

Cryopreservation of bovine embryo by vitrification method

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Abstract

Cryopreservation of bovine embryos by vitrification solutions was developed at the beginning of 1990s for alternation of traditional slow-rate freezing. To apply this method for cryopreservation of bovine embryos in Vietnam, an experiment was conducted at the National Institute of Animal Husbandry. In this study, bovine embryos morulae and blastocyst stages were produced by in vitro fertilization of using oocytes collected by ovum pick-up technique from live cows. Vitrification was performed by equilibration of embryos in vitrification solutions 1 (VS1 contained 10% Glycerol, 0.1M Sucrose, 0.1M Xylose, 1% Poly Ethylene Glycol (w/v)) for 5 min, then in vitrification solutions 2 (VS2 contained 10% Glycerol, 10% Ethylene Glycol, 0.2M sucrose, 0.2M Xylose, 2% Poly Ethylene Glycol (w/v) for another 5 min) and finally in VS3 contained 20% Glycerol, 20% Ethylene Glycol, 0.3M sucrose, 0.3M Xylose, 3% Poly Ethylene Glycol (w/v). Embryos from VS3 were loaded in straws, placed into liquid nitrogen vapour and plunged into liquid nitrogen within 1 min. After a week of preservation in liquid nitrogen, those embryos were thawed and in vitro cultured in medium TCM-199 10% CS for 24h. Total of 160 morulae and blastocysts were frozen by vitrification method. After warming and culturing in vitro for 24h, the survival rate of vitrified embryos was 76.25% (122/160). This result shows that IVF bovine embryos were first frozen successfully by Vitrification method in Vietnam. Vitrification method is a simple and effective method to store morulae and blastocysts of bovine embryos.

Key words: *vitrification solutions, bovine embryo, thawing solution, culture solutions.*

1. Introduction

The ability to cryopreserve sperm and embryos has been applied successfully for more than a decade in over the world. The gene banks of male and female gametes and embryos established in many countries are commercial. Furthermore, in human medicine, pregnancy can be established even after menopause or cancer therapy and various genetic diseases can be screened the analysis of stored embryos before IVF-ET. Cryopreservation of bovine embryos by vitrification solution was applied in many laboratories in the world. Rall et al.[6] were the first to report the vitrification of 8-celled mouse embryos in 1985. Massip et al.[5] also, published the first report on successful vitrification of bovine embryos. Cryopreservation of embryos at the morula and blastocyst stage has become a widely procedure in several species. Bovine morulae and blastocysts have been vitrified successfully and following transfer given birth of normal offspring.(Lazar L et al.)[4]. In Vietnam, an experiment was conducted at National Institute of Animal Husbandry to apply this method for cryopreservation of bovine embryos. Although, in our country, there has been success in cryopreservation by steps wise and one step method, vitrification method for cryopreservation embryos has not been reported. Vitrification method does not induce intracellular ice crystal formation and reduces the damage to embryos. It also is simple, no use of expensive freezing machine, however, requires high skills. The aims of our study were to apply cryopreservation by vitrification method and help decrease the costs of bovine embryos transfer.

2. Materials and methods

2.1. Sources of bovine embryos

In this study, IVF bovine embryos were produced by using oocytes aspirated by ovum pick-up technique from live cows. The number of follicle, oocytes quantity and quality were evaluated. Cumulus-oocyte complexes(COCs) with intact, compact cumulus cells were selected and washed twice in maturation medium.

Groups of 20 selected COCs were transferred to 100 µl-IVM drops in a culture dish covered with mineral oil and cultured for 22-24 h at 38.5⁰C under 5 % CO₂ in air..

Frozen semen obtained from Hostein Frisian bulls were thawed in a water bath at 37⁰C. Spermatozoa were washed twice in BO medium (Brackett and Oliphant), by centrifugation at 1800 rpm for 5 min at 37⁰C each time. The sperm suspension was diluted with the same volume of BO medium supplemented with 20 mg BSA/ml, to give a sperm concentration of 10⁶ spermatozoa/ml. The suspensions were then pre-incubated in 100- μ l drops, following which about 20 oocytes that had been subjected to maturation culture were introduced into each drop. After 5h insemination, oocytes with cumulus cells were washed, denuded and cultured in 100 μ l of CR1aa medium. On day 7, IVF-embryos were collected. Morulae and blastocysts classified with grade A, B were used for freezing by vitrification method.

2.2. Equilibration, freezing and thawing of embryos and culturing in vitro for and 24h

The cryoprotectants used were Glycerol, Ethylene Glycol, Sucrose, Xylose, Polyethylene Glycol. The morulae and blastocyst embryos were freezed by Vitrification method. All steps were carried out at room temperature. Firstly, the good morulae and blastocysts were transferred into medium V1 with 10% Glycerol; 0,1M Sucrose; 0,1M Xylose and 1% Polyethylene Glycol for 5min. Subsequently, embryos were placed into medium V2 with 10% Glycerol; 10% Ethylene Glycol; 0.2M Sucrose; 0.2M Xylose and 2% Polyethylene Glycol for 5min. Finally, embryos were transferred into medium V3 with 20% Glycerol; 20% Ethylene Glycol; 0.3M Sucrose; 0.3M Xylose and 3% Polyethylene Glycol for 1 min (including the time of loading into straw, sealing and plunging into liquid nitrogen). After preservation for a week, frozen embryos were thawed and transferred to the following solutions: 0.5M Sucrose; 0.25M Sucrose and DPBS with 20% CS. Then, embryos were cultured in TCM-199 supplemented with 20% CS. After culturing in vitro for 24h the survival rate and the development to expanded blastocyst stages of embryos were observed.

3. Results

3.1 Evaluation of embryos before freezing and thawing

Table 1- Quality classification of embryos before freezing

Quality of embryos	Morulae		Blastocysts		Total	
	n	%	n	%	n	%
Grade A	65	67,01	56	69,13	121	69,97
Grade B	21	21,64	18	22,22	39	21,91
Grade C	11	11,34	7	8,84	18	10,11
Total	97	100	81	100	178	100

In total of 178 embryos, 160 embryos which were 121 embryos grade A (69,97%), 39 embryos grade B (21,91%) were used for freezing.

Evaluation of embryos after thawing and culturing in vitro 24h

After preservation for a week, frozen embryos were thawed and cultured in vitro and 24 h, the results of survival rate was shown in Table 2

Table 2- The results of survival and development of bovine embryos after thawing-culturing in vitro for 24 h.

No. of frozen embryos	No. of survival bovine embryos and their development after thawing-culturing in vitro							
	No. of survival embryos		No. of Blastocyst		No. of expanded blastocyst		No. of dead and degenerated embryos	
Total	n	%	n	%	n	%	n	%
	160	122	76.25	58	67.44	64	86.49	38

Vitrified embryos which passed through chilling progress were able to develop to blastocyst stages (from morulae) and to expanded blastocyst (from blastocyst) after culturing for 24h. The survival both morula and blastocyst rate was 76.25%. After freezing and thawing, however, there was 23.75% embryos observed degenerated or dead.

Discussion

Vitrification has been used for the cryopreservation of embryos. It has widely used and now becomes a potential alternative to traditional slow-cooling method. Vitrification has been used to cryopreserve the embryos of many mammalian species such as mice (Ishimori et al.,1992)[2], rabbits (Kobayashi et al., 1990)[3], pigs (Vajta et al.,1997)[8], sheeps (Schiewe et al.1991[7]) and cows (Dinnyes et al. 1994)[1]. Vitrification can be defined as a glass-like solidification. The vitrification process completely avoids ice crystal formation. There are some advantages of vitrification: (1) No ice crystallization; (2) Utilizes higher concentration of cryoprotectant that allows shorter exposure times to the vitrification solutions; (3) Rapid vitrification/warming; (4) Small volume used provides a significant increase in the cooling rate; (5) Minimizes osmotic injuries; (6) Reduces the time of the cryopreservation procedure (duration from 2 to 10 min); (7) Very simple protocols; (8) Eliminates the cost of expensive programmable freezing equipment.

The two most important parameters are the cooling rate and the concentration of the cryoprotectants. A practical limit to attainable cooling speed exists, as does a biological limit on the concentration of cryoprotectant tolerated by the cells during vitrification. Therefore, a balance between the maximization of cooling rate and the minimization of cryoprotectant concentration is very important. To achieve high cooling rates requires the use of high concentrations of the cryoprotectant solution, which depresses ice crystal formation. A critical concentration is required for vitrification. Concentration of cryoprotectants in vitrification solutions is one of important factors to affect vitrification efficiency. Considerable efforts were invested to decrease the toxicity of cryoprotectants by applying less toxic and more permeable chemicals, lowering total concentration of chemicals and using two or more cryoprotectants to decrease the specific toxic effect of each. In vitrified embryos, such damage probably results from osmotic shock during the steps of equilibration and dilution of the cryoprotectants.

Sucrose solution is generally used for dilution of post-warming embryos as an osmotic buffer to restrict water permeation and to prevent excessive swelling of the embryos during the removal of cryoprotectant from the cells. Survival of bovine embryos after cryopreservation by vitrification in this study was unaffected by the presence of sucrose in the diluting solution. Morulae and blastocysts were suitable to

cryopreseve embryos. Lazar et al.[5] reported that re-expansion and hatching rates of the blastocysts vitrified on 7 and 8+9 days were 74.6% and 62%, respectively. In this study, we vitrified embryos at morula and blastocyst stages. It helps embryos pass through damages during cryopreservation. The total number of 160 morulae and blastocysts thawed and cultured in vitro in 24h, there were 122 (76.25%) survival embryos. It is concluded that IVF bovine embryos were first freezed successfully by vitrification method in Vietnam. Vitrification method is simple and eassy to apply to store bovine embryos and other species.

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