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PREGNANCY RATE AND SURVIVAL IN CULTURE OF IN VITRO FERTILIZED BOVINE EMBRYOS FROZEN IN VARIOUS CRYOPROTECTANTS AND THAWED USING A ONE-STEP SYSTEM

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ABSTRACT

Bovine oocytes surrounded with compact cumulus cells were cultured for 20 to 22 hours (38.5°C, 5% CO_2) in modified TCM-199 medium supplemented with 5% superovulated cow serum (SCS) and inseminated by in vitro capacitated spermatozoa. Day 7 to 8 embryos were equilibrated for 10 minutes in 1.3 M methyl cellosolve (MC), 1.1 M diethylene glycol (DEG), 1.8 M ethylene glycol (EG), 1.6 M propylene glycol (PG) and 1.1 M 1, 3-butylene glycol (BG) solutions. They were then loaded into 0.25-ml straws, placed into an alcohol bath freezer at 0°C, cooled from 0°C to -6°C at -1°C/minute, seeded, held for 10 minutes, and cooled again at -0.3°C or -0.5°C/minute to -30°C. Straws were then plunged and stored in liquid nitrogen After thawing in 30°C water, the embryos were rehydrated in TCM-199 medium and then cultured for 48 hours in TCM-199 plus 5% SCS. Embryos were considered viable if they progressed to later developmental stages with good morphology. Some of the embryos frozen in each cryoprotectant were thawed and transferred nonsurgically without removing the cryoprotectant. Hatched embryos survived freezing and one-step dilution as follows: EG (50.0 %), MC (53.6%), DEG (56.9%), PG (58.0%) and BG (11.5%). The survival rate of embryos cooled at -0.3°C vs -0.5°C/minute was not significantly different($P>0.05$), however, blastocysts hatched most often ($P< 0.01$) in vitro when cooled at a rate of -0.3°C /minute (64.6%, 31/48) than at -0.5°C/minute (22.6%, 12/53). Pregnancy rates resulting from embryos frozen in the different cryoprotectants were as follows: MC (48%, 10/21); DEG (30%, 3/10); EG (74%, 20/27); and PG (40%, 4/10). These results indicate that MC, DEG, EG and PG have utility as cryoprotectants for the freezing and thawing of IVF bovine embryos.

Key words: cryoprotectants, IVF, bovine embryo, direct transfer

INTRODUCTION

A method of cryopreservation permitting direct transfer of bovine embryos to recipients after thawing would be a

valuable adjunct to commercial embryo transfer procedures, and would present advantages over conventional freezing procedures that require step - wise removal of the cryoprotectant after thawing. Interest in direct transfer systems for embryo marketing programs prompted additional investigation of the problem of rehydrating embryos after freezing and thawing. The primary problem addressed by previous methods of direct transfer is the lack of permeability of bovine embryos to common cryoprotectants. Identifying effective cryoprotectants to which bovine embryos are highly permeable is an alternative approach for developing direct transfer procedures. Several methods for direct transfer of bovine embryos have been previously descrived, and one of the techniques has been a one-step method (1-4). Other method has employed 1.5 M glycerol with 0.25 M sucrose as a cryoprotectant (5). In the above experiments, sucrose was used as an osmotic buffer to maintain osmotic equilibrium. However, these procedures have not been widely accepted within the embryo transfer industry because of the complexity of the process or failure to yield consistently acceptable results. Recently we reported (6) that 1.6 M propylene glycerol can be used effectively as a cryoprotectant for bovine embryos. It permitted thawed embryos to be rehydrated directly in holding medium. A pregnancy rate of 61% was obtained after direct transfer to recipients. Later, Voelkel and Hu (7, 8) reported a pregnancy rate of 50% after direct transfer of frozen-thawed bovine embryos using ethylene glycol. Permiability of sheep and cattle embryos to ethylene glycol and propylene glycol was greater than permiability to glycerol (9), suggesting that these agents may have value in a direct transfer procedure. However, little research on the various cryoprotectants of IVF bovine embryos has been reported. The following experiments were thus conducted to reduce or eliminate the problems caused by removing cryoprotectants from frozen bovine embryos derived by IVF.

The objectives of this study were 1) to determine the optimal cooling rate of -0.3°C vs -0.5°C/minute; 2) to define the optimal concentration of EG, MC, DEG and PG for freezing IVF bovine embryos; 3) to define the degree of damage to embryos by direct rehydration in holding medium after freezing and thawing in a variety of cryoprotectants; and 4) to compare the pregnancy rates following nonsurgical transfer of frozen - thawed bovine embryos without removal of the cryoprotectants.

MATERIALS AND METHODS

Embryos

Ovaries were obtained from a local slaughterhouse. Only oocytes surrounded with a compact cumulus were cultured for 20 to 22 hours (38.5°C, 5% CO_2) in modified TCM-199

supplemented per 100 ml with plucose (55 mg); Na-pyruvate(10 mg); 7.5% NaHCO (1.3 ml) and Insulin (0.5 mg) (10, 11). The medium was also supplemented with 5% superovulated cow serum (SCS) collected on Day 7 from superovulated donors which produced good - quality embryos according to criteria of previous reports (12-14). Frozen - thawed spermatozoa were centrifuged twice in Brackett and Oliphant (BO) media (15) containing 5 mM caffeine and 10 mg/ml heparin and were then incubated for 5 hours at 38.5°C in 5% CO_2 in air (16, 17). Oocytes were cultured for 8 days in TCM-199 supplemented with 5% SCS and Insulin (0.5 mg/ml). On Day 8, we achieved up to 40% blastocysts, for our experiment we use only good quality balstocysts.

Embryo Freezing

Cryoprotectant solutions were prepared in modified-PBS (M-PBS) containing 3 mg/ml (v/w) bovine serum albumin (BSA; fraction V; Sigma, St. Louis, MO, USA). Embryos were exposed to various cryoprotectant solutions at room temperature (25°C). After 10 to 20 minutes of equilibration, the embryos were loaded into 0.25 ml plastic straws. The straws were placed directly into a cooling chamber (NTB - 211, Tokyo-rikakikai, Tokyo, Japan) and kept for 2 minutes, and cooled from 0°C to -6°C at -1°C/minute, seeded, held for 10 minutes, and cooled again at -0.3°C or -0.5°C /minute to -30°C. The straws were then plunged and stored in liquid nitrogen.

In Vitro Viability Assessments

After storage in liquid nitrogen for 2 to 3 weeks, the cryopreserved straws were placed in air for 5 seconds and plunged into a 30°C water bath for 30 seconds for thawing, after which the contents were added to TCM-199 supplemented with 5% SCS and washed several times. Embryos were transferred to a cumulus cell layer in a culture dish containing TCM-199 supplemented with 5% SCS, 5 μg/ml insulin under paraffin oil. All cultures were incubated in a 5% CO_2 in air atmosphere at 38.5°C in an incubation chamber (Sanyo, Tokyo, Japan). After 24 and 48 hours of culture, the number of viable blastocysts and hatched blastocysts, respectively, were recorded. The embyros were considered viable if they progressed to later developmental stages with good morphology

Experiment 1

Blastocysts were placed directly into 1.8 M ethylene glycol (EG) in PBS supplemented with 3 mg/ml(v/w) BSA, 10% SCS and antibiotics to determine the optimal cooling rate of at -0.3°C or -0.5°C/minute after seeding.

Experiment 2

To define the optimal concentration of methyl cellosolve (MC), diethylene glycol (DEG), and EG for freezing bovine

embryos, blastocysts were placed directly into the final 3 concentrations of 1.0 M, 1.3 M, 1.5 M MC, 0.8 M, 1.1 M, 1.2 M DEG or 1.5 M, 1.8 M, 2.1 M EG in PBS supplemented with 3 mg/ml BSA, 10% SCS and antibiotics.

Experiment 3

Blastocysts were placed directly into 1.3 M MC, 1.1 M DEG, 1.8 M EG, 1.6 M PG or 1.1 M 1, 3-butylene glycol(BG) in PBS supplemented with 3 mg/ml BSA, 10% SCS and antibiotics, respectively, to define the degree of damage inflicted to embryos by direct rehydration in holding medium after freezing and thawing.

Experiment 4

Each of 10 to 20 IVF embryos were suspended directly in 1.3 M MC, 1.1 M DEG, 1.8 M EG, or 1.6 M PG in PBS supplemented with 10% SCS and antibiotics. After 10 minutes of equilibration, each embryo was loaded into a 0.25-ml plastic straw and then frozen. After 15 to 30 days of storage in liquid nitrogen, the straws were placed in air 5 seconds and plunged into a 30°C water for thawing. Nonsurgical transfers to synchronous recipients were performed without removing the cryoprotectant. All the freezing and thawing procedures in the experiment were performed by 3 technicians. The success rate of the embryo transfers was estimated by using ultrasonography at 30 and 60 days after transfer.

Statistical Analysis

A statistical evaluation of the differences between treatment groups was made by Chi-square test.

RESULTS

Experiment 1

The effects of blastocyst suspension in 1.8 M EG followed by cooling at -0.3°C and -0.5°C / minute are summarized (Table 1). The survival rate of embryos cooled at -0.3°C vs -0.5°C/minute was not significantly different ($P>0.05$); However, blastocysts hatched more often ($P<0.01$) when cooled at a rate of -0.3°C/minute(64.6%, 31/48) with compared to -0.5°C/minute (22.6%, 12/53).

Experiment 2

As shown in Table 2, the concentration of 1.8 M EG (56.0%), 1.3 M MC (55.0%) or 1.1 M DEG (60%) were superior to other concentrations of like cryoprotectants for preserving hatched embryos subjected to freezing and direct rehydration.

Table 1. Comparison of developmental rates of frozen – thawed IVF embryos cooled at −0.3°C or −0.5°C/minute in ethylene glycol

Cooling rate	No. cultured	No. surviving (%)	Developmental stage(%) Hatched blastocyst	Expanding blastocyst	Blastocyst
0.3°C/ minute	48	46(95.8)	31(64.6)[a]	6(12.5)[a]	9(18.8)[a]
0.5°C/ minute	53	48(90.6)	12(22.6)[b]	13(24.5)[b]	23(43.4)[b]

[a,b] Means with different superscripts in the same column are significantly different(P<0.01).

Table 2. Comparison of developmental rates of frozen–thawed IVF embryos in the various cryoprotectants

Concentration of cryoprotectants	No. cultured	No. surviving(%)	No. of hatched blastocyts(%)
1.0 M methyl cellosolve	15	13(87)	4(27)[b]
1.3 M methyl cellosolve	23	22(96)	12(55)[a]
1.5 M methyl cellosolve	10	10(100)	4(40)[a]
0.8 M diethylene glycol	14	9(64)	4(29)[b]
1.1 M diethylene glycol	18	15(83)	9(60)[a]
1.2 M diethylene glycol	16	13(81)	5(31)[b]
1.5 M ethylene glycol	14	10(70)	6(43)[a]
1.8 M ethylene glycol	16	14(88)	9(56)[a]
2.1 M ethylene glycol	14	9(64)	5(36)[b]

[a, b] Means with different superscripts are different(P<0.05).

Table 3. Comparison of survival rates of frozen–thawed bovine embryos in vitro using various cryoprotectants

Cryoprotectants	No. cultured	No. surviving(%)	No. of hatched blastocyts(%)
1.3 M methyl cellosolve	64(92.8)[a]	37(53.6)[c]	
1,1 M diethylene glycol	51	47(92.2)[a]	29(56.9)[c]
1.8 M ethylene glycol	88	79(89.8)[a]	44(50.0)[c]
1.6 M propylene glycol	50	43(86.0)[a]	29(58.0)[c]
1.1 M butylene glycol	52	34(65.4)[b]	6(11.5)[d]

[a, b,c, d] Values within columuns with different superscripts are significantly different(P<0.05);(P<0.01).

Table 4. Pregnancy rates after nonsurgical transfer of frozen-thawed bovine embryos that were suspended in 4 different cryoprotectants

Cryoprotectant	No. of recipients transferred	No. of pregnancies(%)
1.3 M methyl cellosolve	21	10(48)[a]
1.1 M diethylene glycol	10	3(30)[a]
1.8 M ethylene glycol	27	20(74)[b]
1.6 M propylene glycol	10	4(40)[a]

[a, b] Values within columuns with different superscripts are significantly different(P<0.05).

Experiment 3

Viability (Table 3) of bovine embryos froze in MC, DEG, PG, or EG and rehydrated directly in the holding medium was not significantly different (64/69, 92.8%; 47/51, 92.2%; 43/50, 86%; and 79/88, 89.8%, respectively). All were better ($P<0.05$) than those froze in BG (65.4%). Hatched blastocyst formation was also not significantly different in MC, DEG, PG or EG (37/69, 53.6%; 29/51, 56.9%; 29/50, 58.0%; and 44/88, 50.0%, respectively), but it was poorest ($P<0.05$) in BG (11.5%).

Experiment 4

Pregnancy rates (Table 4) resulting from embryos frozen in different cryoprotectants were as follows: MC(48%, 10/21); DEG(30%, 3/10); EG(74% 20/27); PG(40%, 4/10).

DISCUSSION

The survival rate of embryos cooled at −0.3°C vs −0.5°C/ minute was not significantly different. However, blastocysts hatched most often in vitro when cooled at a rate of −0.3°C/ minute than at −0.5°C/minute. These results indicate that adjustments in the freezing protocol such as seeding temperature, cooling rate, and/or plunge temperature might optimize conditions for using 1.8 M ethylene glycol. Embryos frozen in 1.3 M methyl cellosolve, 1.1 M diethylene glycol, 1.6 M propylene glycol and 1.8 M ethylene glycol tolerated direct rehydration into holding medium in a one-step system, and there was not a significant difference in the survival rate among them. This might be due in part to the high degree of permeability which bovine embryos had to these cryoprotectants. Szell et al.(9) have shown that bovine embryos were more permeable to ethylene glycol than to glycerol. Renard et al. (18) have used propylene glycol effectively for cryopreservation of bovine embryos at a concentration of 2.0 M with sucrose mediated rehydration and for freezing mouse embryos with direct rehydration (19). However, the viability of murine embryos is greater when frozen in ethylene glycol than in propylene glycol (20). It is not clear why other authors were not able to use propylene glycol successfully for freezing and direct rehydration of bovine embryos (6). In the report by Voelkel and Hu (7, 8) propylene glycol was used at a concentration of 1.5 M, ice crystals were seeded at −7°C, the cooling rate was −0.5°C/ minute, and a cooler plunge temperature (−35°C) was used. These differences may have contributed to the variation in results. To our knowledge comparative data for use of methyl cellosolve and diethylene glycol as a cryoprotectant for mammalian embryos have not yet been reported. Embryos were frozen in each cryoprotectant and packaged in 0.25-ml straws containing a central column of each cryoprotectant, with the embryo and a column at each end of the straw containing the holding medium. Embryo frozen in each

cryoprotectant were transferred directly to recipients. When data were pooled across individuals trials, the pregnancy rate of bovine embryos frozen in 1.8 M ethylene glycol (74%, 20/27) was significantly higher ($P<0.05$) than those were 1.3 M methyl cellosolve (48%, 10/21), 1.1 M diethylene glycol (30%, 3/10) and 1.6 M propylene glycol (40%, 4/10), respectively. Therefore, 1.8 M ethylene glycol has utility as a cryoprotectant for the direct rehydration of thawed bovine embryos. Although methyl cellosolve and diethylene glycol produced similar results, our study has shown that 1.8 M ethylene glycol was a more effective cryoprotectant for improving the pregnancy than the others studied. We suggest that 1.8 M ethylene glycol may reduce the toxic effects of cryopreservation in vivo. Ethylene glycol may have potential in direct transfer procedures, in which rehydration occurs in the reproductive tract of the recipient. Previous efforts to develop direct transfer procedures have focused on circumventing the relatively low permeability of bovine embryos to glycerol. It is apparent from the findings reported here that identifying cryoprotectants to which bovine embryos are highly permeable, is another viable strategy for developing direct transfer methods. In this regard, methyl cellosolve and diethylene glycol also appear to be very promising. However, transferable embryos were produced from frozen-thawed bovine oocytes using a low concentration of sucrose and propylene glycol (21). This indicates that such a low concentration of sucrose or trehalose may be necessary for the protection of frozen embryos, even if highly permeable cryoprotectants are employed. Therefore, further refinement and testing of these techniques will be necessary to establish confidence in the direct transfer system.

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