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Analysis of Pluripotent Cell in Post-circumcised Preputial Skin using Cold Transport Methods (Ice and Dry Ice) with Oct-4 Expression

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Abstract

The discovery of pluripotent cells in preputial skin, a previously discarded tissue, means prepuce can become a new source of stem cell banking which can be beneficial for the donor and his family. In transporting preputial skin to the biobank, we wanted to see simpler and inexpensive cold transport methods using dry ice and ice rather than liquid nitrogen to preserve the stem cells. The preputial skins were obtained from mass circumcision with informed consents and transported into the laboratory with dry ice or ice. In the laboratory, the skin samples underwent histotechnique process, Hematoxylin-Eosin (HE) staining, and Immunohistochemistry (IHC) with Oct-4 antibody staining. The data was analyzed using microscope, OptiLab™, Image Raster™, and SPSS. Oct-4 positive expression was counted and the data was examined with SPSS. The mean of Oct-4 expression in samples transported with dry ice was 2.30 and ice was 2.38. Our study resulted in no significant difference between dry ice and ice (P value is 0.901) in the Oct-4 expression. Thus, dry ice and ice have equal function as cold transport method for preputial skin and can be used as a bridge towards liquid nitrogen freezing.

Keywords: Cold transport; Oct-4 expression; Pluripotent cell; Post-circumcised preputial skin

Introduction

Many countries practice circumcision, including Indonesia. In December 2006, WHO estimated >50% of male population got circumcised In Indonesia where it is usually done in childhood [1] Previously, the preputial skin is discarded. However, recently it can be used as a skin graft for example following burn incidents [2].

Oct-4 positive cell expression has been found in the preputial skin, which may show cell pluripotency [3]. Stem cell, a major discussion these past few years, is believed to be the key of future medicine. Stem cell from preputial skin can be beneficial both for the boy and his family for instance stem cell is thought to help severe burns and degenerative disc disorder although further studies are needed [4,5].

Burn can cause scar and its incidence rate is quite high. Approximately 2.4% of trauma cases are burns in America. The incidence is considered to occur more in children rather than adult and about 60% to 65% of the victims are boys [6]. In Indonesia, burn is included in injury (cedera) cases with the prevalence of 8.2% nationally in 2013 [7]. As for his parent or adults; stem cell is important since studies show connection between degeneration of intervertebral disc to Low Back Pain (LBP), which is considered as an universal problem [4]. LBP is one of the world health issues, for example the prevalence of it in Australia has reached 25.6% and in China is 21.7% from the general community [8]. Based on a research done by Community Oriented Program for Control of Rheumatic Disease, Indonesia's prevalence of low back pain is quite high which is 23% in the city and lesser amount (15%) in the countryside [9].

This shows that stem cell banking is ought to be done. Biobank is basically a bank for the biological specimens. The processes of biobank start from collecting, processing, and storing of the specimens for an extended period. Later, the samples will be recorded and the information will be updated to the bank database [10]. The prepuce can be utilized as a stem cell source for banking in the future. With proper tissue transport and handling, the stem cell contained can be transferred in prime condition. From there, it can be processes further.

Cold transport method is a core procedure to preserve the tissue so the stem cell for banking is optimal. A question about which simple method is more advantageous to transport the tissue so that the stem cell could preserved more, emerged on the researcher's mind. Improper transport mayhap will cause it to be defective. In addition, this research attempts to find the low cost, accessible, and least complicated transportation method for stem cell to optimize its condition and function. Thus, through this research, the researcher aimed to formulate a practical cold transport method so preputial skin-derived stem cell banking can be achieved.

Problem identification

In spite of the fact that new researches about stem cells are emerging, this research aims to investigate the affordable, accessible and least complicated transport method.

Research question

Comparing ice to dry ice, which is the better cold transport method for preputial skin to preserve the stem cell, using Oct-4 as one of the stem cell marker?

Hypothesis

Tissue transport using ice is better than dry ice as cold transport method to obtain higher number of Oct-4 positive cells in preserving stem cell in preputial skin.

Objective of the Research

General objective

To optimize transport method for stem cell banking in Indonesia.

Specific objective

To find the easiest and most accessible transport method thus stem cell contained in preputial skin will arrive in ideal condition at desired location.

Literature Review

Transporting methods

Samples obtained need to be relocated for further processes. Low temperature is used as a preserving and transporting method since it helps to slow down the tissue metabolism. In addition, to achieve a satisfactory tissue sample, nucleic acid and protein's integrity has to be maintained using cold temperature. The ideal procedure is to place the tissue in liquid nitrogen in a period less than 30 minutes after sample collection for transferring, and then freeze (-80°C) it when reaching the destination for prolonged storage [11].

This research will use dry ice and ice as the variables. Dry ice consisted of carbon dioxide and commonly used as a transport method possibly since it has lower temperature characteristic rather than the normal ice (dry ice sublimes at approximately -78.5°C at 1 atmospheric pressure) [12]. However, the result from an experiment, conducted by Belitsky, Odam, and Hubley-Kozey with living subject participants, showed that ice can reduce skin temperature more compare to dry ice. Although due to its melting characteristic, ice can drip [13].

Stem cell banking

Identifying the epidermal stem cell is hard because they have no special structure. Morphologically they are similar to basal cell. However, one of the stem cell's characteristic is its slow cell cycle, as a result, labeling their DNA will persevere (the label is diminishing in non-stem cells because they are dividing continuously). Stem cell found in different parts of human body or in different species expresses different markers. Although diverse surface markers are expressed by stem cell, there is no specific marker for it [14].

The facility to store and preserve the biological samples is called as biobank. The process of obtaining the samples are collecting, processing, and storing the biological tissues. One of the most important key points in a biobank is the quality, which has to be maintained as early as possible. Therefore, a clear Standard of Procedure (SOP) should be implemented in these banks [10].

Oct-4

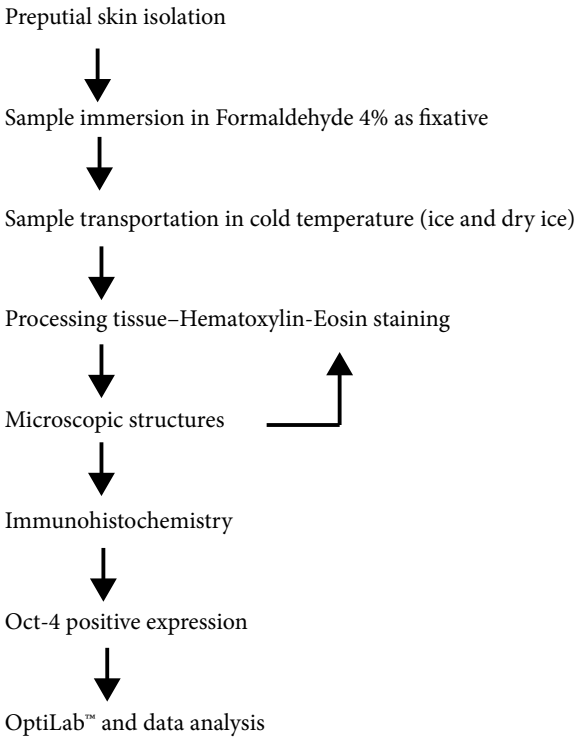
Oct-4 is an abbreviation for Octamer-binding transforming factor 4. It is entitled as the regulator master for the pluripotency of a stem cell. In one of the hypothesis, Oct-4 is associated with cancer, and also differentiation of cells causes the down-regulation of this transcription factor [15]. Other study implied that significant fluctuation, either a decrease or increase in this transcription factor level, will cause the cell to differentiate to different lineage. If mutation occurs in the Oct-4 associated proteins, it can induces a destructive phenotype. In conclusion, the Oct-4 amount ought to be firmly controlled [16]. This research will use Oct-4 as one of the stem cell pluripotency markers.

Oct-4 can be classified into 2 main types; Oct-4A and B. Pluripotent stem cell gene transcriptions are managed by Oct-4A. While the function of Oct-4B has not yet been elucidated. Nowadays, Oct-4 is use for spermatogonial stem cell and progenitor cell marker in mice and its expression can also be found in human testicular tissue [15].

From Anindhita's study, conducted not long ago, eight out of ten male participants aged around seven until sixteen years old are positive for Oct-4 expression on their preputial skin. By Hematoxylin-Eosin along with immunohistochemistry staining utilizing Oct-4, the researcher found about four particular structures where the suspected pluripotent stem cell resides; blood vessel, hypodermis, sebaceous gland, and hair follicle [3].

Methods

Research scheme



Research design

A comparative experimental design was used in conducting this research. The researcher believed that this design was the most appropriate since the goal was to compare which method was better instead of discovering the exact number of cell. There was intervention on the transport method to the sample thus the experimental design was chosen.

Time and location

The time needed to conduct this research was from August 2015 until May 2016 and the research was done in Department of Histology, Faculty of Medicine, Universitas Indonesia. This study was divided into 2 periods; August 2015 (4 weeks) and May 2016 (4 weeks). In between August and May, the researcher was searching for preputial skin samples.

Data source

The preputial skins from twenty different subjects were obtained from mass circumcision event in collaboration with dr. Rorica Tixson (Doctor Share Organzation) and Rumah Sunatan. The Hematoxylin-Eosin stain, Oct-4 antibody, and other material needed for immunohistochemistry were provided from Department of Histology, Faculty of Medicine, University Indonesia. The ice was home-made while the dry ice was bought.

Population and sample

The target population was children (5-15 year-old) who were having circumcision. Twenty samples were needed in this experiment. All subjects must fulfill the inclusion criteria and did not meet any of the exclusion criteria.

Sample size

To calculate the sample size in a qualitative-comparative experimental study, Hardon, Hodgkin, Fresle stated there are no rules for it. However, normally at least ten samples in each group are required [17]. In this experiment, the total samples required were 20 samples; 10 samples were transported with ice while the other 10 by dry ice.

Methods and Analysis of Data

Research methods

Sample preparation: The samples were obtained from the doctors performing the circumcision. Then, the samples were directly put in a fixative solution (Formaldehyde 4%).

Cold transport methods: The shipping box was cool box (33 cm × 24 cm × 26 cm), which can contain up to 15 samples. Three shipping box were used with each sample's area was approximately 6.6 cm × 8 cm. While the samples were being immersed in fixative solution, ten samples were transported with ice and the other with dry ice. The maximum transportation duration was 2.5 hours (author optimization result).

Tissue preparation: At the circumcision location, after the skin was cut, the subjects' skin samples were immersed in fixative solution immediately. In this research, formaldehyde 4% was used. While the samples were in fixative solution, they were being transported (10 with ice and the other 10 with dry ice) inside a cool box for maximum 2.5 hours. After that, the samples were taken out and put in room temperature. For 48 hours post-circumcision, the samples were soaked in formaldehyde [3,18].

The next step was dehydration to extract water inside the tissue. Tissues were bathed in alcohol with increased concentration gradually (70%, 80%, 95%, 100%) for 24 hours each concentration. Samples were soaked again with absolute alcohol 2 more times and continued with xylene (2 times) in order to clear the tissue samples. Tissues were then solidified (embedded) by placing them into cassette bases, which later would be filled with paraffin from inside of the oven and cooled overnight to make a solid block.

The microtome was utilized to slice the tissue paraffin block to thin slices. The slices, which should contain all part of the tissue, were put in a water bath and positioned on the slides. Tissue slides were stained using routine stain or immunohistochemistry. Finally, the stained slides were covered with drops of entellan and thin cover glass.

Hematoxylin-Eosin (HE) staining (routine staining): For this routine staining, the slides were first immersed in xylene solution, alcohol with gradual decrease concentration (100%, 95%, 70%), and aquadest in order for deparaffinization (removal of the wax) and hydration. Tissues were soaked in xylene 3 times with each of the duration 10 minutes, 2 times with absolute alcohol, 2 times with alcohol 95%, once with alcohol 70%, and aquadest. The duration of each alcohol and aquadest immersion was 5 minutes. Next, the slides were stained with Hematoxylin for 2 minutes and 15 seconds. Before the Eosin staining, the glass slides were rinsed with flowing water for 10 minutes. The slides were dehydrated again using alcohol and xylene but in a similar reverse order as the hydration process (2 times alcohol 70% for 3 minutes each, 2 times alcohol 95% for 3 minutes each, 2 times alcohol 100% for 5 minutes each, and 3 times xylene solution; the slides were dipped 10 times in the first xylene and soaked 2 times in the second and third xylene for 10 minutes each). Lastly, the slides were covered [3,18].

Immunohistochemistry: Hydrogen peroxide 3% solution, blocking serum, and Oct-4 antibody were prepared beforehand. Methanol was added to H_2O_2 30% so the concentration of hydrogen peroxide solution reached 3%. Blocking serum 1.5% was made by combining normal donkey serum (75 µL) into working Phosphate Buffer Saline (PBS) (4925 µL). To obtain Oct-4 antibody with 1:10 concentration; the antibody (250 ml) was diluted with blocking serum (2500 ml) [3].

The first step of immunohistochemistry process was immersion in xylene. Specimen slides were submerged in xylene for 3 times as long as 10 minutes for each immersion. After that, the specimen slides were soaked with alcohol (100%, 95%, 80%, and 70%) and continued with aquadest for 5 minutes each. Hydrogen peroxide 3% solution was introduced to all of the slides and incubated for 10 minutes. Next, PBS was used for rinsing the hydrogen peroxide two times, blocking serum was added, and incubated in the moist chamber for one hour. After 1 hour, the slides were rinsed again with PBS twice and finally Oct-4 antibody was added. The slides were then incubated in the moist chamber inside the fridge (4°C) for one night.

The day after, the Oct-4 antibodies on the slides were cleaned with PBS thrice. After that, Biotin conjugated secondary antibody was placed on the specimen and incubated for thirty minutes. Specimen was washed with PBS 3 times, Avidin-biotin enzyme was placed, and incubated for half an hour. PBS was used again to wash the specimen. Diaminobenzidine (DAB) mixture, which was readied before by mixing 9.6 mL distilled water, 30 drops of 10x buffer substrate, 6 drops of 50x DAB, and 6 drops of 50x peroxide substrate, was dropped into the specimen slides and incubated for 30-60 seconds. Next, specimen slides were rinsed with aquadest twice, stained with Hematoxylin for approximately 20 seconds, and aquadest again to rinse. The slides were then washed with alcohol in increasing concentration gradient (70%,

80%, 95%, 100%) and immersed in xylene (3 times, 10 minutes each). Lastly, the subject's specimen slides were cleared and covered.

Microphoto: The images were taken at Department of Histology, Faculty of Medicine, University Indonesia. OptiLab™ application was first installed and the camera was mounted. Five different microscopic field images for each subject were shot and saved. Lastly, Image Raster™ was the application used for tagging the Oct-4 expression. The image file was opened, tagging option was checked and Oct-4 expression on the image can be tagged.

Methods for data analysis

The four structures were identified (blood vessel, hypodermis, sebaceous glands, and hair follicle). Later, the Oct-4 expression on those 4 structures were recorded and compared between the 2 methods.

Data interpretation: The images were captured with OptiLab™ (OptiLab, Indonesia) camera that was mounted to a light Olympus™ microscope (Olympus, Japan). For each subject, a minimum of five random High-Power Fields (HPFs) with the magnification of 400x were taken.

After recording all the samples' picture, Image Raster™ was opened, and all of the images were processed. On each of the image, tagging option was chosen. The tag was given to the nucleus that showed a positive Oct-4 expression. Following the tags, Image Raster would automatically have counted the number of the nucleus being tagged. The Oct-4 positive expressions were then noted and interpreted statistically using SPSS.

In the SPSS, Name-1 was filled with Oct-4 positive expression and Name-2 was filled with group. The group values were labeled; 1 being the dry ice and 2 being the ice group in the variable view. Next, in the data view, the samples' Oct-4 positive expressions were inserted in accordance to their group. The initial test that was conducted was the Saphiro-Wilk's test to know the data normality and the Levene's test to know the data homogeneity. In this research, since the P value in Saphiro-Wilk's test and Levene's test were more than 0.05, the data showed normal and homogeny result, then the analysis was continued with Independent T Test.

Operational definition:

- Cold transport: the cold transport methods used were dry ice and ice. The ice was home made from regular frozen water in plastic. The dry ice was bought.
- Immunohistochemistry: antigen was detected through interaction between antigen and antibody in tissue. The resulted antigen can make a different color indication [19].
- Oct-4 positive expression: visible round dark-brown dots inside a cell

Results

Sample condition after cold transportation

In this research, there were 20 skin samples from different subjects. Ten of the samples which were transported with dry ice were frozen after cold transport duration ended (Figure 1). While, the other half, which was transported with ice, water condensation was found on the outer part of the transporting vessel.

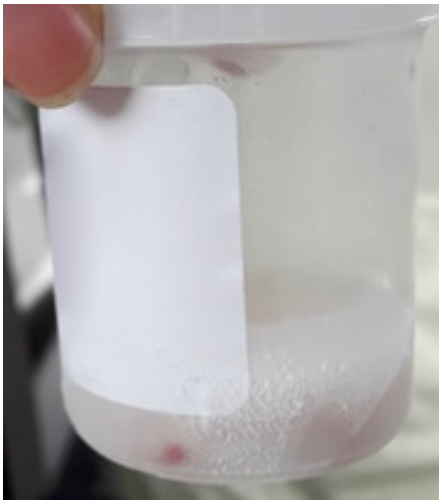


Figure 1: Frozen tissue and fixative solution of subject AY after incubated in cool box filled with dry ice.

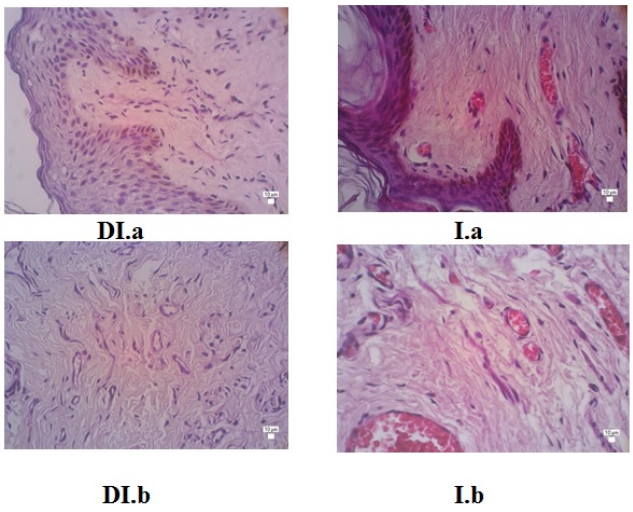


Figure 2: HE staining on post-circumcised preputial tissue with 400x magnification. (DI) The tissue obtained from NIH whose tissue was transported with dry ice. The location of the pictures are (DI.a) dermis and (DI.b) hypodermis (I) The skin stained belongs to FA, a subject from the ice group. The pictures show (I.a) dermis and (I.b) hypodermis part.

Hematoxylin-eosin stain on tissue

HE staining was done before the immunohistochemistry. The goal of this routine staining was to see whether the depth of sample cut was acceptable, by means the structures where Oct-4 expression were found positive in the previous study, can be seen in this staining (Figure 2).

Out of 20 subjects, based on the HE staining, 5 skins had superficial cut; the structures where the Oct-4 expression was located in the previous study were unable to be found. Thus, 5 subjects' skins (RF,

MIY, DA, MA, and AA from ice groups) were cut again deeper. Many blood vessels and hypodermis were found in the subjects' slides. Most of the samples had good intactness; only 2 (TA and GI from the dry ice group) had hypodermis that were not as intact as the other structures. However, hair follicles and glands could only be seen in minimal amount.

Oct-4 expression

Twenty different subjects' prepuces were observed in this research. With age ranging in between 5-12 years old, the subjects were divided into 2 major groups; their prepuces were either transported with dry ice or ice.

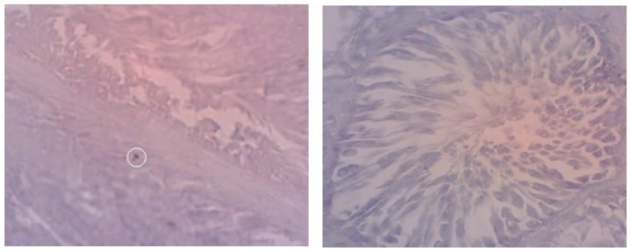


Figure 3: Results of testicular tissue processed by immunohistochemistry. (A) A visible brown dot showed a positive Oct-4 expression. (B) No visible brown dots.

Immunohistochemistry can produce positive Oct-4 expression in testis, which was illustrated in Figure 3A (positive control) where it was visible as round dark-brown dot inside a cell. While in Figure 3B (negative control), the positive signal of Oct-4 cannot be found.

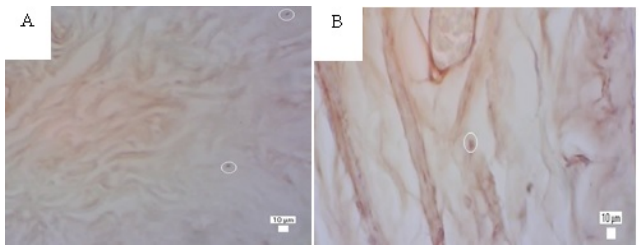


Figure 4: Results of subjects' sample stained with Oct-4 antibody by immunohistochemistry showing positive Oct-4 expression with 400x magnification. (A) Tissue belongs to TA, a member of the dry ice group (B) Tagged Oct-4 expression in MA from the ice group.

Generally, expressions of Oct-4 were positive in most subjects. The positive expressions were found in majority of the subjects' blood vessel lumen and hypodermis. However, one subject had a negative Oct-4 expression in all five HPFs (Figures 4A and 4B).

In each of the subject, Oct-4 expression was searched in 5 different HPFs with 400x magnification. In the dry ice group, all subject tissues contained positive Oct-4 expression (Table 1). While in ice group, one subject (RF) did not show any positive expression (Table 2).

No	Name	Age (years)	HPF					Mean ± SD
			1	2	3	4	5	
1	RS	7	0	3	0	0	3	1.20 ± 1.64317
2	MK	9	6	3	5	2	1	3.40 ± 2.07364
3	AS	9	2	8	2	3	1	3.20 ± 2.77489
4	NIH	8	1	4	2	0	0	1.40 ± 1.67332
5	IR	5	7	2	0	3	2	2.80 ± 2.58844
6	WJ	7	1	0	0	0	2	0.60 ± 0.89443
7	TA	8	1	2	2	2	0	1.40 ± 0.89443
8	GI	6	6	7	1	3	2	3.80 ± 2.58844
9	TI	10	4	3	1	0	4	2.40 ± 1.82659
10	AY	9	3	5	2	3	1	2.80 ± 1.48324
HPF: High Power Field								

Table 1: Detail of the subjects' Oct-4 positive expression in dry ice group.

No	Name	Age (years)	HPF					Mean ± SD
			1	2	3	4	5	
1	RI	11	4	2	6	5	3	4.00 ± 1.58114
2	WH	9	1	4	0	2	3	2.00 ± 1.58114
3	DA	12	1	0	0	0	0	0.20 ± 0.44721
4	FA	9	4	6	5	2	5	4.40 ± 1.51658
5	MIY	5	0	4	2	2	0	1.60 ± 1.67332
6	MA	8	2	7	9	6	0	4.80 ± 3.70135
7	MR	6	4	2	4	3	1	2.80 ± 1.30384
8	AA	10	3	1	0	7	3	2.80 ± 2.68328
9	YU	8	2	1	3	0	0	1.20 ± 1.30384
10	RF	10	0	0	0	0	0	0 ± 0
HPF: High Power Field								

Table 2: Detail of the subjects' Oct-4 positive expression in ice group.

After all of the Oct-4 positive expression values were obtained, the data was processed with SPSS. From the normality test (Saphiro-Wilk's test), the P value for dry ice was 0.499 and ice was 0.648, which were both more than 0.05 (normal). With ten samples each group, the mean ± standard deviation for dry ice and ice were 2.30 ± 1.08012 and 2.38 ± 1.68246 respectively. For the dry ice, the minimum value was 0.60 and the maximum was 3.80 with the median of 2.60. On the other hand, the minimum and maximum value for ice were 0.00 and 4.80 with 2.40 as the median (Figure 5).

Next, the Levene's test to check the homogeneity has P value of 0.151, which was also more than 0.05 (homogenous). Lastly, in the independent T test, the P value was 0.901. The result from this

independent T test was more than 0.05, which means there was no significant difference between ice and dry ice as a cold method.

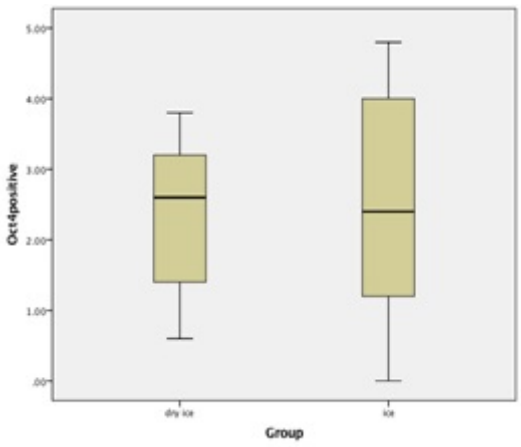


Figure 5: Boxplot on Oct-4 expression in dry ice and ice group.

Discussion

Cold transport method is one of the important steps in tissue banking. Both ice and dry ice can provide the cold environment that the cell needs in order for it to delay its metabolism rate [12]. Day and Stacey stated that when the metabolism is inactive, the tissue could be preserved for a long duration. When the temperature is below 0°C, especially below -135°C, the biology material may only degenerate minimally [20]. Dry ice, theoretically, provides colder temperature than ice, since dry ice itself sublimes at -78.5°C while ice melts into water at 0°C in 1 atm [12].

Our result showed that the low temperature produced by dry ice resulted in the frozen fixative fluid and tissue samples. However, frozen samples were not found in the ice group. Comparing the samples' temperature between the dry ice and ice group in this research, they clearly showed that the dry ice subjects' tissue were exposed to colder environment, thus their metabolism should be slower than the subjects' sample which were transported in ice. As a result, the subjects' tissue transported with dry ice can be preserved more in the bank rather than the tissue from the ice group.

Research conducted by Bussolati, Annaratone, Medico, D'Armento, and Sapino, found that tissue samples which were transported and fixated either in room temperature or cold environment has similar structures and immunohistochemistry product (marker reactiveness). However, fixation in cold temperature (4°C) can help to save the nucleic acid from damages; as formaldehyde, the fixative fluid used in this research destroys the nucleic acid [20].

The results from the HE staining were similar between ice and dry ice. Most of the subjects' tissues were intact, however, 2 subjects whose skin was transported with dry ice have less intactness in the hypodermis. This finding is supported by a paper by Day and Stacey; majority of the tissues were impaired when their temperature is negative or less than 0°C because intracellular ice may manifests and ruins the tissue. Furthermore, faster frozen time can also contribute to the manifestation of intracellular ice [21]. In samples transported with dry ice, their temperature was lower than 0°C thus the formation of this intracellular ice can happen and lessened the intactness of the whole tissue, including the hypodermis.

In the same slides stained with HE, the observer tried to identify the structures where the Oct-4 expressions were found to be positive from the previous study [3]. However, the observer rarely found hair follicle and glands. Thus, she focused more on the cells in the blood vessel's lumen and hypodermis as the location of Oct-4 positive expression. Five of the subject's tissue samples were superficially cut so they needed to be cut a little deeper in order to find the hypodermis and blood vessel.

Issue arises in the transportation is the lag time. Lag time can be interpreted as the duration from the excision of tissue until freezing. This term should be defined more since the tissues that will be excised were already losing much of its nutrient after the clamping of the blood vessels [22]. Without its nutrient, the tissue can undergo necrosis fast. Transport medium can contain different components. For example the Hank's Balanced Salt Solution (HBSS) containing glucose and other important ions needed by the tissue [23]. Thus, changing the fixative solution (formaldehyde 4%) into transport medium so that the tissue is re-nourish can be one of the solutions. However, the transportation time also needed to be considered.

In this research, the optimal concentration of Oct-4 (1:10) has already been used in favor of specific Oct-4 positive expression. Nevertheless, there were still several limitation factors that might cause the initial hypothesis to be dismissed. First of all, there was technical difficulty in processing the tissue and reading the Oct-4 positive expression. When the specimens were being processed in the immunohistochemistry, there were some of the tissues that detached, possibly due to bad coated-slides when mounting process. While in analyzing the visible Oct-4 expression; there were also some problems experienced by the observer in differentiating the Oct-4 positive expression and other histology structures (for example the endothelium of blood vessel) despite the operational definition. Next, the random 5 microscopic fields for 1 subject that the observer took may still be unrepresentative for the amount of Oct-4 positive expression in the tissue.

Histologically, the number of Oct-4 positive expression varied in different subjects. Most, except one subject from the ice group, displayed Oct-4 positive expression in total 5 different HPFs with 400x magnification. However, the independent T Test in SPSS showed that there was no significant difference between the two cold transport methods. Ice and dry ice had equal function as transport method for stem cell banking. Thus, the hypothesis of tissue transportation using ice is better than dry ice to maintain the Oct-4 positive expression in cells was rejected.

Both ice and dry ice are actually required in the standard of procedure of a biobank in the Europe, specifically tumor biobank (TuBaFrost). For example, ice is needed when transporting the tissue from the surgery location to the pathologist. While dry ice can replace the liquid nitrogen in lowering the temperature of the isopentane, a freezing medium. Before the tissue sample is put inside the isopentane for freezing, the isopentane is cooled beforehand by suspending the isopentane vessel on liquid nitrogen or dry ice [24]. Even though dry ice and ice can provide cold temperature and take part in the biobank protocol, more researches are needed especially in comparing ice, dry ice, and liquid nitrogen effect to the tissue, especially to the pluripotent cells in stem cell banking. Since there was no significant difference found between dry ice and ice in this research, the researcher would

suggest the use of either dry ice or ice as a bridging cold transport method for preputial skin from the surgery room to freezing with liquid nitrogen. The dry ice or ice can also be used in transporting tissue samples between centers which do not have liquid nitrogen to those who have.

Conclusion

In this research, there is no significant difference in the cell' Oct-4 positive expression after transporting the preputial skin either with dry ice or ice. However, further studies comparing cold transport methods (dry ice, ice, and even liquid nitrogen) are recommended with several cautions. First, the lag time from clamping the blood vessel until immersion with fixative solution or transport medium, depending on the research or tissue transport goal, should be considered. Secondly, the histotechnique, routine staining, and immunohistochemistry procedure must be done in accurate manner. Third, in recognizing the Oct-4 expression, double blind observers may help. Finally, as there is still no definite stem cell marker for cell pluripotency, other marker beside Oct-4 can be use.

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