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Optimizing cryopreservation of mouse embryonic fibroblast feeder cell layer to improve production of murine embryonic stem cell outgrowth

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Abstract

The inconsistency of efficiency in murine embryonic stem cell (ESC) production might be associated with the differences in preparation and cryopreservation of the feeder cell layer. As the cryopreservation of mouse embryonic fibroblast (MEF) declined the quality of MEF as feeder cell layer, an effective protocol should be determined to produce murine ESC on frozen-thawed feeder cell layer as efficient on fresh feeder cell layer. Under appropriate culture conditions, isolated inner cell mass (ICM) of murine blastocyst will form ESC and be maintained in undifferentiated state. Therefore, the aims of this study were to determine the most optimum freezing density and equilibration duration for cryopreserving MEF feeder cell layer and to determine the effect of fresh and frozen-thawed feeder cell layer on murine ESC production. Freezing density of 5×10^6 cells/mL gave a significantly higher viability rate than 0.5×10^6 cells/mL (68.08% *vs.* 59.78%, $p<0.05$) and comparable with 2×10^6 cells/mL. The viability rates of frozen-thawed MEF derived from 15 minutes equilibration was significantly higher than 20 hours equilibration (79.4% *vs.* 68.08, $p<0.05$). There were no significant differences between fresh and frozen-thawed MEF feeder cell layer for percent successful attachment of blastocysts, consecutive passages of murine ESC up to passage 3. In conclusion, freezing density of 5×10^6 cells/mL and 15 minutes equilibration duration are optimizing the cryopreservation of MEF feeder cell layer to subsequently improve the production of murine ESC.

Keywords: murine embryonic stem cell, mouse embryonic fibroblast, cryopreservation, freezing cell density, equilibration duration.

Introduction

One of the main challenges in culturing murine embryonic stem cells (ESCs) is to maintain the cells in an undifferentiated state. This is achieved by using the feeder cell layer as co-culture. Mouse embryonic fibroblast (MEF) serves as feeder cell layer in ESC culture [1–3] as it forms intracellular junctions such as gap junctions, adherens junctions and tight junctions with ESC [4]. MEF provides a befitting environment for the interplay of signaling networks that regulate the fate of ESC [5, 6].

A significant amount of MEF is the prerequisite factor for culturing ESC to remain at the undifferentiated state [7]. For each subsequent passage of ESC, a fresh confluence feeder cell layer of MEF is required. The dependency on the primary MEF alone is not adequate, consequently, MEF should be passaged and cryopreserved to ensure production of sufficient stock. Thus, cryopreservation and subsequent thawing are important protocols in MEF production that need to be focused in order to produce high quality feeder cell layer [8]. This is in turn enables the successful consecutive culture of ESC. There are a lot of factors that need to be considered before choosing the optimum and reliable MEF cryopreservation protocol such as types of cryoprotecting agent (CPA), molarity of CPA, techniques of cryopreservation, freezing cell density as well as equilibration duration [9–12].

Another challenge that needs to be considered in murine ESC production is the inner cell mass isolation technique as it is an important step in the ESC establishment

[13]. ESC was usually obtained by isolating undifferentiated outgrowths that originated from the inner cell mass of the blastocysts. The murine ESC were expanded, passaged, frozen and tested for pluripotency *via* differentiation. Outgrowths from the inner cell mass of murine blastocysts can be derived through three techniques, namely culture of whole blastocysts, mechanical dissection and laser dissection of inner cell mass from the murine blastocysts, and subsequently cultured to obtain primary outgrowths and consecutive passages [13–15].

Therefore, to improve the production of murine ESC, the present study was designed with the aim to evaluate the freezing density and equilibration duration for cryo-preservation of MEF optimally. This study also investigated the effect of using fresh and frozen-thawed MEF as feeder cell layer as well as the best inner cell mass isolation technique in order to produce murine ESC.

Materials and Methods

Unless stated otherwise, all chemicals were purchased from Sigma-Aldrich Co. (USA). The culturing media were purchased from Gibco (Grand Island, NY, USA). All animals care and conduct were accordance with the guideline of the *Institutional Animal Care and Use Committee* (IACUC), University of Malaya, Malaysia.

Preparation of MEF culture

Fetuses at 13.5 to 15 days *post coitus* were processed by removing head, tail, limb and internal organs and

subsequently washed and minced into smaller pieces in 0.25% trypsin EDTA (ethylenediaminetetraacetic acid) solution by using a sterile blade. The minced tissues were then stirred in 0.25% trypsin EDTA solution with the aid of magnetic stirrer for 20 minutes for completing the mincing process. The mixture of minced tissues were then filtered and centrifuged at 2000 rpm for five minutes. The supernatant was removed, the cell pellets were resuspended with DMEM (Dulbecco's modified Eagle's medium) + 10% fetal bovine serum (FBS) + 3× Penicillin–Streptomycin (PS) and seeded to culture dish. The primary MEF were cultured in presence of 5% CO_2 in a maximum humidified atmosphere of a CO_2 incubator, at 37^0C, until reaching 80% confluency. The MEF were consecutively subpassaged until passage 2 in DMEM + 10% FBS + 1% PS medium.

Cryopreservation of MEF

Passages 1 and 2 of the MEF cultures were cryopreserved at three different freezing cell densities ($0.5×10^6$, $2×10^6$ and $5×10^6$ cells/mL) by using freezing technique at two equilibration times (20 hours *vs.* 15 minutes). Briefly, the confluence cell cultures were trypsinized. The freezing medium [60% DMEM medium + 20% FBS + 20% dimethylsulfoxide (DMSO)] were added into the cell mixture and subsequently were transferred into cryovials at different freezing cell density and inserted into the cryocanes. For 20 hours equilibration, the cryocanes were placed into -80^0C freezer for 20 hours before stored in liquid nitrogen whereas in 15 minutes equilibration, the MEF were frozen at -80^0C by using liquid nitrogen gaseous for 15 minutes before directly plunged into the liquid nitrogen (-196^0C) for 15 minutes. The frozen MEF were stored in the liquid nitrogen tank minimum two days before used.

Cell viability evaluation

Cell count was carried out before cryopreservation and after thawing of MEF to evaluate the viability of the MEF cell passages before and after cryopreservation. The cell suspension was allowed to mix with 0.4% Trypan blue. Counting of the viable and non-viable cells was done by using hemocytometer. The number of viable cells was determined by using the following formula:

$$c = \frac{d(n \times 10^4)}{5}$$

where, c – number of cells, d – dilution factor, n – number of unstained cells.

Inactivation of feeder cell layer

The confluent MEF was inactivated with DMEM + 10% FBS supplemented with 5 µg/mL Mitomycin C for three hours. The feeder cells were prepared one day before and incubated with ESC culture medium at least three hours before being used for subsequent ESC production.

Production of murine blastocyst

In this study, murine blastocyst was produced through *in vivo* fertilization followed by *in vitro* culture of the two-cell embryos to blastocysts. These blastocysts subsequently were subjected for inner cell mass (ICM) isolation for producing ESC on the inactivated MEF feeder cell layers.

Briefly, murine females of 6–8 weeks old were super-ovulated with an intraperitoneal injection of pregnant mare's serum gonadotrophin (PMSG; 10 IU) followed by an intraperitoneal injection of human chorionic gonado-trophin (hCG; 10 IU) 48 hours later. Each female murine was mated with a fertile male for mating and vaginal plug was checked on the next morning for mating confirmation. For two-cell embryos recovery, oviducts of superovulated murine females were flushed with Hepes Whitten's medium using a 32G flushing needle connected to a 1 mL syringe. Collected embryos were washed and cultured in the Whitten's medium microdroplets under mineral oil at presence of 5% of CO_2 in a maximum humidified atmosphere of a CO_2 incubator, at 37^0C, for *in vitro* development until blastocysts.

Isolation of inner cell masses from blastocysts

Three different ICM isolation techniques were used in present study (a) whole blastocyst culture, (b) mechanical dissection and (c) laser dissection. Consecutively, the ICM cells were cultured on the MEF feeder cell layer to obtain primary ICM outgrowths. For whole blastocyst culture, *zona pellucida* of blastocyst was chemically digested by using 0.5% pronase. Then, whole blastocysts were plated on inactivated MEF in a maximum humidified atmosphere of 5% CO_2 in CO_2 incubator, at 37^0C, for cell attachment and culture. After 6–8 days later, the growing colonies were individually dissociated into clumps after treating with 0.05% trypsin EDTA. The resultant small clumps containing approx. 20–50 cells were transferred to a new well with a fresh feeder cell and medium. The new colonies were inspected daily and subcultured at an interval of approx. 6–10 days according to their size and growth rate, and medium were changed on alternate day.

Mechanical dissection technique involved removal of *zona pellucida* of blastocyst by using 0.5% pronase. However, instead of directly culture the whole blastocyst on the feeder layer, the area between the ICM and troph-ectoderm (TE) on blastocyst were dissected using two 30G needles for isolation of the ICM and subsequently transferred onto an inactivated feeder cell with sufficient gap in between them.

Laser dissection technique was adapted from Goh *et al.* (2012) [13] and carried out with the aid of micro-manipulator system. Briefly, the blastocyst was held by holding pipette with the ICM was positioned at the nine o'clock position. The laser was shot along the way between the ICM and TE cells. After shooting, the biopsy needle was sucked on the other side of the blastocyst to remove the *zona pellucida* and TE from the ICM. The isolated ICM was cultured onto the inactivated feeder cells and the primary outgrowth of the culture was observed.

Isolation and passages of embryo-deserved cell lines

The primary ICM outgrowths were sub-cultured using trypsinization procedures by 0.05% trypsin EDTA. After around 4–6 days of culture, the primary ICM outgrowths were picked and sub-cultured into new feeder cells. The ICM that were selected had characteristics included dome shape colony surrounded with primitive endoderm, homo-geneous and higher nuclear to cytoplasm ratio. They had

a slightly refracted and were located directly next to the outgrowths. The suitable outgrowths were picked before differentiation occurred.

Confirmation and characterization of ESC by alkaline phosphatase (AP) staining and immuno-fluorescence staining of ESC protein markers

For AP staining, ESC was fixed with 4% paraform-aldehyde for 30 minutes, washed five times and added to substrate for covering the cells prior 30 minutes incubation in dark environment. After incubation, the cells were washed and stained with AP staining. The staining was carried out to determine the AP activities in ESC, which detected as purple color after staining. For characterization, the immunofluorescence staining was performed. Briefly, the ESC were fixed with 4% paraformaldehyde for 30 minutes, washed and added to blocking solution (10% FBS + PBS) prior leave it in room temperature for two hours. After two hours, blocking solution was removed and the diluted (1:250) primary antibody (Oct-4, SSEA 1, SSEA 3, SSEA 4, TRA-1-80 and TRA-1-60) was added and incubated overnight at 4^0C. After incubation, the primary antibody was removed and ESC was washed prior incubated with diluted secondary antibody (1:1000) in dark environment for two hours. Lastly, after secondary antibody removal and ESC was washed, 5 μg/mL Hoechst 33342 was added and incubated five minutes in dark condition. The staining cell was observed under fluorescent microscope.

Experimental design

Experiment No. 1: Effect of freezing density and equilibration duration for cryopreservation of mouse embryonic fibroblast cell

In this experiment, MEF at passages 1 and 2 were cryopreserved using three freezing densities of 0.5×10^6, 2×10^6 and 5×10^6 cells/mL to test the viability rates for comparing the cryopreservation efficiency. The viability rates of frozen-thawed MEF were compared between the three cell freezing densities. Next, to evaluate the effect of equilibration duration for cryopreservation MEF, the 20 hours equilibration duration was compared with the 15 minutes equilibration duration. Both equilibration durations were carried out at cell density of 5×10^6 cells/mL using both passages 1 and 2 MEF. The cells were then thawed and the viability rate was calculated and compared between the two-equilibration duration.

Experiment No. 2: Effect of isolation of inner cell mass technique of blastocyst and subsequent culture using fresh and frozen-thawed MEF feeder cell layer on murine ESC production efficiency

This experiment was designed to evaluate the effect of fresh and frozen-thawed MEF as feeder cell layer on the efficiency of ESC production as well as to compare the efficiency of murine embryonic stem cells derived from three different ICM isolation techniques, namely whole blastocyst culture, mechanical dissection and laser dissection. The ICM outgrowths were isolated and passaged up to passage 3. The success rates of blastocyst attachment, rate of primary ICM outgrowth that can be successfully cultured in an undifferentiated state to passages 1, 2 and 3 were compared between the three ICM isolation techniques as well as the fresh and frozen-thawed MEF as feeder cell layer.

Statistical analysis

Data were analyzed using SPSS software (ver. 23.0, IBM, USA). For Experiment No. 1, the viability rate of fresh and frozen-thawed between the three freezing densities were analyzed using one-way ANOVA (analysis of variance) followed by Duncan Multiple Range Test. The difference of the viability rate between equilibration duration was analyzed using Student's unpaired *t*-test. For Experiment No. 2, the differences of rates of ICM attachment, primary ICM outgrowth as well as the rate of successful consecutive ESC passages between the three isolation ICM techniques (whole blastocyst culture, mechanical dissection and laser isolation) on fresh and frozen-thawed MEF were analyzed using one-way ANOVA followed by Duncan Multiple Range Test. Significance was determined when $p < 0.05$.

⮺ Results

The viability and mortality rates of MEFs after frozen-thawed at three different cell densities displayed in Table 1. Freezing density of 5×10^6 cells/mL gave a significantly higher viability rate than 0.5×10^6 cells/mL (68.08% *vs.* 59.78%; $p < 0.05$) and comparable with 2×10^6 cells/mL. Therefore, the freezing density of 5×10^6 cells/mL was used for subsequent density in order to evaluate the equilibration duration efficiency (Figure 1).

As shown in Table 2, the viability rates of frozen-thawed MEFs from both 20 hours equilibration and 15 minutes equilibration were significantly reduced from the fresh MEFs (68.08% and 79.4% *vs.* 80.09% and 95.4%, respectively; $p < 0.05$). The viability rates of frozen-thawed MEFs derived from 15 minutes equilibration was significantly higher than 20 hours equilibration (79.4% *vs.* 68.08; $p < 0.05$).

Table 3 shows the percent successful attachment of ICM and successful consecutive passages of murine ESC lines based on three different ICM isolation techniques on fresh and frozen-thawed MEF. On fresh MEF, the ICM attachment rate was significantly lower when isolated by using laser dissection than whole blastocyst culture and mechanical dissection (73.24% *vs.* 81.05% and 80.6%; $p < 0.05$). Consecutively, no significant effect of ICM isolation techniques on primary ICM outgrowth percent as well as successful passages 1 and 2 of ESC ($p > 0.05$). However, ESC at passage 3 derived from ICM isolated by whole blastocyst culture was significantly lower than two other techniques (22.86% *vs.* 39.36% and 38.77%; $p < 0.05$).

On frozen-thawed MEF, ICM isolated by whole blastocyst culture had significantly lower for the percent ICM attachment (52.39% *vs.* 76.92% and 74.51%; $p < 0.05$) and percent of primary ICM outgrowth (71.69% *vs.* 78.16% and 83.33%; $p < 0.05$) compared to mechanical dissection

and laser dissection techniques. Although no significant differences between three ICM isolation techniques on percent successful passages 1 and 3 (*p*>0.05), laser dissection technique gave highest successful passage murine ESC lines at passage 2 (59.91% *vs.* 48.77% and 39.44%; *p*<0.05) compared with mechanical dissection and whole blastocyst culture techniques.

On both fresh and frozen-thawed MEF feeder cell layer, high percent of ICM attachment with no significant decrease of primary ICM outgrowth for all three techniques (*p*>0.05). However, significant decrease was observed on the successful consecutive passages (passages 1 to 3) for all ICM isolation techniques (*p*<0.05) as showed in Table 3. There were no significant differences between fresh and frozen-thawed MEF feeder cell layer for percent successful attachment of blastocysts, consecutive passages of murine ESC up to passage 3 (Figure 2).

To determine the ESC outgrowth is true and pluripotent, the AP staining and immunostaining by ESC protein markers were used. Murine ESC stained with AP staining showed purplish color whereas specific ESC markers were found in murine ESC such as Oct-4 and SSEA 1 in this experiment (Table 4). There were no color shown for TRA-1-60 and TRA-1-81 ESC markers in murine ESC. Therefore, it could be confirmed that the presence of true murine ESC and pluripotent of murine ESC have been found (Figure 3).

Table 1 – *Percent of viability and mortality (mean ± SEM) of MEFs after frozen-thawed at three different cell densities*

Freezing density (×10^6 cells/mL)	Percent viability (mean ± SEM)	
	Fresh (*n*)	Frozen-thawed (*n*)
0.5	80.09±3.32^b (16)	59.78±1.93^ax (16)
2	80.09±3.32^b (16)	63.5±1.96^axy (16)
5	80.09±3.32^b (16)	68.08±1.97^ay (16)

SEM: Standard error of mean; MEFs: Mouse embryonic fibroblasts; ^abMeans with different superscripts in a row within a group were significantly different (*p*<0.05); ^xyMeans with different superscripts in a column within a group were significantly different (*p*<0.05); *n*: No. of samples.

Table 2 – *Percent of viability and mortality of MEFs after frozen-thawed using the slow freezing and quick freezing techniques*

Equilibration duration	No. of samples	Percent viability (mean ± SEM)	
		Fresh	Frozen-thawed
20 hours	16	80.09±3.32^ax	68.08±1.97^bx
15 minutes	12	95.4±0.67^ay	79.4±2.69^by

SEM: Standard error of mean; MEFs: Mouse embryonic fibroblasts; ^abMeans with different superscripts in a row within a group were significantly different (*p*<0.05); ^xyMeans with different superscripts in a column within a group were significantly different (*p*<0.05).

Figure 1 – *Mouse embryonic fibroblast culture after frozen-thawed: (a) 50% confluency and (b) 80% confluency of MEF culture (×10).*

Table 3 – *Percent of successful attachment of inner cell mass and consecutive passages of murine embryonic stem cell lines (%, mean ± SEM) based on three different inner cell mass isolation techniques on fresh and frozen-thawed mouse embryonic fibroblasts*

MEFs	ICM isolation technique	No. of blastocysts	Percent ICM attachment (*n*)	Percent primary ICM outgrowth (*n*)	Percent successful consecutive passage (*n*)		
					P1 (*n*)	P2 (*n*)	P3 (*n*)
Fresh	Whole blastocyst	971	81.05±11.1^cy (777)	79.05±2.54^cx (606)	75.34±3.34^cx (444)	43.86±4.99^bx (207)	22.86±4.31^ax (71)
	Mechanical dissection	725	80.6±1.37^dy (583)	78.8±1.21^dx (463)	72.36±1.46^cx (340)	59.58±2.55^by (212)	39.36±3.37^ay (96)
	Laser dissection	1183	73.24±1.54^cx (862)	75.89±1.48^cx (659)	70.58±1.74^cx (459)	52.95±2.02^by (250)	38.77±2.81^ay (107)
Total		2879	77.82±0.97	77.72±1	72.5±1.26	52.52±1.88	34.47±2.06
Frozen-thawed	Whole blastocyst	1467	52.39±1.95^bx (788)	71.69±2.24^cx (561)	69.75±3^cx (397)	39.44±4.15^ax (173)	28.60±5.09^ax (60)
	Mechanical dissection	1337	76.92±1.2^dy (1041)	78.16±1.7^dy (832)	65.88±2.18^cx (566)	48.77±2.49^bx (333)	35.29±3.2^ax (159)
	Laser dissection	1148	74.51±1.86^cy (828)	83.33±1.94^dy (616)	66.76±2.87^bcx (384)	59.91±2.71^by (207)	40.42±5.16^ax (87)
Total		3952	70.87±1.2	77.67±1.21	66.93±1.54	48.77±1.87	34.74±2.41

SEM: Standard error of mean; MEFs: Mouse embryonic fibroblasts; ICM: Inner cell mass; P1, P2, P3: Passages 1, 2, and 3; ^abcdMeans with different superscripts in a row within a group were significantly different (*p*<0.05); ^xyMeans with different superscripts in a column within a group were significantly different (*p*<0.05); *n*: No. of samples.
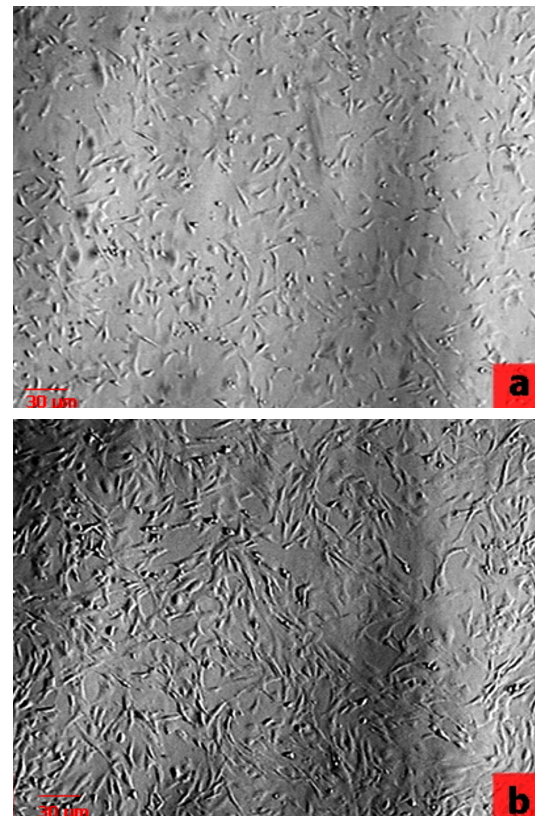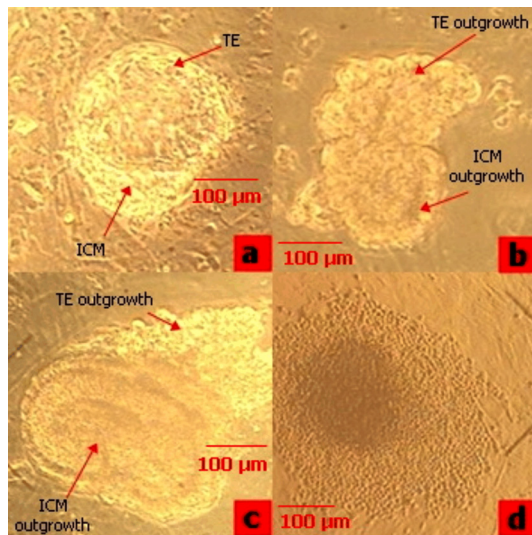
Figure 2 – *Trophectoderm (TE) and inner cell mass (ICM) outgrowth extension from (a) Day 2, (b) Day 3 and (c) Day 5 murine blastocyst. (d) Characteristics ring structure of outgrowths from attached blastocyst (×20).*

Table 4 – *Confirmation and characterization pluri-potency of murine ESCs*

Pluripotent marker	Result
AP	+
SSEA 1	+
SSEA 3	-
SSEA 4	-
TRA-1-80	-
TRA-1-60	-
Oct-4	+

ESCs: Embryonic stem cells; AP: Alkaline phosphatase; SSEA: Stage-specific embryonic antigen; Oct-4: Octamer-binding transcription factor-4.
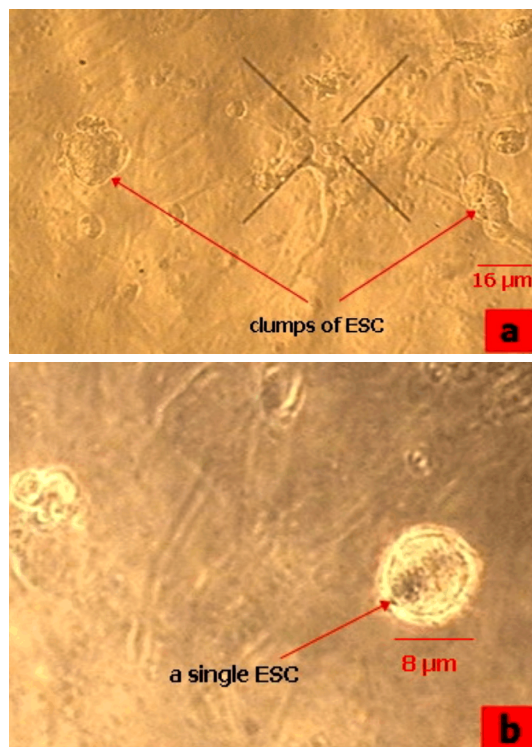

Figure 3 – *(a) Clump of embryonic stem cells (ESCs) with a large, dome-shaped colony (×20). (b) A single ESC with round shape and refractive edge (×40).*

Discussion

An effective cryopreservation protocol must be able to maintain the quality of the MEF and ensure high viability rate after thawing. Inconsistent production of ESC is related to differences in MEF preparation, including the cryo-preservation as it greatly influences the cellular quality [8].

The current finding suggested that the most optimum freezing density for cryopreserved MEF is 5×10^6 cells/mL as this density obtained higher viability rate compared to other densities. The growths in culture of mammalian cells are known to be prevented when cell density falls below a certain critical level, and the growth is due to the loss of cell substance *via* leakage from the cells, resulting in reduction of attachment and degeneration of cytoplasmic membrane [16]. The loss is compensated when cultured at high densities due to mutual feeding among the cells [17]. However, the high density of the cells leads to overcrowding and impairs the growth rate and overall quality of cells.

When evaluating the equilibration duration to freeze MEF, 15 minutes equilibration duration is superior to 20-hour duration in term of the viability rate the frozen-thawed. This finding is contradicted with the results obtained from previous mammalian cell freezing studies [18–20]. Long equilibration duration, which have low cooling rate, permits the water diffusing out of cells, whereas, the short duration of equilibration disallowed the water diffusion out of the cell which resulting in formation of intracellular ice crystal [20]. This causes damage to cell organelles and lead to cell apoptosis. However, Mazur *et al.* proposed on '2 factor' hypothesis on cell injury during cryopreservation, as the optimum equilibration duration or cooling rate varies among cell types [21]. Prolonged exposure to hypertonic freezing medium induced osmotic stress, which resulted in ire-versible damage to the cell integrity [22]. This finding was also supported by Higgins *et al.*, who obtained higher post-thawed viability rate on rat neural cell when freezing at faster cooling rate [23].

The present results showed no significant differences ($p>0.05$) in rates of attachment of ICM, primary outgrowth of ICM, successful consecutive passages of murine ESC culture on fresh and frozen-thawed MEF feeder cell layer. Although there is concerned on lost of factors in cryopreserved MEF in triggering the ESC growth and inactivating their differentiation [8, 18], the current data suggested that there was no significant difference in term of efficiency in murine ESC production when using both fresh and frozen-thawed MEF. This showed that the present freezing protocol which included the usage of 20% of DMSO as cryoprotectant did not give any detrimental effect on the optimum production of murine ESC.

ICM isolation technique is an important step to ensure the success of murine ESC establishment. In the present study, the whole blastocyst culture gave the lowest inner cell mass attachment rate and primary inner cell mass outgrowth rate on frozen-thawed MEF feeder cell layer as well as low successful rate of passage 3 on fresh MEF feeder cell layer compared to mechanical dissection and laser dissection inner cell mass isolation techniques. This may be due to TE cells were cultured along with the ICM,

which often disturb the ICM growth [24, 25]. In addition, the intact embryos where trophoblastic cells induce inner cell mass differentiation to be three embryonic germ layers by suppressing Oct-4 and Nanog expression levels [24]. Therefore, complete removal of trophoblastic cells provides more advantages to ICM growth to become ESC, which could be conducted by using either mechanical or laser dissection techniques. This has been proved that mechanical isolation of the inner cell mass has previously been successfully used in the derivation of two cell lines [25–28] and successful derivation by using laser dissection on murine and human ESC [29, 30].

Conclusions

The present study suggested that the freezing density of 5×10^6 cells/mL and the equilibration duration of 15 minutes are the most optimum protocols for cryo-preservation of the MEF. Both of fresh and frozen-thawed MEF could be utilized as feeder cell layer for producing murine ESC outgrowth. The manual and laser dissection techniques were suggested to isolate the murine ICM for optimum production of murine ESC outgrowth.

Conflict of interests

The authors declare no conflicts of interest for this research article.

Acknowledgments

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