservation with 5% DMSO + 5% sucrose.

Honey contains disaccharides, such as trehalose, which is often used as a cryoprotectant. Studies have shown that the addition of trehalose to the cryopreservation medium of various cells, including hematopoietic and mammalian germ cells, can increase the viability and colony-forming ability post-cryopreservation. The addition of trehalose combined with DMSO to cryopreservation media has been shown to be beneficial for post-thawing survival and maintenance of various stem cell functions (Ha et al., 2016). Trehalose can form hydrogen bonds with the phospholipid heads of cell membranes in order to provide membrane stability (Sum et al., 2003). The ability of trehalose in maintaining the structure of cell membranes is due to trehalose having the ability to expand and contract its glucose ring and adjust to the exact dimensions between the lipid head groups. This makes trehalose more efficient than sucrose and other disaccharides (Lee et al., 2014).

During the thawing process, the potential for cell swelling exists. The process of melting extracellular ice crystals can lead to a reduction in the concentration of solutes surrounding the cell, resulting in a decrease in extracellular osmolarity. This shift prompts water from the external environment to infiltrate the cell, consequently causing it to swell. To counteract this phenomenon, it becomes crucial to employ a cryoprotectant mixture capable of upholding an equilibrium in solute concentration both outside and inside the cell. This equilibrium aids in preventing cell swelling subsequent to thawing (Gilfanova et al., 2021). The superior capacity of honey in thwarting cell swelling in comparison to sucrose can be attributed to its broader spectrum of sugar varieties, encompassing monosaccharides, disaccharides, and polysaccharides (Cheepa et al., 2022). The high sugar content in honey can maintain the concentration of solutes outside the cell during the thawing process to maintain extracellular osmolarity and prevent the movement of water from outside the cell into the cell (Mijovic and Phampilon, 2007).



(ns) There is no significant difference between before and after cryopreservation (p>0.05)



(ns) There is no significant difference between before and after cryopreservation (p>0.05)

Fig. 5. Decreasing of CD34+ percentage in fresh samples and after cryopreservation (a); Mean percentage of CD34+ after cryopreservation (b). There is no statistically significant difference between DMSO 10% and DMSO 5% + Sumbawa honey 5% group (ns, p>0.05). There is no statistically significant difference between DMSO 5% + Sumbawa honey 5% and DMSO 5% + sucrose 5% group (ns, p>0.05). Also there is no statistically significant difference between DMSO 10% and DMSO 5% + sucrose 5% group (ns, p>0.05)

Cryopreservation can cause cell membrane damage by reactive oxygen species (ROS), such as lipid peroxidation, protein oxidation, and DNA damage (Liu et al., 2021). Lipid peroxidation refers to the decomposition of lipids into aldehydes such as 4-hydroxynonenal (4-HNE) and malondialdehyde (MDA). Lipid peroxidation greatly affects cell function because lipids are important components of cell membranes (Tsikas, 2017). CD34+ surface protein attaches to the surface of cell membranes (Sidney et al., 2014). If the integrity of the cell membrane is disturbed, CD34+ protein can be released from the cell, causing a decrease in the percentage of CD34+ cells analyzed using flow cytometry. ROS production can be prevented or reduced by the addition of antioxidants (Ahmed et al., 2018; Len et al., 2019). Honey is known to contain various antioxidants including vitamin C, vitamin E, flavonoids, and glutathione reductase (Lu et al., 2015). The supplementation of various types of antioxidants, such as vitamin E and vitamin C, in the storage medium resulted in an increase in the ability to maintain DNA integrity and increase cell viability (Fujita et al., 2005; Kalthur et al., 2011). Vitamin E (alpha-tocopherol) is an antioxidant that can break the very strong lipophilic bonds in ROS between fatty acids in cell membranes. Vitamin E interacts with and neutralizes free radicals, converting them into tocopheroxyl radicals (Kalthur et al., 2011). Vitamin E can interact with vitamin C in a "vitamin E recycling" reaction. Tocopherol hydroxyl groups react with peroxyl radicals to form lipid hydroperoxides and tocopheryl radicals. Tocopheryl radicals react with vitamin C and oxidize vitamin E again so that vitamin E can return to its reduced state. The interaction between vitamin E and vitamin C can make the antioxidant function of vitamin E continue to be restored by vitamin C (Traber and Stevens, 2011).

The limitation of this study is that it did not measure ROS levels or intracellular antioxidant enzyme activity. Therefore, it is necessary to measure ROS levels and intracellular antioxidant enzyme activity in future studies.

4. Conclusion

Our findings suggest that Sumbawa honey may be a potential extracellular cryoprotectant that reduces DMSO concentration during HSC cryopreservation by maintaining HSC viability and morphology after cryopreservation.

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Conflict of interest

The authors declare there is no conflict of interest in this study.

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