Mouse Blastocyst Vitrification Compared with the Conventional Slow-Freezing Method

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Abstract

To compare mouse blastocyst survival after cryopreservation with vitrification and the slow-freezing method, one-hundred and forty-eight *in vitro* mouse blastocysts were randomly frozen by the two methods: vitrification and conventional slow-freezing. After being thawed, the blastocysts were assessed for survival and hatching rate. The survival rates of blastocysts cryopreserved by vitrification and slow-freezing were 68.33 and 65.52 per cent ($p = 0.89$), whereas hatching rates were 51.22 and 44.74 per cent, respectively ($p = 0.64$).Therefore, vitrification of blastocyst-stage-embryos may be a useful, economic method for freezing the excess blastocysts in some centers where blastocysts are routinely transferred.

Key word : Mouse Blastocyst, Cryopreservation, Vitrification, Conventional, Slow-Freezing Method

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Blastocyst transfer was claimed to generate high pregnancy and implantation rates, while lowering multiple gestation following *in vitro* fertilization (IVF), due to the reduced number of blastocysts transferred[1-3]. Therefore, blastocyst cryopreservation has become increasingly important in assisted repro-

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ductive techniques and reliable protocols are needed. Human blastocyst cryopreservation was first reported in 1985 by Cohen et al[4], using 10 per cent glycerol alone as cryoprotectant in a 10 step-procedure with a survival rate of 52 per cent (n = 23). Owing to sub-optimal culture conditions at that time, only 25 per cent of human zygotes reached the blastocyst stage *in vitro*[5], resulting in abandonment of blastocyst cryopreservation for years. Recently, the development of sequential culture medias for blastocysts has improved their survival rate and the tendency to transfer embryos in the blastocyst stage is more favorable[3]. However, previous studies showed that cryopreservation of mouse blastocysts had very low viability compared with other embryo developmental stages[6].

Successful cryopreservation depends at least in part on the mode of the freezing-thawing procedure[7]. Slow controlled-rate cryopreservation is conventionally achieved *via* commercially available computer-controlled-freezing systems which requires expensive equipment and is time consuming. Vitrification was described as a simple method of directly transferring embryos into liquid nitrogen after a brief exposure to a cryoprotectant solution[8]. Due to the cost-benefit of vitrification process, it is worth comparing the efficacy of freezing and thawing with conventional slow-freezing method and vitrification.

MATERIAL AND METHOD

Colony-raised ICR-female and male mice (Institute of Researched Animals, Mahidol University, Thailand) were maintained on a 12 h light and 12 h dark cycle (light activated at 0700h). Female mice 6-8 weeks old were superovulated with an intraperitoneal injection of 10 IU pregnant mare gonadotrophin (Sigma Chemical, St Louis, MO) at 1300 h. A second injection of 10 IU human chorionic gonadotrophin (hCG) (Sigma Chemical, St. Louis, MO) was given intraperitoneally 48 hours later. The females were then placed with males of the same strain. On the following morning, the vaginal plug was checked for evidence of mating. At 44 hours from hCG administration, the mated females were killed and their oviducts were excised. Two-cell embryos were obtained by oviduct flushing, as described by Hogan et al[9]. The embryos were then washed twice and placed in culture media : Human Tubal Fluid (HTF) (in house prepared) supplemented with 0.5 per cent Bovine Serum Albumin (BSA) (A3311, Sigma Chemical, St. Louis, MO).

The two-cell mouse embryos were cultured in groups of 8-10 in 20-microlitre (μl)-drops of culture media at 37°C in a humidified atmosphere of 5 per cent CO_2 in air. Blastocyst development was assessed after 96 hours of culture. All potentially surviving blastocysts were randomly allocated to either the slow-freezing or vitrification group.

Slow-freezing and thawing method

The two step slow-freezing method modified from the protocol of Menezo et al[7] was used for the slow-freezing procedure. Briefly, the embryos were exposed to 5 per cent glycerol (G5516 Sigma, St. Louis, MO) in a phosphate buffered solution (PBI in House prepared) supplemented with 10 per cent Serum Substitute Supplement (SS, Irvine Scientific, Santa Ana, California, USA) for 10 minutes in room temperature, then transferred to 9 per cent glycerol in PBI supplemented with 10 per cent SS and equilibrated for another 10 minutes.

During this second step, the embryos were loaded in 0.25 ml straws (Institute de Medicine Veneterinare straws, Bicef, L'Aigle, France). About 2-3 embryos were loaded into each straw.The straws were heat-sealed at the end which was filled with the polyvinylpyrolidone powder (PVP-40 Sigma, St. Louis, MO) and the other end was occluded with a plug. Then the straws were placed into the Kryo 10 freezing machine (Planer, Middlesex, UK). The programme for freezing was that from room temperature to -6°C, the straw was cooled at the rate of -2°C/minute. After holding at -6°C for 30 seconds, manual seeding was performed. The straws were then cooled at a rate of -0.3°C/minute from -6°C to -37°C. After that, they were plunged into liquid nitrogen.

The thawing procedure for slow-freezing protocol was performed by picking up the straws from the liquid nitrogen and holding the straws in the vapour for 40 seconds, followed by another 40 seconds in air and then plunged into a water bath of 30°C for a few seconds until the ice melted. The embryos suspended in melted cryoprotectant were pushed from the straws into a small petri-dish. Two-step thawing solution using 0.5 M sucrose (S1888, Sigma, St. Louis, MO) in PBI supplemented with10 per cent SS for the first 10 minutes and 0.2 M sucrose in PBI supplemented with10 per cent SS for another 10 minutes. The embryos were washed twice in PBI. Finally, they were placed in a 20 μl-drop of HTF supplemented with 0.5 per cent BSA covered with mineral oil at 37°C in

a humidified atmosphere with 0.5 per cent CO_2 in air About 8-10 embryos were placed in each drop.

Vitrification and thawing method

The vitrification process followed the protocol previously described by Lane et al[3]. Briefly, blastocysts were equilibrated prior to freezing procedures which was carried out at room temperature with 10 per cent (v/v) dimethyl sulfoxide or DMSO (Sigma, St Louis, MO) diluted in PBI supplemented with10 per cent SS and10 per cent ethylene glycol (E-9129, Sigma, St. Louis, MO) for 2 minutes, then transferred to 20 per cent DMSO plus 20 per cent ethylene glycol diluted in PBI with10 per cent SS and 30 per cent Ficoll (w/v) (F-4375, Sigma, St. Louis, MO) and 0.65 M sucrose for 30 seconds; during which the embryos were loaded onto an electron microscopic grid (600 mesh, copper-EM grid; G 600TT: Ted Fella Inc, Redding, CA, USA). The mean number of blastocysts loaded onto one grid was two or three.They were then plunged directly into liquid nitrogen. The total time elapsing from the immersion of blastocysts in cryprotectant to the plunge of grid into liquid nitrogen was about 20 seconds.

Thawing procedure for vitrification was carried out at room temperature by 4-step dilution. Firstly, the grids were transferred as quickly as possible into the first thawing solution which contained PBI supplemented with10 per cent SS plus 0.25 M sucrose for 2 minutes. By doing so, the embryos fell from the grid down into the solution. Then they were transferred to the second solution containing PBI supplemented with10 per cent SS plus 0.125 M sucrose for 3 minutes, followed by the third one containing PBI with10 per cent SS plus 0.05 M sucrose for another 3 minutes. Finally, they were transferred to the last solution containing PBI with10 per cent SS for 5 minutes, and then placed in the culture media similar to the slow-freezing method.

In both groups, after 24 hours of culture, the thawed blastocysts were examined for re-expansion of the blastocoele, and later hatching blastocysts (survival and hatching rate). The damaged ones were not recruited.

Statistical analysis

Differences in viability after cryopreservations with the two techniques were compared by χ^2-test with Yates correction.

RESULTS

A total of 182 zygotes were collected from mice, of which 148 blastocysts (81.3%) were obtained from embryo culture. (The rest were damaged.) An equal amount of blastocysts (n = 74) were randomly frozen by using the two study methods. Fifty-eight and 60 blastocysts from the slow-freezing method and vitrification were recovered for viability assessment, respectively, the rest were lost or damaged during the procedure.

The survival and hatching rates of mouse blastocysts frozen and thawed with slow-freezing method were 65.52 and 44.74 per cent, whereas in the vitrification group they were 68.33 and 51.22, respectively (Table 1).

When the degree of blastocyst expansion was considered, there was a tendency that expanded blastocyst was the best blastocyst stage to be frozen in both groups as shown in Table 2.

DISCUSSION

Several studies found that blastocysts are easy to be frozen and thawed because of the lower cytoplasmic volume of the cells resulting in a higher nucleocytoplasmic ratio that is suitable for the dehydration process of crypreservation[7,10,11]. The higher cell number of the trophectoderm is the other reason that allows good embryo recovery even if some cells

Table 1. Survival and hatching rate of slow-freezing and vitrification.

	Vitrification n = 60	Slow freezing n = 58	P-value
Survival rate %	68.33 n = 41	65.52 n = 38	0.89
Hatching rate %	51.22 n = 21	44.74 n = 17	0.64

Table 2. Survival and hatching rate according to degree of expansion.

Blastocyst stage	Survivals				Hatching			
	Vitrification (n = 60)	%	Slow-freezing (n = 58)	%	Vitrification (n =21)	%	Slow-freezing (n =17)	%
Early blastocyst andblastocyst	50.00	6/12 [a]	33.33	2/6 [a]	0.00	0/6 [b]	0.00	0/2 [b]
Full blastocyst	65.00	13/20 [a]	69.23	9/13 [a]	46.15	6/13 [b]	33.33	3/9 [b]
Expanded blastocyst	78.57	22/28 [a]	67.64	23/34 [a]	68.18	15/22 [b]	43.47	10/23 [b]
Hatching/ Hatched blastocyst	0.00	0/0 [a]	66.67	4/6 [a]	0.00	0/0 [b]	100.00	4/4 [b]

Note [a] number of blastocysts survived per total number frozen.
[b] number of blastocysts hatched per total number survived.

have been destroyed during cryopreservation. Many factors affect the survival of the frozen-thawed blastocysts; such as freezing methods, types and concentration of the cryoprotectants, time and temperature exposed during the process as well as the quality of the blastocysts. Vitrification has an advantage of being a simple method using no complicated equipment. However, vitrification can damage the membranous structure of the cells, as demonstrated in bovine embryos(12), including the loss of microvilli, disruption of the plasma membrane, mitochondrial changes and swelling of the endoplasmic reticulum, whereas nuclei and junctional region seemed to be resistant to cryoinjury. On the contrary, it is thought that the use of vitrification would be the preferred method of cryopreservation over the slow-freezing method because of the lack of ice crystal formation(13). Furthermore, vitrification is substantially better for cells that have high chilling sensitivity(5,14).

In the present study, the authors demonstrated that mouse blastocysts can be successfully vitrified by loading on an EM grid and plunging directly into liquid nitrogen. Blastocysts were capable of re-expansion and hatching after vitrification at a similar rate to those frozen by the conventional slow-freezing method. It is worth noting that there were different stages of mouse blastocysts developed at 96 hours after fertilization. The developmental rates determined blastocyst quality(15), which might be the reason for lower survival and hatching rates in the early blastocyst stage. Furthermore, expanded blastocyst seemed to be the most suitable stage to be frozen by both the vitrification and slow-freezing method as shown in Table 2.This may be explained by the higher nucleocytoplasmic ratio of the blastocyst in the later stage.

Compared to sophisticated and expensive, conventional slow-freezing method, vitrification provides a technique that is both successful and easy to perform and might be widely applicable to all mammalian gametes and embryos.

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การแช่แข็งตัวอ่อนหนูระยะบลาสโตซิสท์ด้วยวิธีวิทริฟิเคชั่น เปรียบเทียบกับวิธีมาตรฐานแบบทีละขั้น

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การแช่แข็งตัวอ่อนระยะบลาสโตซิสท์ยังเป็นปัญหาที่น่าสนใจ และมีการศึกษาปรับปรุงกันอยู่อย่างต่อเนื่อง เพราะแนวโน้มของการเลี้ยงตัวอ่อนถึงระยะนี้เพิ่มมากขึ้น ในกรณีของการเพิ่มอัตราการตั้งครรภ์ และลดความเสี่ยงของการตั้งครรภ์แฝด ผู้วิจัยจึงได้ทำการศึกษาการแช่แข็งตัวอ่อนหนูระยะบลาสโตซิสท์ด้วยวิธีวิทริฟิเคชั่น เปรียบเทียบกับ วิธีมาตรฐาน แบบทีละขั้น พบว่า ผลของการแช่แข็งตัวอ่อนทั้งสองวิธีไม่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ คืออัตราการอยู่รอดของบลาสโตซิสท์หลังแช่แข็งและละลายแล้ว ด้วยวิธีวิทริฟิเคชั่น เปรียบเทียบกับ วิธีมาตรฐาน แบบทีละขั้นเท่ากับ ร้อยละ 68.33 และ 65.52 ($p = 0.89$) อัตราการฟักตัวของบลาสโตซิสท์เมื่อเลี้ยงต่อหลังแช่แข็งและละลายแล้ว เท่ากับ ร้อยละ 51.22 และ 44.74 ($p = 0.64$) ตามลำดับ ดังนั้นวิธีวิทริฟิเคชั่น ซึ่งทำได้ง่าย ไม่สลับซับซ้อน ไม่ต้องใช้เครื่องแช่แข็งราคาแพง น่าจะเป็นวิธีที่ดีวิธีหนึ่ง สำหรับแช่แข็งตัวอ่อนระยะบลาสโตซิสท์

คำสำคัญ : แช่แข็งตัวอ่อนหนูระยะบลาสโตซิสท์, วิทริฟิเคชั่น, วิธีมาตรฐาน แบบทีละขั้น

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