CRYOPRESERVATION OF BOVINE IVF EMBRYOS USING EGG YOLK IN THE FREEZING MEDIUM

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ABSTRACT

The study was aimed at developing a freezing protocol for in vitro produced bovine blastocysts using different concentrations of egg yolk (EY) in the freezing medium. In experiment I, the survival rate of embryos in the medium-C, (72%) containing 20% EY was significantly higher ($p<0.01$) than in medium-A, (34.6%) (no contents of EY and polyvinylpyrrolidone (PVP). There was no difference between media C and B (60.7%) (containing 5% PVP), and also between media B and A. This indicate that egg yolk contributed to protection of the blastocyst at this stage. In experiment II, Post-thaw survival rates were 14/29 (48.2%), 27/38 (71.1%), 18/25 (72.0%), 13/34 (38.2%) of 5, 10, 20 and 30% EY respectively. Survival rates in EY 10 and 20% were significantly higher ($p<0.01$) than that of 30%. There was no difference ($p>0.01$) between 5, 10 and 20% EY, neither between 5 and 30% EY . With regard to the hatching , the 5, 10, 20 and 30% EY resulted in 4/14 (28.6%) , 19/27 (70.4%) , 11/18 (61.1%) and 4/13 (30.8%) respectively. Both 10 and 20% EY were significantly higher ($p<0.01$) than 5 and 30%. These results indicated that use of 10 or 20% egg yolk in freezing media resulted in an optimal post-thaw survival and hatching of bovine blastocysts.

INTRODUCTION

Long term preservation of mammalian embryos can be accomplished by freezing and storage at ultra low temperatures where cellular functions are reduced greatly or suspended (Fiser and Macpherson, 1977). The hypothesis that temperature reduction itself is one of the stresses caused by freezing has been partly confirmed in plants and bacteria (Linden *et al.*, 1973; Lyons, 1973). This stress may cause a physical state change in membrane lipids, such as phase transition or lateral phase transition or lateral phase separation (Qiunn, 1985). Various cryoprotectants have been used for embryo freezing such as glycerol, polyvinylpyrrolidone (PVP), sucrose, dimethyl sulfoxide, dimethyl glycol, propylene glycol, ethylene glycol, monomethyl ether, with various methods (Scneider and Mazur, 1984; Massip *et al.*, 1993; Takagi *et al.*, 1993; Suzuki *et al.*, 1995).

The remarkable cryoprotective properties of EY have been known for some time. The discovery that egg yolk has a beneficial effect on fertility in semen extenders (Phillips and Lardy, 1940) led to its widespread use in bull semen extenders. So for, Egg yolk (EY) has been extensively applied in mammalian semen extenders for protecting spermatozoa against chilling and freezing injuries (Martin, 1963; Mayer & Lasely, 1945; Watson & Martin, 1975; Wilmut & Polge, 1977). To our knowledge, freezing of bovine embryos using egg yolk in the freezing media has not been made previously. Therefore, the present study was conducted to determine the influence of egg yolk on freezing of bovine IVF embryos.

MATERIALS AND METHODS

Oocyte Recovery and Maturation

Bovine ovaries (Friesian, Japanese black, Japanese brown) were obtained from the slaughterhouse and were brought to the laboratory within 3 hour of collection in phsiological saline (0.9% (w/v) NaCl) supplemented with penicillin-G (100 IU/ml) and streptomycin sulfate (0.2 mg/ml) at 30 to 32°C. Cumulus-Oocyte Complexes (COCs) were aspirated from small antral follicles (2 to 5 mm in diameter) with an 18-G needle attached to a 5ml syringe in modified-PBS (m-PBS, Embryotec, Nihon Zenyaku Co., Fukushima, Japan) supplemented with 50 ul/ml Gentamycin Sulfate (Sigma Chemical Co., St.

Louis MO., USA). After collection, COCs were rinsed once with m-PBS and three times with maturation medium (TCM-199, Earle`s salt; Gibco, Grand Island, NY, USA) supplemented with 5% Day-7 superovulated cow serum (SCS; Matsuoka *et al.*, 1992), 0.01 mg/ml follicle stimulating hormone (FSH; Denka Pharmaceutical Co., Kawasaki, Japan), and 50 ug/ml Gentamycin (Sigma Chemical Co., St. Louis MO, USA) in culture dish (35 mm diameter, Falcon, Bacton Dikinson Co., Ltd. Oxnard, CA, USA). Thereafter, the selected COCs with an intact cytoplasm and surrounded by unexpanded cummulus cells over more than one-third of their surface were placed into the maturation medium covered with mineral oil (Squibb & Sons, Inc. Princeton, NJ USA) and cultured for 21-22h at 38.5°C in 5% CO_2 in air.

***In vitro* fertilization (IVF)**

Samples of frozen semen from a Chimera bull (Boediono *et al.*, 1993) were thawed in a water-bath (37°C), washed 2 times using 2.5 mM caffeine in Brackett and Oliphant`s medium (Caff-BO, Brackett and Oliphant, 1975) by centrifugation at 500 g for 5 min. The sperm pellet was resuspended in Caff-BO supplemented with 1% bovine serum albumin (BSA, Initial Fractionation by Cold Alcohol Precipitation, Sigma, USA) and 20mg/ml heparin (Shimizu Pharmaceutical Co. Ltd. Shimizu, Japan) to yield a final sperm concentration of $5x10^6$/ml. A 100 ul aliquot of sperm suspension was overlaid with mineral oil and incubated for 1 hour at 38.5°C in 5% Co_2 in air prior to insemination. Oocytes matured in vitro were then transferred into sperm droplets (30 to 50 oocytes/micro drop) for insemination.

***In vitro* culture (IVC)**

After a sperm-oocyte incubation of five hours, the oocytes with cumulus cells were removed from the fertilization medium, washed 2-3 times by repeated pipetting and transferred into a polystyrene dish (4-well multidish, Nunclon, Roskilde, Denmark) containing culture medium (TCM-199) supplemented with 5% SCS, 5 μg/ml insulin (Wako Pure Chemical Industries. Ltd. Osaka, Japan) and 50ul/ml Gentamycin for further development. After 48 hours of culture, the adherent cumulus cells surrounding the embryos were removed by repeated pipetting, while the monolayer of cumulus cells attached to the bottom of the culture dish was not disrupted and used as co-culture. The culture medium was replaced with new medium every 96 h of the culture.

Freezing thawing and culture

Morphologically normal blastocysts and expanded blastocysts which had developed to that stage on days 7, 8 and 9 following in vitro fertilization were used for freezing. Embryos were suspended in Dulbecco`s phosphate buffered saline (D-PBS; Gibco, NY, USA) supplemented with 10% BSA and 1.8 M ethylene glycol (EG; Wako Pure Chemical, Osaka, Japan), PVP (30%) 5% and 5, 10, 20 or 30% egg yolk. Trehalose, a carbohydrate, was added in all the cryoprotectant solutions in order to prevent the osmotic shock. The cryoprotectants were added at room temperature (20 to 25 °C). After 10 to 20 min. equilibration, the embryos were loaded into 0.25 ml plastic straws and Placed directly into a 0°C alcohol bath chamber (ET-1 program Freezer FHK, Fujihara Industries Co., Ltd. Tokyo, Japan). Then the embryos were cooled from 0°C to - 7°C at a rate of 1°C/min. and seeded at -7°C. After seeding, the straws were held for 10 min. at -7°C, and then cooled at a rate of 0.3°C/min. to -30°C. Finally, the straws was plunged and stored in Liquid Nitrogen (LN2) for one week to one month.

Embryos were thawed by placing the staws in a 30-32°C water bath, and the contents drained into a sterile petri dish. The embryos were then placed into the culture medium for rehydration and washed 3 times. Embryos were cultured on feeder layer of bovine cumulus cells in TCM-199 supplemented with 5% SCS and 5μg/ml insulin at 38.5 °C in 5% CO_2 in air. Embryos were evaluated microscopically at 24 and 48 hours interval. Survival rates were assessed at 48 h culture, by re-appearance of the blastocoele and/or attainment of a fully expanded stage (including the hatching and hatched blastocyst).

A total of 245 blastocysts (day 7, 8 and 9) were used for freezing in two different experiments. In experiment I, the medium containing 20% egg yolk (medium-C) was compared to medium-A, containing no PVP and no egg yolk, and medium-B containing 5% PVP and no egg yolk (Table 1). In experiment II, affect of various levels of egg yolk on the survival and hatchability of blastocysts was seen. Experiment I was repeated four times and experiment II was repeated three times. The data were subjected to x2 (Chi-square) analysis.

RESULTS

The results of experiment I (Table 2) indicated that egg yolk could protect the blastocysts against freezing shock. The results of experiment II are shown in Table 3.

Post-thaw survival in 10 and 20% EY was significantly higher ($p<0.01$) than that in 30% EY. Hatchability of surviving embryos was not different ($P>0.01$) between 10 and 20% EY. Hatchability in 10 and 20% EY was significantly higher ($P<0.01$) than in 5 and 30% EY. These results indicated that very low or very high percentages of egg yolk resulted in detorious effects on post thaw survival and hatching of IVF embryos.

DISCUSSION

Egg yolk supplementation improved to a limited extent the viability of porcine expanded and hatched blastocysts exposed to 0°C for 10 min. (Fujino *et al.*, 1993). Our results also show that EY can protect the blastocyst from some stresses due to rapid temperature reduction from room temperature (20 - 25°C) to 0°C upto -196°C, when supplemented in PBS + BSA + EG + trehalose. When embryos are put into the cryoprotectant medium, substantial amount of ethylene glycol penetrates into the embryonic cells, while at the same time trehalose helps to dehydrate the embryonic cells, which is a process for successful cryopreservation (Saha *et al.*, 1994). Egg yolk is a large, interface-seeking molecule, and it is suggested that EY coats the cells immediately following thawing giving them mechanical protection against cell-lysis due to the osmotic stresses. The EY component has sufficient buffering capacity to return the pH to neutral. The precise mechanism by which EY protects spermatozoa subjected to cold shock is, however, largely unknown, but three possibilities should be considered: (i) chelation of Ca ion (ii) modification of lipid fluidity and hence phase separation events, and (iii) stabilization of protein elements in the membrane lipid matrix (Watson and Martin, 1975). The protection by large molecular weight substances, such as EY, suggests that these substances act the external cell surface, implying that the primary damage induced by cold shock occurs in the plasma membrane.

Foulkes (1977) reported that one egg lipoprotein fraction was particularly effective in preventing injury to bovine spermatozoa during dilution and freezing. Egg yolk lipoproteins are the critical components for the beneficial effects, with a synergistic effect due to the TES-Tris buffer component (Jeyendran *et al.*, 1995). A 2-hour incubation of sperm samples with TEST-yolk buffer containing medium enhances the binding capacity of human spermatozoa in approximately 20% of the patients considered to have male factor infertility (Gamzu *et al.*, 1994). The variations in the survival rates noted may be attributed to differences in the breeds (Japanese black, J. brown, Holstein) used in the two studies. Differences between EY and PVP media in the post-thaw survival were not significant. The optimal protection from the freezing shock was observed at concentrations of 10 or 20% egg yolk. However, the use of 5 and 30 % EY depressed the post-thaw survival and subsequent hatching. The viability of blastocysts frozen in EY-medium remains to be tested in the field by transfer of embryos to recipient cows.

Table1. Composition of three different freezing media.

Ingredients	Medium		
	A	B	C
Bovine Serum Albumin	+	+	+
Trehalose	+	+	+
m-PBS	+	+	+
Ethylene-Glycol	+	+	+
Polyvinylpyrrolidone	-	+	-
Egg Yolk (20%)	-	-	+

Table 2: Comparison of the post-thaw survival rate of the IVF-produced blastocysts among the different media.

Media	No. of blastocyst treated	No. of Survival (%)
A	26	9/26 (34.6)b
B	28	17/28 (60.7)ab
C	25	18/25 (72.0)a
Control	40	40/40 (100.0)a

Values within columns with different superscripts are significantly different ($P<0.01$)

Table 3: Effect of egg yolk percentage in freezing medium on the post-thaw survival and hatching rate of IVF- produced blastocysts.

Egg yolk	No of blastocyst	Survival (%)	Hatched (%)
5	29	14/29 (48.2)ab	4/14 (28.6)b
10	38	27/38 (71.1)a	19/27 (70.4)a
20	25	18/25 (72.0)a	11/18 (61.1)a
30	34	13/34 (38.2)b	4/13 (30.8)b

Values within columns with different superscripts are significantly different ($P<0.01$)

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