ANIMAL EXPERIMENTATION

Cryopreservation of Mouse Embryos at Morula/Compact Stage

JUN TAO,^{1,2} ROBERT TAMIS,¹ and KATHARINE FINK¹

Submitted: July 6, 2000

Accepted: November 8, 2000

Purpose: The objective of this article was to study mouse morula/compact stage embryo postthaw survival rates and postthaw developmental potential.

Methods: Mouse morula/compact stage embryos were classified into 3 substages. Their morphological behavior during the freezing and thawing process were examined. Postthaw survival rates and blastocyst formation rates were compared between the three substages and the pronucleate and the 2-cell stage embryos. Finally, Postthaw fully compacted and late compact embryos were transferred to foster mothers.

Results: Blastomeres of early compacting embryos separated from each other during the freezing process, whereas in fully compacted and late compact stages, the blastomeres remain compacted. Fully compacted and late compact stage embryos had higher Postthaw survival rates than other stages, though those were not statistically significant ($P > 0.05$). These two substages had significantly higher blastocyst formation rates than Postthaw pronucleate and early compacting embryos ($P < 0.01$). A total of 72 Postthaw fully compacted and late compact embryos were transferred and 20 live offspring were delivered.

Conclusions: After passing early compacting stage, fully compacted and late compact stage mouse embryos have high Postthaw survival rates and significantly higher blastocyst formation rates. Successful cryopreservation of embryos at these two substages has a significant value in embryo

selection. The results obtained in this study may provide useful information for human assisted reproductive technology.

KEY WORDS: Cryopreservation; embryo transfer; morphological study; morula/compact embryo.

INTRODUCTION

Successful mouse embryo freezing and thawing was reported by Whittingham *et al.* in 1972 (1). Since then, embryos of many other mammalian species, including the human being, have been successfully cryopreserved (2–3). It is always a great challenge to obtain a high survival rate and a high implantation or pregnancy rate after transferring postthaw embryos. To improve the survival rate after cryopreservation, there are numerous papers discussing different cryoprotectants (4–6), different embryonic stages for freezing (7–9), and different freezing/thawing methods (10–12). Routinely, embryos are frozen at the pronucleate and the 2-cell to 8-cell stages, and acceptable survival rates have been achieved. However, the embryonic genome activates after 4- to 8-cell stage, and the capability of the fertilized egg or early cleaved embryo to reach blastocyst stage is limited. It has been realized that morphologic features of embryos at early stages cannot provide accurate information to predict developmental potential, which is at least partially due to the embryonic genome not being activated. Therefore, significant attention has also been focused on blastocyst stage freezing (5,8,13–15). The blastocyst embryo is the last stage

¹ Arizona Institute of Reproductive Medicine, 3411 North 5th Avenue, Suite 207, Phoenix, Arizona 85013.

² To whom correspondence should be addressed at, 5333 W Mercury PL, Chandler, Arizona 85226.

before implantation and the inner cell mass can be examined in the expanded blastocyst stage, and therefore provides the best opportunity for embryo selection. Nevertheless, the postthaw survival rates of blastocyst cryopreservation are not always encouraging (7,8,13,16). Morula/compact stage embryos, though not as suitable as blastocyst stage embryos in terms of embryo selection, when compared to pronucleate and early cleaved embryos, should have better prognostic value. However, freezing embryos at morula/compact stage is not an area that has been fully investigated. This paper classifies mouse morula/compact embryos into three substages (early compacting, fully compacted and late compact stages) and compares the postthaw survival rates and blastocyst formation rates between these three substages and the pronucleate and the two-cell stage embryos. It also studies the posttransfer developmental potential of some postthaw fully compacted and late compact embryos that were transferred to foster mice.

MATERIALS AND METHODS

Embryo Preparation

Five- to seven-week-old female B6C3 F1 mice (Charles River Laboratories, Wilmington, MA) were induced to superovulate by injections of 5 IU pregnant mare serum gonadotropin (PMSG, Sigma, St. Louis, MO) and 5 IU human chorionic gonadotropin (hCG, Sigma) 48 h later. The female mice were then caged with 12–20-week-old Swiss Webster male mice (Charles River Laboratories) and mating was confirmed next morning by visible vaginal plug. Mated female mice were killed by cervical dislocation and pronucleate embryos were collected from the ampullae of the excised oviducts. Adherent cumulus cells were then removed by 0.1% hyaluronidase (Sigma) and the pronucleate were transferred to P1 culture medium (Irvine, CA), which was supplemented with 10% Serum Substitute Supplement (Irvine, CA).

Pronucleate embryo freezing took place approximately 2–3 h after retrieval. Two-cell stage embryos were frozen in the morning of the following day (day 2). Late day 3 or the early morning of day 4, morula/compact stage embryos were frozen based on the following categories: 1) *Early compacting stage*, in which the arrangement of the blastomeres started to compact making the cell boundary obscure, but each individual blastomere was still distinct; 2) *Fully compacted stage*, in which the blastomeres were “fused” together. The whole embryo appeared as a big sphere-

shaped cell mass with a relatively smooth profile. At this stage it is difficult to identify any individual cells. Embryos that appeared as the fully compacted before freezing but in which blastomeres separated during the freezing process were also classified as the early compacting stage in this study; 3) *Late compact stage*, at which stage a small cavity, from approximately 8 μm to less than one third of the whole embryo, was observed. Because the majority portion of the cell mass was still compacted and the cavity disappeared during the freezing process, these embryos were classified as the late compact stage rather than very early blastocyst stage in this study. The cell membrane of the blastomeres again appeared to be distinct though cells were still tightly arranged. The number of blastomeres was increased and some spindle-shaped blastomere could be observed.

Freezing and Thawing Procedure

Modified Testart's freezing method was used on all embryos at various embryonic stages (17). Briefly, the embryos were first transferred to a Hepes-buffered HTF medium supplemented with 10% SSS at room temperature for 5–10 min. Then embryos were moved to a 0.5 M 1,2-propanediol (Sigma)/0.05 M sucrose (Sigma) solution for 5 min followed by a 1.0 M 1,2-propanediol/0.075 M sucrose solution for another 5 min. Finally, the embryos were transferred to a solution with 1.5 M 1,2-propanediol/0.1 M sucrose and loaded into 0.25 ml straws (TS Scientific, Perkasi, PA). An average of 10–15 embryos was placed in each straw. The freezing process was performed using a Cryogenesis freezer (Biogenics, Model CL-863, Napa, CA). A standard procedure was used, that is, $-2^{\circ}\text{C}/\text{min}$ from 18°C to -7.5°C , followed by seeding and holding for 10 min. Then the temperature was lowered to -30°C at the rate of $-0.3^{\circ}\text{C}/\text{min}$. From that point, the cooling rate was $-10^{\circ}\text{C}/\text{min}$ to -140°C . The embryos were then transferred to liquid nitrogen for at least 1 week before thawing.

The thawing procedure was performed using the following procedure: The straws were taken out of the liquid nitrogen and set at room temperature for 30–40 s in a horizontal position. Then the straws were transferred to a 30°C water bath for about 15–20 s. After being released from the straw, embryos were first transferred to a 1.0 M 1,2-propanediol/0.2 M sucrose solution for 5 min, followed by a 0.5 M 1,2-propanediol/0.15 M sucrose solution for 5 min. Finally, the embryos were transferred to a 0.1 M sucrose solution for 5 min. After rinsing two to three times

in Hepes-buffered HTF medium, embryos were cultured in P1 medium with 10% SSS.

Embryo Transfer

Swiss Webster male mice underwent vasectomy at 12–14 weeks of age. The sterility of the vasectomized male was confirmed by mating with female mice 8 weeks after the surgery with no pregnancies achieved. Seven- to eight-week-old Swiss Webster female mice were chosen as the foster mothers. Pseudopregnant recipient mice were prepared by caging the female mice with the above vasectomized male mice and observation of the vaginal plug the following morning. When mating was confirmed, the female mice were moved to a separate cage. About 40 h later, morula/compact embryo transfer took place. Embryo thawing was performed 3–4 h before the transfer procedure. Embryos used for transfer were not included in postthaw survival and blastocyst formation rates analysis because the embryos would be transferred to foster mothers before reaching blastocyst stage. A total of 88 fully compacted and late compact embryos were thawed; 81 survived of which 72 embryos were transferred.

The embryo transfer procedure was performed under general anesthesia by administration of Pentobarbital sodium injection solution (Abbott Laboratories, North Chicago, IL) intraperitoneally with the dose of 2.3 mg/mouse. The number of embryos transferred to each female mouse is shown in Table I. Embryos were transferred into either one side or both sides of the uterus. A total of five transfer procedures were successfully conducted.

Statistical Analysis

Postthaw survival rates and blastocyst formation rates were compared between different groups

Table I. Results of Mouse Postthaw Fully Compacted and Late Compact Stage Embryo Transfers

Animal	Postthaw survival		Transfer no.	Live birth
	Fully compacted	Late compact		
1	7/7	8/9	15	4
2	17/17	12/13	20	0
3	6/7	2/2	8	4
4	8/8	8/8	16	12 ^a
5	7/10	6/7	13	0

^a Plus one still birth.

from the pronucleate stage to late compact stage. Data analysis was conducted with Chi-squared tests and statistical differences regarded as significant if $P < 0.05$.

RESULTS

Morphological Changes During the Freezing Process

Morphologically, pronucleate and two-cell stage mouse embryos demonstrated typical volumetric changes during freezing process, that is, after pronucleate and cleaved embryos were exposed to cryoprotective solutions, cells underwent osmotic contraction. This osmotic contraction was demonstrated on an individual cell basis, which means that each cell reacted as a single unit. Therefore, when cells contract, distance between cells of the cleaved embryos increased and individual cell profile was more distinct.

Morula/compact embryos exhibited a reversed morphological response to osmotic pressure changes. In the early compacting stage, blastomeres started to shrink after exposure to freezing solutions. This shrinkage was also demonstrated on the individual cell basis, which made the blastomeres separate from each other. Therefore, the embryos appeared to be reverting back to a premorula stage, that is, eight-cell stage (Fig. 1). The extent of the cell separation increased as the osmotic pressure in the freezing media increased. After embryos were equilibrated in 1.5 M 1,2-propanediol/0.1 M sucrose, the blastomeres in some embryos appeared to become completely separated. therefore, at this stage, response of the blastomeres to the osmotic pressure changes during the freezing process was similar to early cleaved embryos, that is, each cell acted as a single unit, which made the distance between cells increase as each blastomere got smaller. It was also observed that in some embryos the blastomeres were only partially separated. In those cases a certain degree of attachment among blastomeres still remained.

Fully compacted embryos did not show apparent morphological changes. The entire embryo appeared to act as a single unit after exposure to freezing solutions, which means that there was no blastomere separation, except the whole cell mass had slight volumetric contraction (Fig. 2). After equilibration in 1.5 M 1,2-propanediol/0.1 M sucrose solution, this compacted embryo still remained as a sphere-shaped cell mass. However, it was common that the blastomeres of some “compacted” embryos separated during the freezing process. These embryos may have

Fig. 1. Reversed morphological change of mouse early compacting embryos during a freezing process. A. Before freezing, blastomeres started to compact. Individual cell profile can be observed. However, cell boundaries between cells were obscure. B. After exposure to 1.0 M 1,2-propanediol/0.075 M sucrose solution for 5 min, blastomeres were separated which made embryos appear as at the eight-cell stage.

been between the early compacting and the fully compacted stages. Since the blastomeres separated or partially separated during the freezing process, these embryos were classified as early compacting stage for this study.

For the late compact stage embryos, an apparent reversed morphological change was also demonstrated. After exposure to freezing media, the late compact embryos seemed to revert back to the fully compacted stage due to the blastomeres shrinkage and the tight arrangement of cells. There was no blastomere separation observed in the late compact stage. Small cavities, which showed in late compact stage embryos, became smaller or even nonnoticeable during the freezing

Fig. 2. Morphological changes of mouse fully compacted and late compact stage embryos during a freezing process. A. Before freezing, four embryos at fully compacted stage and one at late compact stage with a cavity (arrow). B. After exposure to 0.5 M 1,2-propanediol/0.05 M sucrose for 5 min, four fully compacted embryos did not show apparent morphological changes, except the whole cell mass had slight volumetric contraction. However, the late compact stage embryo exhibited a smaller cavity (arrow). C. After exposure to 1.0 M 1,2-propanediol/0.075 M sucrose solution for 5 min, the cavity in the late compact stage embryo disappeared which made the embryo appear as a fully compacted embryo.

Fig. 3. Postthaw survival rates of mouse embryos at various embryonic stages. No significant difference between any two groups ($P > 0.05$).

process, which made these embryos appear as the fully compacted embryos (Fig. 2).

Embryo Thawing and Survival

Embryos in which more than half of the blastomeres remained intact after thawing were considered as surviving. Figure 3 shows postthaw survival rates when mouse embryos were frozen at the various stages. Fully compacted and late compact stage embryos exhibited higher postthaw survival rates (94.3% and 95.4%, respectively) when compared with the pronucleate, the 2-cell, and the early compacting stages (91.8%, 92.7%, and 88.6%, respectively), but there was no statistical significance ($P > 0.05$). Of the 114 early compacting embryos thawed, it was observed that 25 embryos exhibited blastomere lysing, of which 4 had all cells lysed and 21 had some cells lysed, referred to as partial survival. Of these 21 partial survival embryos, 12 had equal to or more than

half of blastomeres remaining intact, and so these embryos were considered as surviving though with some blastomeres lost. The remaining nine embryos were considered as nonsurviving due to more than half of the blastomere lysing. In contrast, of the 250 fully compacted and the late compact embryos thawed, a total of 13 embryos had cell lysing, of which 11 had no cell survival at all. In one embryo, only one cell survived (Fig. 4). In another embryo, all blastomeres lysed right after thaw, but a small portion seemed to recover later. However, there was no further development of either embryo the next day. It appears that postthaw survival in the fully compacted and the late compact stages showed a trend of "all or none" pattern, that is, either all blastomeres survived or they all lysed (Fig. 4).

Morphological Changes During the Thawing Process

During thawing, the morphological changes of morula/compact embryos were opposite to those

Fig. 4. During the thawing process fully compacted embryos stayed as a sphere-shaped multicellular mass. Cytoplasm became more granular, cell number was significantly increased. Four surviving embryos that had no cell lysis and two nonsurviving embryos where almost all blastomeres were lysed showing a trend of “all or none” survival pattern.

observed during the freezing process. Morula/compact embryos quickly recovered to their original prefreezing status during the thawing process. For example, separated blastomeres in the early compacting embryos usually started to compact again as the osmotic pressure in the medium got relatively lower during the thawing. Before reaching the last step of thawing, transferring to P1 culture medium, it was common for the embryo to return back to the early compacting stage. Fully compacted embryos did not exhibit dramatic morphological change. During the thawing process, fully compacted embryos still remained as a multicellular mass, but the cytoplasm became slightly granular. The cell boundaries seemed more visible (Fig. 4). It was common that these embryos entered the early blastocyst stage soon after the thawing process was complete. For the late compact stage embryos, some signs, such as spindle-shaped blastomere could be observed at the end of the thawing. Granular cytoplasm was common and the cell boundary became clearer. The small cavities, which had existed before freezing, could be observed once again during the thawing process or soon after thawing was complete.

At the beginning of the thawing process, it was observed that 37 of the 250 (15%) fully compacted and late compact embryos showed membrane surface bubbles. These surface bubbles started from tiny bubbles and then fused together to form larger ones. Several large surface bubbles could fuse again

and eventually surround the whole embryo. This surface bubble had a distinct membrane surrounding it with a clear inside content. If the size of the bubble was less than 20 μm , it was defined as a small bubble. The small surface bubbles usually disappeared during the thawing process and seemed to have no adverse effect on survival. However, 13 embryos had several large surface bubbles, >20 μm , and 11 of these embryos lysed at the end of the thawing procedure. It was difficult to judge whether the surface bubble was the first sign of embryo lysing because some embryos were found to have lysed when first observed, before any morphological changes were noticeable.

Blastocyst Formation

Figure 5 shows the blastocyst formation rates from the embryos frozen at various embryonic stages. Fully compacted and late compact stage embryos have significantly higher blastocyst formation rates when compared with the pronucleate embryos ($P < 0.01$) and the early compacting embryos ($P < 0.01$). Embryos frozen at the 2-cell stage also have higher blastocyst formation rate when compared with the pronucleate embryos ($P < 0.05$) and the early compacting embryos ($P < 0.05$).

Embryo Transfer

Table I presents the procedure and the results of embryo transfers. Three out of five pseudopregnant mice became pregnant. A total of 72 embryos were transferred with 20 live births and one still birth. All the 20 mice delivered alive, 11 males and 9 females, were observed up to 4–5 weeks and judged to be normal and healthy. All offspring were black in color, which excluded the possibility that these offspring were genetically related to the foster mothers (white) and the vasectomized male mice (white).

DISCUSSION

The main challenge of embryo freezing is the survival rate after thawing. Traditionally, most mammalian embryos are frozen at either pronucleate or early cleaved stages, that is, 2-cell to 8-cell stages (7–9,18). However, embryos at these stages cannot provide sufficient information for embryo selection. Before embryonic genome activation, early embryogenesis is under the control of the maternal genome. Therefore, the prognostic value of morphological

Fig. 5. Blastocyst formation rates of postthaw mouse embryos at various stages. * Significantly different from the pronucleate and the early compacting stages ($P < 0.05$). ** Significantly different from the pronucleate and the early compacting stages ($P < 0.01$).

grading is discounted. Thus, there have always been attempts to freeze embryos at the blastocyst stage (4–5,13–15), but the outcome after thawing is not always encouraging (7,8,13,16). Based on our literature search it seems that very few articles have extensively studied morula/compact stage embryo freezing. Theoretically, the morula/compact embryo has a better prognostic value compared to early cleaved embryos because of the activated embryonic genome. In this article, we focused on the morphological behaviors of morula/compact embryos during freezing and thawing processes and studied postthaw survival rates, blastocyst formation rates, and the outcome after embryo transfer.

In this study, mouse morula/compact stage embryos were classified into three substages. It was found that embryos at the fully compacted and the late compact stages have similar morphological responses to osmotic pressure alterations as well as similar post-

thaw survival rates and blastocyst formation rates. These two stages have higher postthaw survival rates though there was no statistical significance when compared with other groups. Blastocyst formation rates in these two groups were significantly higher than postthaw pronucleate ($P < 0.01$) and early compacting stage ($P < 0.01$) but not significantly higher than 2-cell stage ($P > 0.05$). These results suggested that the postthaw fully compacted and late compact embryos had better growth potentials than did earlier stage embryos and that the normal offspring delivered also indicated the great growth potential of these postthaw embryos.

During the freezing process morula/compact embryos exhibited some reversed morphological changes. These changes are believed to be caused by osmotic pressure alterations. Maybe for the same reason, during the thawing process, embryos changed back to their original status quickly. The difference

between the early compacting embryos and the fully compacted or the late compact embryos is that after equilibration in 1.5 M propanediol/0.1 M sucrose solution the former separated into individual cells but the latter two remained as a multicellular mass. This difference may be responsible for different survival rates after thawing.

Similar results, that the mouse morulae exhibited high survival rates after rapid freezing have been reported by a limited number of authors (16,19,20). However, morula/compact embryos were never classified into substages as this article does. The results of this study indicate that at different substages, morula/compact embryos exhibited different morphological behaviors and had different postthaw survival rates and blastocyst formation rates. Several possible explanations have been considered. First, early compacting embryos could be relatively slow growing embryos, therefore their quality might be poorer than faster growing compacted or late compact stage embryos. However, this seems not the case, because early compacting embryo freezing was performed in the late afternoon of day 3 and fully compacted and late compact embryos freezing was conducted in the morning of day 4. Therefore, it is embryo age that is more important. Second, the partial survival of blastomeres in some early compacting embryos might affect growth potential because 12 "surviving" embryos had some blastomeres that were lysed. Third, after the blastomeres are fully compacted, the embryo may provide better selection value. Nevertheless, this is unlikely to play a major role in the current experiment because postthaw 2-cell stage embryos also yield higher postthaw survival and blastocyst formation rates compared with the early compacting stage embryos. Fourth, early compacting and fully compacted or late compact embryos may have different cell membrane permeability to the cryoprotectant, which caused blastomeres to become separated in the former but to remain in a tight arrangement in the latter. Fifth, cryopreservation-caused cell membrane damage in the early compacting embryos is possible. Morphologically, blastomeres of early compacting embryos shrink during the dehydration process that exists after the embryos are exposed to cryoprotectant. Because each cell shrinks as a single unit, this cell volume change subjects the blastomere membrane to a stretching force due to the established partial connection between cells. It is highly doubtful that the cell membrane underwent this stretching without damage. This possible cell membrane damage may result in cell lysis in the later thawing process.

Further studies will be needed to understand these considerations.

Tissue or multicellular system freezing is usually more difficult compared to single cell freezing. One of the reasons is that when a single cell is surrounded by a freezing solution the cell can be efficiently exposed to a cryoprotectant and dehydrated. Whereas in a multicellular system, cells at different locations are exposed to the cryoprotectant at different gradients and dehydrated at different rates. The second is that water accumulated in intercellular space of a multicellular system will freeze and significantly increase in volume. This volume change in intercellular space is sufficient enough to injure adjacent cells (21). Nevertheless, the fully compacted embryo may not have intercellular space but direct membrane-membrane contact. It is possible that because the lack of intercellular space, blastomeres not only access to cryoprotectant efficiently, but also avoid possible injury caused by intercellular ice.

Morphologically, after reaching the fully compacted and the late compact stages, extensive cell connection had been established, which left no gap between cells. During the freezing process, these embryos exhibited a very unique phenomenon in which the embryos remained as a compacted multicellular mass rather than separated into individual cells. This phenomenon indicates that the whole embryo responses to the osmotic pressure change as a single unit, like a single cell. There are two possible hypotheses for this phenomenon. First is the extensive cell connection established after becoming compact prevents cell separation. Second is that the cell membrane permeability may have changed, which facilitates cryoprotectant penetration of the cell membrane. This phenomenon may be also related to the "all or none" survival pattern in the fully compacted and the late compact stage embryos. Since blastomeres did not separate from each other, cell membranes between blastomeres were arranged in a tight-contacted status and could have formed a network-like structure, which might function as a skeleton for embryos. This double cell membrane formed "embryonic skeleton" may play a role to strengthen embryo integrity and provide better tolerance to osmotic pressure changes.

High postthaw survival rates and high blastocyst formation rates from the fully compacted and the late compact embryos have a significant meaning for embryo selection. When embryo cryopreservation can be successfully conducted at this stage, the embryos chosen for the freezing will have a better growth potential

when compared with earlier stage embryos because of the activated embryonic genome. Blastocyst stage embryos though provide better selection information, however, the relatively poor cryopreservation outcome (7,8,13,16), the possible of high vulnerability to injury during embryo transfer procedure, and more labor and incubator space requirements may discount that value. In this study, 94.7% and 96.4% of surviving fully compacted and late compact embryos, respectively, reached the expanded blastocyst stage. These results support another option for the stage of embryo freezing.

In conclusion, this study, for the first time, classified mouse morula/compact embryos into three substages and discovered that they responded to osmotic pressure changes in different patterns. In the early compacting embryos, blastomeres were separated during the freezing process, whereas fully compacted and late compact stage embryos remained at compacted status, which may be the main reason why different survival and blastocyst formation rates yielded. The “all or none” trend of survival observed in the fully compacted and the late compact embryos suggest that blastomeres at these two substages responded to osmotic pressure changes as a single unit. Results of this study indicate that the fully compacted and the late compact mouse embryos can be frozen successfully and achieve good growth potential. Compared to the expanded blastocyst freezing, these two substages may have some advantages in terms of postthaw survival rate, less vulnerability to injury, and less labor and incubator space required. These advantages may have great value for clinical applications.

REFERENCES

- Whittingham DG, Leibo SP, Mazur P: Survival of mouse embryos frozen to -196°C . *Science* 1972;178:411–414
- Trounson A, Mohr L: Human pregnancy following cryopreservation, thawing and transfer of an eight-cell embryo. *Nature* 1983;305:707–709
- Cohen J, De Vanc GW, Elsner CW, Fehilly CB, Kort HI, Massey JB, Turner TG, Jr: Cryopreservation of zygotes and early cleaved human embryos. *Fertil Steril* 1988;49:283
- Li R, Trounson A: Rapid freezing of the mouse blastocyst: Effects of cryoprotectants and of time and temperature of exposure to cryoprotectant before direct plunging into liquid nitrogen. *Reprod Fertil Dev* 1991;3:175–185
- Nowshari MA, Brem G: Effect of cryoprotectants and their concentration on postthaw survival and development of expanded mouse blastocysts frozen by a simple rapid-freezing procedure. *Theriogenology* 1998;50:1001–1013
- McWilliams RB, Gibbons WE, Leibo SP: Osmotic and physiological responses of mouse zygotes and human oocytes to mono- and disaccharides. *Hum Reprod* 1995; 10:1163–1171
- Ciriminna R, Schillaci R, Cefalu E, Agrifoglio V: What is the most idoneous developmental stage for embryo-freezing. *Acta Eur Fertil* 1994;25:173–176
- Troup SA, Matson PL, Critchlow JD, Morroll DR, Lieberman BA, Burslem RW: Cryopreservation of human embryos at the pronucleate, early cleavage, or expanded blastocyst stages. *Eur J Obst Gynec Reprod Biol* 1990;38:133–139
- Demoulin A, Jouan C, Gerday C, Dubois M: Pregnancy rates after transfer of embryos obtained from different stimulation protocols and frozen at either pronucleate or multicellular stages. *Hum Reprod* 1991;6:799–804
- Van der Elst JC, Nerinckx SS, Van Steirteghem AC: Slow and ultrarapid freezing of fully grown germinal vesicle-stage mouse oocytes: Optimization of survival rate outweighed by defective blastocyst formation. *J Assist Reprod Genet* 1993;10:202–212
- Jones HW Jr, Veeck LL, Muasher SJ: Cryopreservation: The problem of evaluation. *Hum Reprod* 1995;10:2136–2138
- Martinez AG, Matkovic M: Cryopreservation of ovine embryos: Slow freezing and vitrification. *Theriogenology* 1998;49:1039–1049
- Stein A, Fisch B, Tadir Y, Ovadia J, Kraicer PF: Cryopreservation of rat blastocysts: A comparative study of different cryoprotectants and freezing/thawing methods. *Cryobiology* 1993;30:128–134
- Menezo Y, Nicollet B, Herbaut N, Andre D: Freezing cocultured human blastocysts. *Fertil Steril* 1992;58:977–980
- Shaw JM, Diotallevi L, Trounson AO: A simple rapid 4.5 M dimethyl-sulfoxide freezing technique for the cryopreservation of one-cell to blastocyst stage preimplantation mouse embryos. *Reprod Fertil Dev* 1991;3:621–626
- Nakagata N: Survival of mouse morulae and blastocysts derived from in vitro fertilization after ultra rapid freezing. *Jikken Dobutsu* 1993;42:229–231
- Lassalle B, Testart J, Renard JP: Human embryo features that influence the success of cryopreservation with the use of 1,2-propanediol. *Fertil Steril* 1985;44:645–651
- Veeck L, Amundson C, Brothman L, DeScisciolo C, Mahoney M, Muasher S, Jones H: Significantly enhanced pregnancy rates per cycle through cryopreservation and thaw of pronuclear stage oocytes. *Fertil Steril* 1993;59:1202–1207
- Aksoy M, Takahashi Y, Hishinuma M, Elsheikh AS, Tanaka H, Kanagawa H: Influences of retrieval stages and glutathioneaddition on Postthaw viability of quick frozen mouse morula during in vitro culture. *Theriogenology* 1999;51:681–687
- Takahashi Y, Kanagawa H: Effect of equilibration period on the viability of frozen–thawed mouse morulae after rapid freezing. *Mol Reprod Dev* 1990;26:105–110
- Rubinsky B, Pegg DE: A mathematical model for the freezing process in biological tissue. *Proc. Roy. Soc. London (Biol.)* 1988;234:343–358