

The Effects of Different Cryopreservation Techniques on the Survival Rate of In Vitro Produced
(IVP) and Biopsied Bovine Blastocysts

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Abstract

This study's objectives were to determine if the biopsy of expanded blastocysts prior to cryopreservation affected the survival rates and determine if there is a difference between slow-freezing in 10% glycerol, slow freezing in ethylene glycol, and vitrification in terms of survival rate of the embryos. Blastocoeles were reduced prior to cryopreservation by means of biopsy. Embryos for slow freezing were exposed to ethylene glycol (SynGRO Ethylene Glycol with Sucrose, BIONICHE, Pullman, WA, USA) or 10% glycerol (SynGRO 10% Glycerol, BIONICHE, Pullman, WA, USA) as cryoprotectants. Vitrified embryos were exposed to 10% and 40% ethylene glycol. Survival rate of biopsied embryos was similar than non-biopsied embryos (77.8 vs 63.9%, $p > 0.05$). There was no significant difference between the different cryopreservation methods ($p > 0.05$).

Introduction

Current innovations *in vitro* production (IVP) allows for the efficient production of large numbers of bovine embryos, leading to a commercial application of cryopreservation of as a means of storage and transportation of genetic material.

Basile J. Luyet and Eugene L. Hodapp first recorded the cryopreservation technique of vitrification as successful in preserving gametes in their publication on frog sperm in 1938 [1]. Since their success, various modifications to the vitrification process have led to it being one of the two basic techniques used in the cryopreservation of embryos [2]. The other commonly used method is slow freezing, often considered the conventional form of cryopreservation [3].

It has been found that water content and membrane permeability of the embryo are crucial factors in the success of cryopreservation by reducing the amount of cryoinjury, damage to the embryo by chemical or physical stress introduced during the cryopreservation process [4]. The most common form of cryoinjury to embryos is the occurrence of intracellular formation of ice crystals and toxicity from cryoprotectants [5]. The most effective method to preventing cryoinjury by ice crystal formation and toxicity is to remove water from the embryo before and during the cryopreservation process. This can be achieved by optimizing the cooling rates and the addition of non-embryotoxic macromolecules to increase the osmotic action of the cells, allowing for an ideal balance of water flow out of the cell and cryoprotectant into the cell [6].

The biopsy or blastocoele reduction of blastocysts prior to cryopreservation has been indicated as having an effect on survival rates (re-expansion of blastocyst post thaw). Biopsied IVP bovine embryos cryopreserved utilizing the conventional slow freezing technique had a greater rate of survival in 0.7M glycerol and 0.05M sucrose than non-biopsied IVP blastocysts [3]. Previous studies had indicated that in both mouse and human embryos, collapse of the

blastocyst via the reduction of the blastocoel via micro-needle manipulation or laser pulse had improved survival rates of blastocysts during vitrification [7,8].

The effectiveness of cryoprotectants and cryopreservation techniques is also a concern for survival rates of bovine embryos. It has been previously determined that both ethylene glycol and glycerol are effective cryoprotectants for slow freezing of IVP embryos [9]. Permeability of the cell membranes of the embryo to different cryoprotectants can have a significant effect on the survival rate of the cell post-thaw [4].

Unlike slow freezing, which relies on an equilibrium and dehydration of the cells with ice crystal formation, vitrification success relies on the lack of ice crystal formation [1]. The prevention of crystal formation is achieved by cooling an embryo in the presence of a high concentration of cryoprotectant very rapidly in a small volume to creating a glass-like physical state [6].

The objective of this study was to evaluate the effects that different cryopreservation methods and embryo biopsy had on the survival rate of expanded bovine blastocysts. The cryopreservation survival rates of biopsied blastocysts were compared to non-biopsied blastocysts that did not undergo any form of micromanipulation. Three methods of cryopreservation were compared: slow freezing in the presence of 10% glycerol, slow freezing in the presence of ethylene glycol, and vitrification in ethylene glycol.

Materials and Methods

In-Vitro Production

Ovaries from various breeds of adult cows were collected on the Central Valley Meats (Hanford, CA) plant floor and transported at 30-35°C to the laboratory within 4 hours. Ovaries were transported in an insulated thermos which contained palpations sleeves filled with sterile saline with antibiotics. Upon arrival at the laboratory, ovaries were washed thoroughly with sterile saline (38.5°C) to remove impurities and clotted blood. Oocytes were aspirated from visible follicles using an 18G needle and pump with OPU media and heparin (Partnar, Port Huron, MI). Aspirated oocytes were collected in flacon tubes and maintained at a temperature of 38.5°C.

In vitro maturation (IVM). Promptly after aspiration, oocytes were washed through emP₃ complete flush media several times and placed under mineral oil in M-CDM media (CDM plus 0.5% fatty acid-free BSA and hormones). Embryos were then incubated for 23 hours at 38.5°C, 5% CO₂, 5% O₂ and RH% 85.

In vitro fertilization (IVF). After IVM the plates containing the cumulus oocyte complexes (COCs) were removed from the incubator and placed on to warm plates set to 38.5°C. The COCs were washed through F-CDM (CDM + 0.5% heparin) and moved to warmed 4-well plates holding 450µl of F-CDM under mineral oil. While embryos were transferred to IVF plates, frozen sperm from Jersey and Holstein bulls was thawed and processed and extended so that a concentration of 1million cells/ml was used to fertilize each well of no more than 40 oocytes. Fertilized IVF plates were placed in to the incubator and for 16 hours at 38.5°C, 5% CO₂, 5% O₂ and RH% 85. Following incubation, presumptive zygotes (PZ) were removed from the

fertilization media and placed in snap tube filled with 70µl of vortex media (ViGro Holding Plus, BIONICHE, Pullman, WA). Tubes were placed on the vortex at full speed for 2 minutes. Fluid containing PZ was removed from the snap tube and pipetted onto a large petri dish. PZ were washed through 6-7 clean drops of embryo wash media (Minitube of America, Verona, WI), all non-viable PZ (empty zona pelucidas and atretic zygotes) were not picked up.

In vitro culture (IVC). After IVF, PZ are pipetted into of culture BBH7 media (BoviPure under oil. For each culture droplet, 1.5µl of culture media was used for each oocyte on 35mm plates. Oocytes were first washed through a drop containing embryo wash media on the culture plate prior to transfer into the culture droplet. These zygotes were then incubated at 38.5°C, 5% CO₂ , 5% O₂ and RH% 85 for 5 days, until embryos matured into day 7 expanded blastocysts.

Embryo Biopsy

Of the 126 blastocyst used in this study 54 were biopsied before cryopreservation, . Biopsy was performed by puncturing the ZP and removing a small amount of cells from the trophoderm of the blastocyst by micro-needle manipulation. The blastocoele cavity would reduce following biopsy. Non-biopsied cells were characterized by an intact ZP and no manipulation of inner cell mass.

Cryopreservation of Blastocyst

Three methods of cryopreservation were utilized in this experiment to preserve blastocysts. IVC cultured were examined and blastocysts were separated from unsuccessful and young zygotes. Groups of 4-13 blastocysts were then processed through appropriate cryoprotectants and loaded into straws.

Slow freezing in ethylene glycol (SF-EG). The first method of cryopreservation used was slow freezing in ethylene glycol (EG). Blastocysts were pipetted from the IVC plates into the first well of a 4-well plate containing 500µl of holding media (HM) (SynGRO Holding Media, BIONICHE, Pullman, WA, USA). Blastocysts were then moved to the second well containing 300µl of EG (SynGRO Ethylene Glycol with Sucrose, BIONICHE, Pullman, WA, USA). Within 10 minutes of transfer from HM to the EG, all blastocyst were washed through a second well of 300µl EG and loaded into plastic freezing straws in 100µl of EG. Straws were loaded to have an air column on either side of the embryo containing fluid column (Fig. 1) and sealed with a plastic plug. After 10 minutes from the initial transfer of blastocysts from HM to EG, straws were placed into freezing platform submerged in a liquid Nitrogen (LN₂) bath of the slow freezing machine and program was run. After 2 minutes of freezing, cooled forceps were used to initiate ice crystallization in the embryo-containing fluid column by application to the straw at the air column directly above it. Controlled-rate freezer is allowed to run, decreasing the temperature at a rate of -0.5 °C/min to -36.5°C. Straws a removed from the freezing platform and rapidly dunked into the LN₂ where they are transferred into canes and stored in LN₂ tanks until thaw.

Slow freezing in 10% glycerol (SF-10G). The second method used was slow freezing in 10% Glycerol. A similar protocol was used as in the first method SF-EG with the use of 10%

Glycerol (10G) (SynGRO 10% Glycerol, BIONICHE, Pullman, WA, USA) as a cryoprotectant in the place of EG.

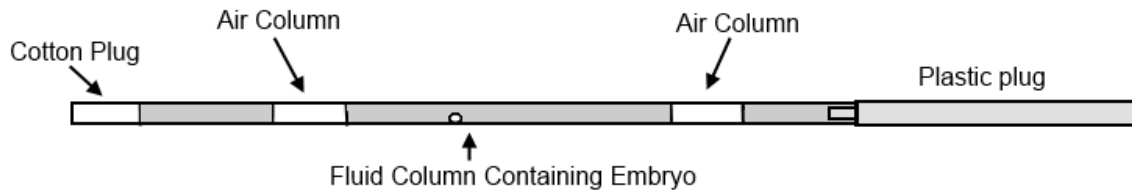


Figure 1. Diagram displaying the layout of freezing straw setup for both SF-EG and SF10G, center column of fluid contains the 100 μ l of cryoprotectant (EG or 10G) and an embryo (not to scale).

Vitrification in Ethylene Glycol (V-EG). Blastocyst for vitrification are processed differently than those for slow freezing. Blastocysts are transferred to the first well of a 4-well plate containing 500 μ l of embryo thaw holding media (M199 with HEPES, 20% FCS, 25 μ l/10ml gentamycin). Blastocysts were moved to the second well containing 500 μ l of 10% EG for 5 minutes. Blastocyst is then washed through a 20 μ l drop of 40% EG and 0.6 M galactose and deposited onto the plate into a 1 μ l drop. An Open Pull Straw (OPS) is used to pick up the embryo via capillary action. The OPS containing an embryo is then submerged vertically into LN₂.

Thawing of Blastocysts

A different thawing/warming procedure was used for SF-EG, SF-10G, and V-EG. For V-EG, tip of OPS is submerged directly into first well of a 4-well plate containing 500 μ l a solution of 1M galactose (W1) (ViGRO Thaw 1 plus, BIONICHE, Pullman, WA, USA). After 3 minutes, embryos are transferred to the second well containing 0.5M galactose (W2) (ViGRO Thaw 2 plus, BIONICHE, Pullman, WA, USA) and then the third well containing 0.25M galactose (W3) (ViGRO Thaw 3 plus, BIONICHE, Pullman, WA, USA) for 3 minutes each. Thawed embryos are final transferred into the fourth well containing 500 μ l of HM. SF-10G blastocysts were thawed in a very analogous manner.

For SF-10G straws are submerged into 30 $^{\circ}$ C water after allowing the straw to equilibrate in the air for 10 seconds. After 1 minute of the warm water bath, the plugs were removed and the straw content was unloaded into a clean petri dish and embryos were pipetted into the first well of the four well plate. Blastocysts were rotated through W1, W2, and W3 for 5 minutes each before transfer into HM. In contrast, SF-EG blastocysts were thawed using direct transfer. After straw had been submerged in 30 $^{\circ}$ C water as with SF-10G, contents were directly deposited in the holding media. Plates were then placed into the incubator at 38.5C, CO₂ 5.1 and RH% 85 to allow for re-expansion of the Blastocyst's cell mass.

Blastocyst Evaluation

Survival of a blastocyst was determined by the evidence of complete re-expansion of the blastocoel or hatching of the blastocyst within 24 hours post thaw. Survival was recorded as the number of blastocysts that exhibited re-expansion or hatching as compared to the total number frozen. Groups of biopsied and non-biopsied blastocysts were processed and evaluated separately. Because of the small sample size, Fisher's exact test was used to evaluate the differences between each group. A $P < 0.05$ was chosen as the alpha to indicate a statistical significance between data sets.

Results

There was a total of 124 blastocysts were evaluated and used in this experiment. The parental bull and cow breeds and ages were not specified.

Survival Rates of Biopsied vs. Non-Biopsied Blastocysts

Biopsied and non-biopsied embryo survival rates were evaluated as a total over all three methods of freezing. Survival rates were calculated as number of survived blastocysts as a percent of total frozen for each group (Table 1).

For each freezing procedure the biopsied blastocysts had a high rate of survival than the non-biopsied embryos (Fig. 2). SF-EG biopsied embryos ($66.7\% \pm 8.5$) survived at a greater rate than non-biopsied embryos ($61.5\% \pm 27.8$) however the percentage of surviving embryos was different by only 5.2%. SF-10G biopsied embryos ($88.9\% \pm 5.6$) similarly did better than non-biopsied embryos ($72.0\% \pm 5.5$) with a greater percentage of difference (16.9%) than that of SF-EG. Biopsied V-EG embryos ($77.8\% \pm 8.0$) survived as a much greater rate than non-biopsied ($58.8\% \pm 12.4$) with a difference of 19.0% (Table 1). As a total of all tests, biopsied oocytes ($77.8\% \pm 5.2$) survived at a greater rate than non-biopsied embryos ($63.9\% \pm 3.3$) but not significantly ($p=0.29$) (Fig. 3).

Table 1. Survival rates of biopsied and non-biopsied blastocysts.

Method	Biopsied		Non-Biopsied	
	Survival, N (%)	Total, N	Survival, N (%)	Total, N
SF-EG	12 (66.7)	18	8 (61.5)	13
SF-10G	16 (88.9)	18	18 (72.0)	25
V-EG	14 (77.8)	18	20 (58.8)	34
Total	42 (77.8)	54	46 (63.9)	72

Blastocyst survival rate was evaluated by the count, N, and a percent (%) of slow freezing in ethylene glycol (SF-EG), slow freezing in 10% glycerol (EG-10G), vitrification in ethylene glycol (V-EG) and as a total of all blastocysts frozen and thawed.

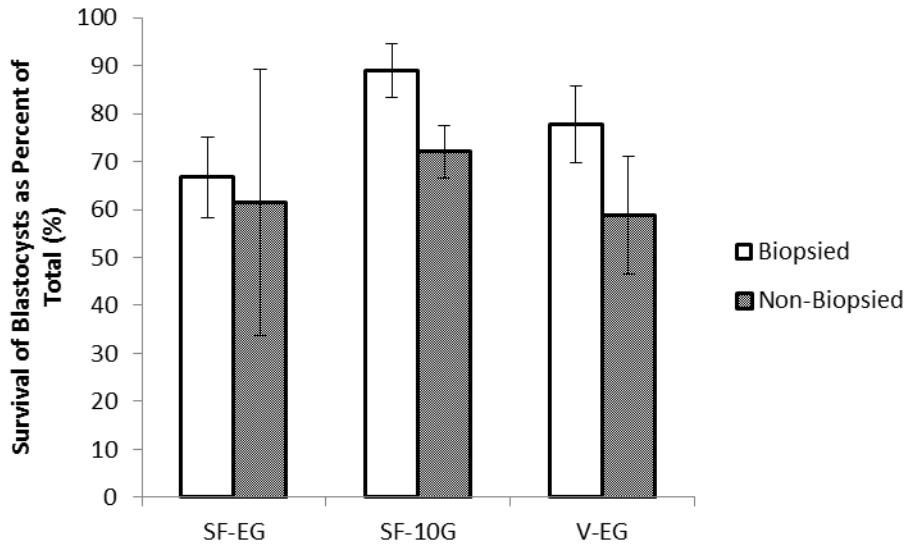


Figure 2. Average survival rate of embryos of embryos biopsied and non-biopsied as a percent of total embryos frozen by slow freezing in ethylene glycol (SF-EG), slow freezing in 10% glycerol (EG-10G), and vitrification in ethylene glycol (V-EG).

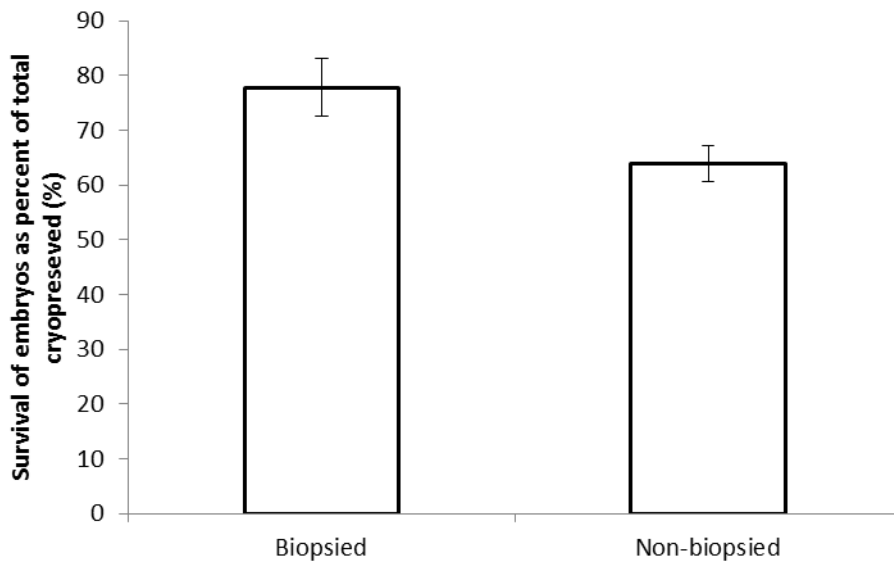


Figure 3. Average survival rate of biopsied and non-biopsied embryos as a percent of total embryos frozen by all three methods of cryopreservation (SF-EG, SF-10G, V-EG). Error bars represent the standard error of the mean.

Effects of Freezing Methods on the Survival Rates of Blastocysts

Fisher's exact test was used to determine whether or not the data was evidence of a significant difference between survival rates of the freezing methods. Each of the three methods was compared against the two other tests.

By just examining percentages, SF-10G had a greater rate of survival after thaw than both SF-EG (79.1>64.5%) and V-EG (79.1>65.4%) (Fig. 4). However, with such a small sample size, it cannot be assumed that SF-10G has a significantly greater freezing rate than either SF-EG or V-EG without performing Fisher's exact test.

Although more embryos survived using SF-10G (34/43, 79.1% \pm 3.3) than SF-EG (21/31, 64.5% \pm 9.0) the difference was not statistically significant (74, $p=0.1920$). The SF-10G survival rate was also greater than the V-EG survival rate (34/52, 65.4% \pm 7.8), similarly the difference was not great enough to be considered significant (95, $p=0.1735$). The survival rates of SF-EG and V-EG expressed the least amount of difference, a factor of 0.9%, which was not significant (83, $p=1.0000$) (Table 2).

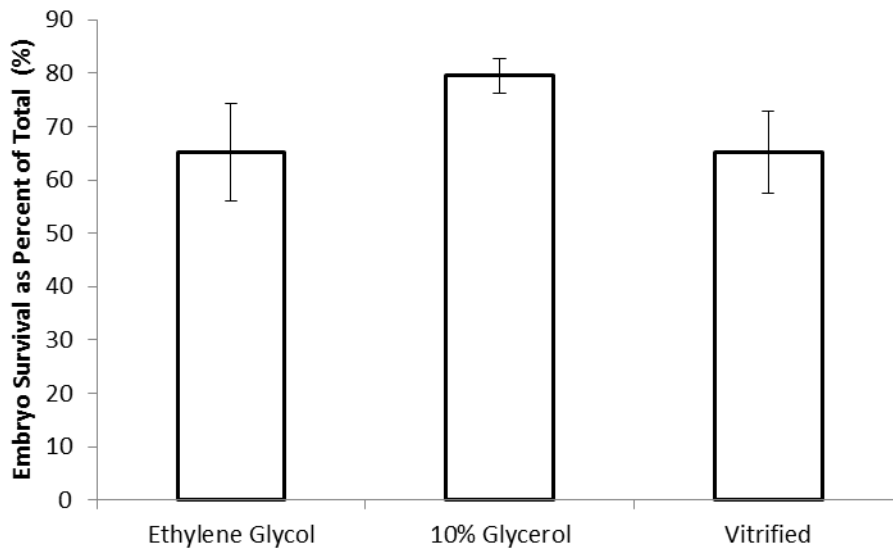


Figure 4. Average survival rate of embryos using slow freezing in ethylene glycol (SF-EG), slow freezing in 10% glycerol (EG-10G), and vitrification in ethylene glycol (V-EG) as freezing methods. Value of y-axis is percent of embryos that survived freezing out of total embryos frozen using the particular method. Error bars represent the standard error of the mean.

Table 2. Survival of blastocysts using different cryopreservation methods

	SF-EG, N (%)	SF-G10, N (%)	V-EG, N (%)
Survived	20 (64.5)	34 (79.1)	34 (65.4)
Total Cryo-Preserved	31	43	52

Blastocyst survival rate was evaluated by the count, N, and a percent (%) of slow freezing in ethylene glycol (SF-EG), slow freezing in 10% glycerol (EG-10G), and vitrification in ethylene glycol (V-EG).

Discussion

Mouse and human embryos have displayed an increase in vitrification survival rate when the blastocoele is artificial reduced [7,8]. In this experiment 54 of 126 blastocysts were biopsied. Piercing of the zona pelucida (PZ) causes the blastocyst to reduce in volume by the rapid movement of fluid out of the blasocoele.

Though the sample size was small, the results of this experiment are consistent with previous experiments. Biopsied blastocysts survived at a greater rate than non-biopsied embryos following vitrification (77.8>58.8%). Similarly, biopsied blastocysts that were cryopreserved using slow freezing in both EG and 10G had higher survival rates than non-biopsied (88.9>72.0%; 66.7>61.5%). The results suggest during cryopreservation and thawing, biopsied expanded blastocysts yield a greater survival rate than non-biopsied blastocysts (77.8>63.9%). These results are consistent with previous reports of bovine biopsied blastocysts that displayed both better hatching rates and a greater number of cells when blastocoele was punctured prior to vitrification [10]. This is most likely due to the increases distribution of cryoprotectant throughout the blastocysts as well as the reduction of water within the blastocoele to reduce chances of damage by ice crystal formation [8]. A punctured ZP may also lead to a reduced risk of toxicity by allowing cryoprotectant to flow out of cell more readily [7,8]. It is possible that hatching rate of blastocysts also increases due to the puncture site causing a weak point in the ZP. The same implication cannot be applied to all embryo stages, as it has previously been demonstrated that biopsy of morulas and early blastocysts does not increase their survival rate [3].

No significant difference in survival rate was found between SF-EG, SF-10G, and V-EG ($p>0.05$). Previous experimentation on bovine embryos has shown vitrification to be a superior process of cryopreservation over conventional slow freezing. Conflicting data can easily be attributed to different protocols used in slow freezing and vitrification used. Differences in freezing rate or in storage temperature have been known to have significant effects on blastocyst survival rate [11]. The small sample size of this report can also be attributed to the deviation of the results.

It should be noted that forskolin was added to the media at day five of IVP for 54 out of the 126 blastocysts used in this study. Forskolin is known to improve survival rates of cryopreserved bovine embryos without having deleterious effects on embryo development or quality [12]. However no difference between the survival rates of embryos cultured in the presence and absence forskolin was identified in the results. L-carnitine was also added to the culture media to help improve survival rates. Previous experimentation has demonstrated that culturing bovine embryos in the presence of L-carnitine improves the development, cryotolerance and the quality of IVP blastocyst [13].

In conclusion, biopsy of bovine expanded blastocysts produced *in vitro* prior to cryopreservation increases the post-thaw survival rate. Further experimentation with the inclusion of pregnancy rate would be needed to determine the true success of biopsied over non-biopsied embryos.

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