The Effect of Cryopreservation Method to the Quality of Bovine Cumulus Cells Primary Culture

by Gratiana E Wijayanti

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Abstract. Cumulus cells are play a role as nucleus donor in Somatic Cell Nuclear Transfer (SCNT) technology. This study was conducted to find a suitable method for cryopreserving cumulus cells in aim to provide donor cell stock of primary cell culture. The freezing temperature was made at three levels: -20°C, -80 °C, and -196 °C (liquid nitrogen), while cryopreservation was performed by adding cryoprotectants (CPs) that made from 10% Ethylene Glycol (EG) or 10% Dimethyl Sulfoxide (DMSO) in Dulbecco's Phosphate Buffer Saline (DPBS) + 20% New Born Calf Serum (NBCS) medium. The results showed that the freezing temperature, cryoprotectants and their interaction had no significant effects on the cell concentration ($p>0.05$). The cell viability efficiency was decreased significantly when the cell were freezing at -20°C compared to freezing temperature at -80°C and -196°C ($p<0.05$). The highest cell viability efficiency was obtained from the cell preserved at -196°C using 10% DMSO. The regression test showed that the decrease in temperature will increase the viability efficiency. In conclusion, cumulus cell primary culture can be cryopreserved in temperature of -80°C and -196°C with CPs of 10% DMSO or 10%EG.

1. Introduction

Cumulus cells are known for their capability as donor nucleus for Somatic Cell Nuclear Transfer (SCNT) technology. Nevertheless, this technique is hampered by a concern regarding suitability of donor cell stock. A long-lasting nuclear donor cell preservation is needed for research and commercial programs [1,2]. In this regard, production of ready-to-use donor cells such as cumulus cells is crucial. This ready-to-use cells can be provided through cell culture and donor cell stock in form of cryopreserved cells [3]. Nowadays, the use of freeze-donor cells in SCNT technology was commonly accepted due to the no-prevented action on pre-implantation development of the clones [2].

The basic principle of cryopreservation including the temperature reduction, cellular dehydration, freezing and thawing need to be handled properly to ensure cellular survival of somatic cells [1,4]. Based on previous report, the bovine cumulus cells has been succesfully cryopreserved using the vitrification technique on cumulus cells that cultured for several passages. The vitrification medium that used was comprised of 40% v/v EG + 18% w/v Ficoll + 0.3 M sucrose + PBS [3]. However, the cryopreservation on bovine cumulus cells from primary cell culture has never been reported. The use of cryoprotectant for preserving primary cell culture could be used to minimize the loss of its pluripotency due to prolonged culture time [5]. It has been also reported that the cell preservation can be conducted by

adding 10% (v/v) DMSO and EG as cryoprotectant (CP) to the culture medium, 90% FBS, or normal saline containing serum albumin followed by cooling process [6,7]. The aim of this study was to find suitable conditions for cryopreservation of bovine cummulus cells from primary culture.

2. Materials and methods

2.1. Cumulus cells preparation

Oocytes were obtained from bovine ovaries that collected from an abattoir (Bogor). The cumulus cells were collected from *in vitro* maturated oocytes (metaphase-II stage), then they were separated mechanically (pippetting method) from the cumulus oocyte complex (COC).The cells were washed in DPBS (Gibco) with two times centrifugation (2000 rpm;10 minutes) (Thermo Scientific Heraeus Pico 17), then washed in culture medium composed of Dulbecco's Modified Eagle's Medium (DMEM, Sigma Aldrich) supplemented with 0.04 M sodium bicarbonate (NaHCO3, Merck), 10% NBCS (Gibco), 10% non-essential amino acid (NEAA, Sigma Aldrich), and 125 µL/100 ml gentamycin (Gibco). The cells were cultured in 35 mm × 10 mm culture dish (Corning), subsequently incubated at 38.5°C in 5% CO_2 (Thermo Scientific) for 7 – 14 days, enabling to form a confluent monolayer. The culture was observed on day-3 and the day in which the cells reached <90% confluency (mostly on day 7 of culture). The confluence culture was trypsinized and evaluated for the cell concentration and cell viability.

2.2. Cumulus cells cryopreservation

The cells were suspended in cryomedium, DPBS supplemented with 20% NBCS, 125 µL/100 ml gentamycin and 10% of EG (Merck) or 10% of DMSO (Sigma-Aldrich), then transferred into 2 ml-Nalgene cryogenic tubes. The tubes were then cooled at three levels as follows: -20, -80, and -196°C (liquid nitrogen). The tubes in first group were placed in a freezer at -20°C (Ing Climas), while the second group was stored in Nalgene Mr. Frosty™ (Thermo Scientific) containing isopropanol, then cooled to -80°C in a freezer. Last, the cryogenic tubes in third group were placed in Mr. Frosty™ at -80°C (Ing Climas) for overnight, then plunged to liquid N_2 on the following day for 1 week. For thawing, the tubes containing cells were warmed up in waterbath (Memmert) at -37°C (1-2 minutes). The thawed cells were washed in culture medium containing 0.25 M or 0.5 M sucrose for 3 and 5 minutes then centrifuged two times (2000 rpm for 5 minutes), which aimed to eliminate the cryoprotectant from cells. The post-thawing cell viability and cell concentration were evaluated.

2.3. Data Analysis

The efficiency of viability and concentration was calculated as follows: $post\ cryopreservation\ \times 100\%/pre\ cryopreservation.$ The result of efficiency was tested for their normality using Kolmogorov-Smirnov and their homogenity using the Levene test followed by two-way ANOVA for the normally distributed data. The relationship between freezing temperature and viability efficiency was analysed using Pearson correlation.

3. Results

The evaluation on day-3 period showed that the cumulus cell primary cultured attached to the substrate in the form of a fibroblast-like feature. The size of cells is about 10 µm, while they appeared as elongated and bipolar cells, and their nuclei occurred in the center of cytoplasm. Afterwards, clear cytoplasmic extension was observed in some cells (Figure 1). On day-7, cultured cells were reached confluence. The cell morphology seemed change into an epithelial-like feature, while the cells appeared as polygonal without clear cytoplasmic extension, and their nuclei were located at the center of cytoplasm (Figure 2.).

The result was also supported by the growth curve of primary culture (Figure 3). The constant increase of cell concentration was observed from day-5 to day-7. The culture reached confluency on day-7 with cells occupied >90% of the culture substrate. After that, the cell concentration showed no

changes until day-8. A significant decrease in cell concentration was observed on day-9. In this regard, cell confluency began to decline until day-11. The result also demonstrated that Population Doubling Time (PDT) was recorded at 90 hours (3 days 8 hours) after incubation.

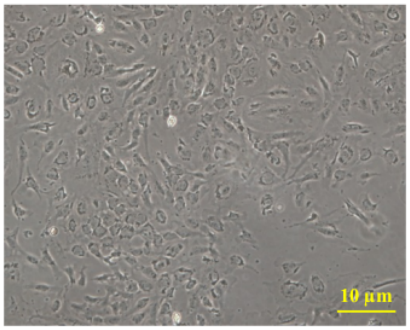

Figure 1. Micrographical morphology of bovine cumulus cells primary culture on day-3 (50×).

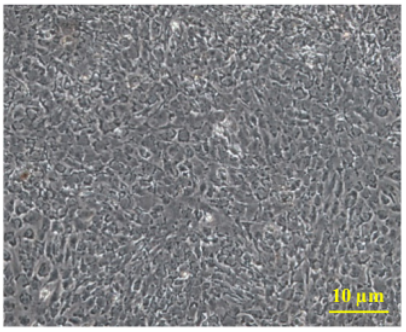

Figure 2. Micrographical morphology of bovine cumulus cells primary culture on day-7 (50×).

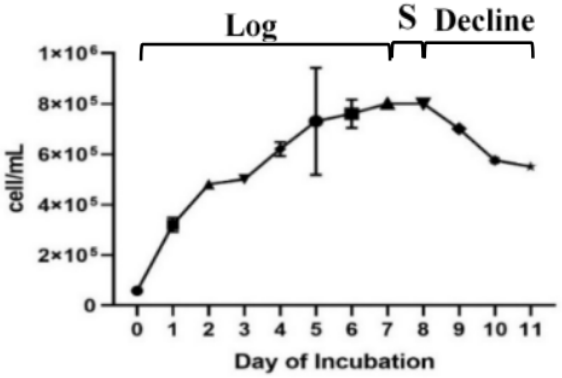

Figure 3. The growth curve of bovine cumulus cells primary culture

There was no significant difference in cell concentration efficiency for all of the factors studied. Similarly, no interaction was found between factors affecting the concentration and viability efficiency (Table 1). The results also exhibited in significant difference between 10% EG and 10% DMSO as cryoprotectant. Based on the freezing temperature treatment, cells exposed to -20°C in both types of CPs showed the highest decrease in cell concentration (72% – 96% of concentration efficiency).

However, the treatment resulted in noticeable difference in viability efficiency compared to cells treated at -196°C ($P<0.05$), regardless of CPs used. The cells treated at -80°C showed similar results in comparison with those treated at -20°C and -196°C. The lowest cell concentration efficiency was found in cells treated at -20°C using 10% EG; conversely, the highest one was achieved at -80°C using 10% EG. In terms of cell viability efficiency, the lowest percentage was found in cells treated at -20°C using 10% EG, while the greatest one was observed in cells treated at -196°C using 10% DMSO. The regression test revealed that within the range of tested freezing temperature (up to -196°C) the decrease of freezing temperature by 1°C resulted in an increase in viability efficiency, as represented by following

equations:y = -0.0007x + 0.7737 for 10% EG and y = -0.0009x + 0.7723 for 10% DMSO (Figure 4 & Figure 5). These result indicated that the effect of temperature on the efficiency of viability was also influenced by the type of cryoprotectant.

Table 1. Effect of cryopreservation method on bovine cumulus cells primary culture concentration and viability

T (°C)	CP	CE (%) ± SD	VE (%) ± SD
-20	EG	72 ± 0.06	78,37 ± 0.11[a]
	DMSO	96 ± 0.03	75,14 ± 0.18[a]
-80	EG	99 ± 0.01	83,95 ±0.11[ab]
	DMSO	97 ± 0.01	90,20±0.11[ab]
-196	EG	89 ± 0.04	90,77 ± 0.29[b]
	DMSO	92 ± 0.04	92,64 ± 0.23[b]

Note: T = freezing temperature, CP = cryoprotectant, CE = cell concentration efficiency, VE = viability efficiency. a, b within column, values with different superscripts are significantly different ($p<0.05$).

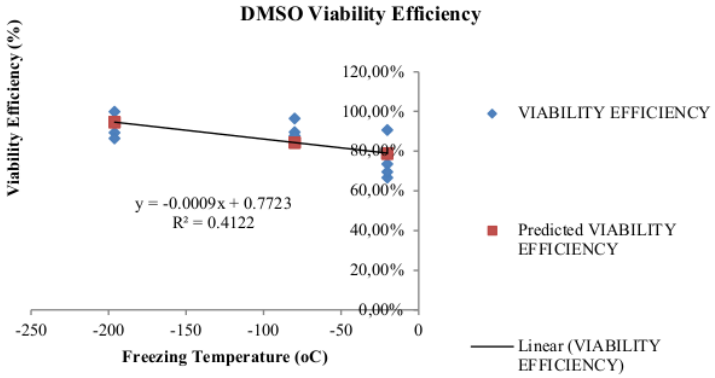

Figure 4. Regression for freezing temperature on viability efficiency in 10% DMSO

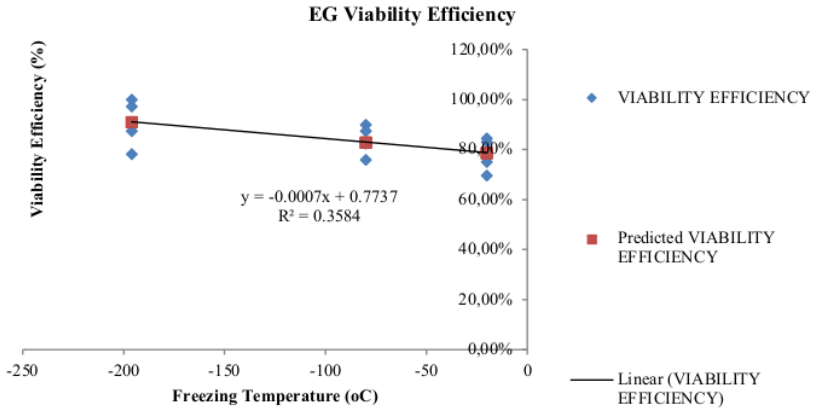

Figure 5. Regression for freezing temperature on viability efficiency in 10% EG

4. Discussion

Our experimental data demonstrated a significant decrease of CE and VE under condition of -20°C. When cells are not in an equilibrium environment, they react through creating a flux of permeating substances like water that leads to alteration of volume. During cryopreserved, the cells undergo critical points, which may cause lethality during cooling and warming process at temperature between -15°C and -60°C [8]. Afterwards, spontaneous crystal formation occurs at around -18°C during freezing storage [9,10,11]. In this regard, the loss of survival ability resulted from inappropriate level of CP and cooling rate [11]. Besides, thawing process seemed to be unsuccessfull to recover the cellular structures which led to changes in cell volume. Cryoprotectant was still trapped inside the cells when the cells undergo change of volume. During the centrifugation process, the cells were unable to settle in the bottom of the centrifuge tube, consequently they were discarded together with the supernatant during medium substitution.

The freezing temperature of -80°C was usually applied for transition temperature for cell line cryopreservation [12]. Compared to hematopoietic cells cryopreservation using similar freezing temperature with 10% DMSO as the cryoprotectant, the result was quite similar in which the freezing temperature unaltered the phenotypic of the cells [9]. The depletion of tissue or fragments stored in a deep freezer (-80°C) for 4 weeks of storage, being lower than those placed in liquid nitrogen. The temperature in which water can maintain its vitreous ice structure is below -160°C [13]. For this reason, cryoprotectant is incorporated in order to preserve such vitreous structure, enabling to provide preservative effects on cells when stored below -80°C. Overall, the cryopreserved cells were having a decrease of viability. The decrease of cell viability was due to an increase in cell death caused by cryopreservation and thawing process [5].

Based on the results, CP showed no significant difference in two parameters discussed. This is associated with similar mode of action by both cryoprotectants. They work by penetrating the cells, then promote formation of hydrogen bonds with water molecules inside the cells, leading to slowdown the temperature and prevent crystallization [1]. Removal of CPs could be perfectly carried out after thawing process, since this allowed to prevent toxicity to the cells [13]. The results showed that EG had the least residual amount compared to DMSO after thawing procedure. The slow freezing technique also produces the least amount of residual due to the small amount of CPs used at the very first place. Previously, other studies discussed about embryo cryopreservation [14,15], finding that embryo stored

using EG as CP showed higher viability rate compared to that stored using DMSO. However, in this research, DMSO might still leave higher residual after the thawing than EG, which caused cytotoxicity to the cells. Both of the CPs were cytotoxic at room temperature [16].

Although employing similar type of cells, the results of the experiment may vary due to difference in methods (such as presence of vitrification) and cryprotectants [3]. The results indicated that the concentration of vitrified cells gave effect to the susceptibility of vitrification. Higher cell viability can be obtained from the culture with a higher number of cells. In this research, such pattern was shown on cells treated at -80°C and -196 °C. However, as presented in Table 1, the high cell concentration of -20°C treatment did not accompanied by a high viability efficiency. In conclusion, the freezing method became one of most crucial factors as it remarkably determined the quality of the bovine cumulus cells primary culture during cryopreservation. The use of slow freezing methods, i.e. freezing at -80°C and -196°C, was suitable for bovine cumulus cryopreservation, while cryoprotectant of 10% EG and 10% DMSO showed satisfying effects.

Acknowledgments

We would like to express our sincere gratitude to drh. Faisal Amri Satrio, M.Si and Achmad Setiyono, S.Pt. for providing ovaries and cumulus cells; Paskah Partogi Agung, M.Si for evaluating the manuscript, and Pusat Unggulan Iptek (PUI) Bioteknologi Peternakan Sapi Potong dan Sapi Perah (Beef and Dairy Cattle Center of Excellent) for facilitating publication at 4th APIS seminar.

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