Vitrification of in vitro-produced bovine embryos matured in modified TCM-199 medium

Asiye İzem SANDAL*, Özen Banu ÖZDAŞ

Department of Reproduction and Artificial Insemination, Faculty of Veterinary Medicine,
İstanbul University, Avcılar, İstanbul, Turkey

Abstract: In vitro-produced bovine embryos matured in modified Tissue Culture Medium 199 (TCM-199) were vitrified at the 7th and 8th days of culture, and development at 48 h after warming was recorded. Ovaries were obtained from a local abattoir, and the oocytes were recovered by slicing method and divided into two main groups. The first group included cysteamine in the TCM-199 (Group A) and the second did not (Group B). They were incubated for 24 h at 38.8 °C in an atmosphere of 5% CO_2 in humidified air. Matured oocytes were fertilized with $1-2 \times 10^6$/mL of frozen-thawed bull semen. At the end of the period the embryos were divided into two subgroups in order to be developed. Therefore, four groups were established: Group AI-SOF (synthetic oviduct fluid), Group AII-CR1aa (Charles Rosencrans), Group BI-SOF, and Group BII-CR1aa. The rates of blastocyst development and expanded blastocysts were detected as 20%, 18.1%, 24.6%, and 22.5% and survival rate as 5.5%, 23.6%, 0%, and 5.2%, respectively, showing statistically significant superiority in Group AII-CR1aa at the level of P < 0.05. In conclusion, supplementing cysteamine into maturation media with subsequent culture in AII-CR1aa had a positive effect on blastocyst survival after vitrification.

Key words: Bovine, embryo, cysteamine, maturation, vitrification

1. Introduction

Cryoprotectant and antioxidant substances have often been supplemented into media for in vitro production and vitrification of bovine embryos in the last 10 years (1–3). It has been established that oxygen pressure in the oviduct and uterus is lower than atmospheric oxygen pressure (4). In vitro embryo production of mammals under atmospheric oxygen pressure is being used routinely, but during embryo culture this high pressure leads to the formation of reactive oxygen species (ROS) (5,6). Harmful effects of ROS are DNA damage, lipid peroxidation, oxidative modifications of proteins, and spermatozoon–oocyte fusion inhibition (7). As well as the known negative effects of ROS, in some circumstances cell apoptosis is another important physiological factor (5,8). One of the most important endogen sources of ROS is oxidative phosphorylation. Inhibition of oxidative phosphorylation decreasing ROS has a positive effect on in vitro embryo development (9). The most important factor that leads to an increase in the formation of ROS is the exogenous oxygen pressure. Oxygen pressure in the oviduct is only one-fourth of atmospheric oxygen pressure. In in vitro-produced bovine embryos under low oxygen tension (5%–

7%) it has been reported to increase resistance to freezing and the nonprotein structure of the synthesis of one of the sulfhydryl compounds, glutathione (GSH) (10,11).

Antioxidants such as β-mercaptoethanol, cysteamine, cystine, cysteine, N-acetyl-L-cysteine (NAC), and superoxide dismutase (SOD) are used frequently in order to protect in vitro-produced bovine embryos against oxidative stress (2,12). It is known that antioxidants have positive effects on embryo development, but some researchers suggest that these positive effects can only be effective under certain conditions (8,13). Studies revealed that positive effects of antioxidants can occur under 20% oxygen tension (13). Various oxygen pressures have been tested in different culture or maturation conditions by researchers in in vitro production of bovine embryos. For example, oviductal or granulosa cells used in coculture environments of 20% O_2 pressure, or non-coculture that does not contain somatic cells in environments of 5% oxygen pressure, were found to increase results (8,14). There are some critical points during oocyte or embryo freezing with vitrification. The first is the freezing temperature; by using liquid nitrogen, a freezing temperature of –196 °C can be achieved. The second and the most important point

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* Correspondence: izem@istanbul.edu.tr

for vitrification is the amount of freezing medium. Smaller volumes of medium increase the success of vitrification due to toxic effects on the embryo by cryoprotectants. It has been concluded that decreasing the vitrification volume minimizes the probability of glass fracture (15,16).

Cryoprotectants such as glycerol, ethylene glycol, propylene glycol, and DMSO act by passing through the cell membrane (17). Glucose, sucrose, fructose, and trehalose are known as extracellular cryoprotectants and they show their effects by increasing the surface area with the interaction of phospholipids that can cause membrane damage during freezing and warming (18,19). Frozen and thawed embryo survival and pregnancy rates after embryo transfer with vitrification methods are more commonly recommended by researchers (20,21). Although a limited number of studies have been conducted on cysteamine supplemented into oocyte maturation medium with subsequent culture into SOF medium (22) and vitrification, according to the current literature there is no research that we can compare with CR1aa medium (23). Therefore, in this study, two different culture media, SOF and CR1aa, were used.

2. Materials and methods

2.1. Oocyte recovery and selection

Eleven replicas of 329 ovaries were obtained and 2710 bovine oocytes were collected; from these oocytes, 2357 were used for maturation (86.97%). Bovine ovaries of Holstein cows were obtained from a local abattoir and transported to the laboratory in phosphate-buffered saline (PBS) supplemented with penicillin (250 IU/mL), streptomycin (125 μg/mL), and neomycin (125 μg/mL) at 30–35 °C within 2–3 h of slaughter (24). The cumulus oocyte complexes (COCs) were recovered by slicing antral follicles at 2–8 mm in diameter (25). COCs were assessed morphologically and only those that had compact three or more complete layers of cumulus cells and fully grown oocytes with homogeneous cytoplasm were selected for maturation (26).

2.2. In vitro maturation (IVM)

Selected oocytes were washed three times in HEPES-buffered tissue culture medium (TCM-199), washed once in IVM medium, and placed in petri dishes that contained 700 μL of the same medium. Oocytes were divided into two groups. Cysteamine was added to the first group of oocytes in TCM-199 medium (Group A) while in the second group it did not contain cysteamine (Group B), and they were incubated for 24 h at 38.8 °C in an atmosphere of 5% CO_2 in humidified air.

2.3. In vitro fertilization (IVF)

After maturation the oocytes that had expanded cumulus oophorus cells were accepted as matured and they were transferred into IVF-TALP medium (14,27) that was

recently incubated in a gas environment for Groups A and B. For fertilization, two 0.25-mL straws of Holstein bull sperm were thawed. Spermatozoa were centrifuged in Sperm-TALP medium at $1500 \times g$ for 5 min twice and were counted in Thoma preparation to $1-2 \times 10^6$/mL for fertilization. They were then incubated for 18–24 h for fertilization at 38.8 °C in 5% CO_2.

2.4. In vitro culture (IVC)

Matured bovine oocytes in TCM-199 medium with or without cysteamine were tested in SOF and CR1aa medium (22,23). For this reason both groups were divided into two subgroups and a total of 4 culture groups were formed: Group AI-SOF, Group AII-CR1aa, Group BI-SOF, and Group BII-CR1aa. The presumptive zygotes were vortexed in tubes containing HEPES-buffered TCM-199 to remove the surrounding cumulus cells and/or spermatozoa and cultured in droplets of 100 μL (20–30 eggs/drop) containing SOF and CR1aa media with 5% FCS added at 38.8 °C in a humidified atmosphere of 5% CO_2 and 5% O_2 for 7–9 days.

2.5. Cleavage controls

Cleavage was assessed on day 2 of IVC. At the end of 48 h cleaved embryos were transferred to petri dishes containing fresh culture medium droplets according to their groups for separation from the uncleaved embryos.

2.6. Vitrification, warming, and culture

Before vitrification, embryos were rinsed with PBS supplemented with 20% FCS. Embryos were vitrified in straws (19). Briefly, blastocysts and expanded blastocysts were initially incubated in 1.5 M ethylene glycol (EG) and 1 M dimethyl sulfoxide (DMSO) in a holding medium (VS1; PBS supplemented with 20% FCS) for 3 min. Embryos were then transferred to 2.5 M EG, 2 M DMSO, and 0.5 M sucrose vitrification medium (VS2; PBS supplemented with 20% FCS) for 30 s. Embryos (three or five per straw) were loaded by placing the middle of the straw into the droplet. The straws were then immediately submerged into liquid nitrogen and subsequently stored there.

Warming was performed by placing the middle of the straws directly into holding medium containing 1 M sucrose (PBS supplemented with 20% FCS) for 3 min. Embryos were then transferred into medium containing 0.5 M sucrose for 1 min. After warming, embryos were rinsed with HEPES-buffered TCM-199 and cultured in SOF and CR1aa media supplemented with 5% FCS at 38.8 °C in a humidified atmosphere of 5% CO_2 and 5% O_2 for 48 h to assess postcryopreservation survival. It was defined as the reexpansion of blastocysts (28).

2.7. Statistical analysis

The chi-square statistical analysis method was used for the results obtained in this study and $P < 0.05$ was considered significant.

3. Results
The cleavage rates were 47.9% (Group AI-SOF), 50.1% (Group AII-CR1aa), 52.2% (Group BI-SOF), and 49.4% (Group BII-CR1aa), respectively, and no statistical significance was detected between the groups (Table 1).

From embryos that were matured with or without cysteamine in the TCM-199 medium at the end of their culture in SOF or CR1aa medium, 154 blastocysts and 98 expanded blastocysts were obtained, totally 252 embryos (Table 1). In Group A1, the rate of blastocyst stage was 10.8%, while in B1 it was 15.9%; there was a difference detected in percentages, although it was not statistically significant. In Group AII the blastocyst rate was 9.2%, while in Group BII it was 16.1% and no statistical significance was detected (P < 0.05) between them. Vitrification capacity of blastocysts was found to be 74% (114/154) and for expanded blastocysts it was 98.9% (97/98). As shown in Table 2, from vitrified embryos after warming, in Group AI from 21 blastocysts 18 blastocysts (85.7%) and from 26 expanded blastocysts 18 expanded blastocysts (69.2%) were transferred to culture medium in order to observe their survival for 48 h of incubation. Respectively, these rates were 73.9% (17/23) and 80.7% (21/26) in Group AII, 70.2% (26/37) and 81.4% (22/27) in Group BI, and 75.7% (25/33) and 72.2% (13/18) in Group BII.

At the end of this period in Group AI totally 2 embryos were continuing to develop (5.5%); one of them was

hatching and one of them had hatched. In Group AII 9 embryos had developed after warming (23.6%); 5 of them were recorded as expanded, 1 of them was recorded as hatching, and 3 of them was recorded as hatched. In Group BI after warming, none of the embryos continued their development (0%). Finally, in Group BII at the end of the development stage, 1 expanded blastocyst and 1 hatching blastocyst were detected (5.2%).

4. Discussion
Many researchers have suggested that antioxidant substances have positive effects on oocyte maturation and embryo development, but the mechanisms of how antioxidants act are not well known.

In studies without supplementary antioxidant substances, matured and cultured embryos from different animal species and on different media reaching the blastocyst stage were 7.2% for bovines in TCM-199 and 6% in SOF, while in pigs it was 23.3% in SOF (2,10,29). As shown in Table 1, our cleavage rates were 47.9% (Group AI-SOF), 50.1% (Group AII-CR1aa), 52.2% (Group BI-SOF), and 49.4% (Group BII-CR1aa). There was, however, no statistically significant difference between the rates of these groups. Singhal et al. (29) reported the cleavage rate of buffalo oocytes as 60.7% when they were supplemented with 50 µM cysteamine. Oyamada and Fukui (10) investigated the effect of epidermal growth factor and

Table 1. The effect of cysteamine on in vitro development of bovine embryos.

Maturation medium	Total oocyte number	Culture groups	Maturated oocyte number	Cleaved oocyte number (%)	Blastocyst number (%)	Expanded blastocyst number (%)
Cysteamine (+)	1176/1370	Group AI (SOF)	594	285 (47.9)	57 (20)	26 (9.1)
		Group AII (CR1aa)	582	292 (50.1)	53 (18.1)	26 (8.9)
Cysteamine (–)	1181/1340	Group BI (SOF)	589	308 (52.2)	76 (24.6)	27 (8.8)
		Group BII (CR1aa)	592	293 (49.4)	66 (22.5)	19 (6.4)

Table 2. Development of vitrified bovine embryos after warming.

Groups	Vitrified embryo number		Warming embryo number		Survival at 48 h			Total embryo number at 48 h (%)
	Blastocyst	Expanded blastocysts	Blastocyst	Expanded blastocysts	Expanded blastocysts	Hatching blastocysts	Hatched blastocysts	
Group AI (SOF)	21	26	18	18	0[b]	1	1	2[b] (5.5)
Group AII (CR1aa)	23	26	17	21	5[a]	1	3	9[a] (23.6)
Group BI (SOF)	37	27	26	22	0[b]	0	0	0[b] (0)
Group BII (CR1aa)	33	18	25	13	1[b]	1	0	2[b] (5.2)

[a, b]: Significant difference between values in the same column without a common letter (P < 0.05).

cysteamine on the maturation of bovine oocytes. In the group for which they added 100 µM cysteamine, they found the cleavage rate to be 62.4%, while in the group for which they added epidermal growth factor and cysteamine, they found that the cleavage rate increased to 63.2%. However, there was no statistical significance. These results are in line with our studies. After warming the highest rate of embryo development was 23.6% in Group II. With this result, statistical significance was detected between the other groups (P < 0.05). As seen in Table 2, the most important point is that in Group BI after warming the lack of any progress was detected at the end of 48 h of incubation. In Group AII the rate of reaching the blastocyst stage after warming was 23.6%, very close to the rate of Mucci et al. (28) (26.7%) in the 48 h after warming. In Group BI rate of reaching the blastocyst stage was detected as 0%, but Gomez et al. (30) detected

the rate of reaching blastocyst stage after warming in SOF medium as 35.2% at the 7th day. Only in Group I was the development rate detected as high (23.6%) after warming. This proves that under certain conditions and in different media antioxidants can demonstrate their positive effects (1,2,5).

In conclusion, the addition of cysteamine to maturation medium had no difference for development for embryos that were cultured in SOF medium, but in CR1aa medium cysteamine increased the rate of blastocysts and expanded blastocysts. Furthermore, it rendered embryos more resistant to freezing.

Acknowledgment

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