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Improvement of bovine *in vitro* embryo production by fetal calf serum and cysteamine supplementation and investigation of freezability

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Abstract: The aim of this study was to investigate the effects of cysteamine (Cys) and fetal calf serum (FCS) in synthetic oviduct fluid (SOF) and charles and rosenkrans (CR1aa) on the bovine in vitro embryo production and its ability of freeze. The oocytes were divided into two groups and allowed to mature in TCM-199, with and without cysteamine. They were divided into 4 subgroups according to whether they contain Cys and FCS in 2 different culture media (SOF and CR1aa). Accordingly, 8 groups were formed as SOF+FCS+Cys, SOF+Cys, SOF+FCS, SOF, CR1aa+FCS+Cys, CR1aa+Cys, CR1aa+FCS and CR1aa. Embryos were cultured for 7-9 days and were frozen using vitrification method. Development of embryos was observed during the first 24 hr post-thaw period. The highest rate of compact morula was 20% in SOF+FCS+Cys and 19.4% in CR1aa+FCS+Cys. The rate of blastocyst in SOF+FCS and CR1aa+FCS were found 17.9% and 15.4% respectively and the difference between groups was statistically significant (P<0.05). During the 12 hr vitality assessment, the highest rate was determined in SOF+FCS (47%). The results indicate that FCS has a positive effect in reaching compact morula and blastocyst regardless of the presence of the culture medium or antioxidant used. There was no statistically difference between the values of post-thaw embryos.
Keywords: Bovine, cysteamine, embryo, fetal calf serum, vitrification.

Sığır in vitro embriyo üretiminin fetal buzağı serumu ve sisteamin katkısı ile iyileştirilmesi ve dondurulabilirliğinin araştırılması

Özet: Bu çalışmanın amacı, sisteamin (Cys) ve fetal buzağı serumu (FCS)'nun sentetik oviduct sıvısı (SOF) ve charles ve rosenkrans (CR1aa) medyumlarında in vitro sığır embriyo üretimi ve donma kabiliyeti üzerindeki etkilerini araştırmaktır. Toplanan oositler iki gruba ayrıldı ve sisteaminli ve sisteaminsiz TCM-199 medyumunda maturasyonuna izin verildi. 2 farklı medyumda (SOF ve CR1aa) kültüre edilmek üzere Cys ve FCS içerip içermediklerine göre 4 alt gruba ayrıldı. Buna göre 8 alt grup; SOF+FCS+Cys, SOF+Cys, SOF+FCS, SOF, CR1aa+FCS+Cys, CR1aa+Cys, CR1aa+FCS ve CR1aa olarak oluşturuldu. Embriyolar 7-9 gün boyunca kültüre edildi ve vitrifikasyon yöntemi kullanılarak dondurulduru. Çözdürme sonrası ilk 24 saat boyunca embriyonik gelişim gözlendi. Kompakt morula oranı en yüksek SOF+FCS+Cys'de %20 ve CR1aa+FCS+Cys'de %19,4 olarak bulundu. SOF+FCS ve CR1aa+FCS'deki blastosist oranı sırasıyla %17,9 ve %15,4 olarak saptandı ve gruplar arasındaki fark istatistiksel olarak anlamlıydı (P<0,05). En yüksek 12 saatlik canlılık değerlendirme oranı SOF+FCS'de (%47) belirlendi. Elde edilen sonuçlar, kullanılan kültür ortamının veya antioksidanın varlığına bakılmaksızın FCS'nin kompakt morula ve blastosiste ulaşmada olumlu bir etkiye sahip olduğunu göstermektedir. Çözdürme sonrası embriyonik gelişimleri arasında istatistiksel olarak bir fark yoktu.
Anahtar sözcükler: Embriyo, fötal buzağı serumu, sığır, sisteamin, vitrifikasyon.

Introduction

The success of reproductive technologies widely used in animals mainly depends on the cryopreservation of gamete cells and the embryo. Due to advances in technology and science, the vitrification method has gained popularity instead of slow freezing, which has been a standard method used in various animal species (27).

During the period of in vitro mammalian embryo culture, atmospheric oxygen tension is routinely used, and this leads to the generation of reactive oxygen species (ROS) (14, 31). DNA damage, lipid peroxidation, oxidative modifications of proteins, and inhibition of oocyte and spermatozoon fusion are among the known detrimental effects of ROS (2). Moreover, ROS may develop under some conditions physiologically as an important factor in programmed cell death (apoptosis) (31, 34). ROS may originate directly from male and female gametes or embryos in different stages of embryonic development, but it may also originate as a result of environmental conditions (9). The most important endogenous source of ROS is oxidative phosphorylation. Inhibition of oxidative phosphorylation reduces ROS generation and has a positive effect on in vitro embryo development (29). Oxygen tension is the most important factor that increases ROS generation. The oxygen tension within the oviduct only equals to ¼ of the atmospheric oxygen tension. It was reported that the synthesis of glutathione (GSH), one of the non-protein sulfhydryl compounds, and freezing resistance in in vitro-produced bovine embryos increased under low oxygen tension (5-7%) (21, 22). Antioxidants such as β-mercaptoethanol, cysteamine, cystine, cysteine, N-acetyl-L-cysteine (NAC), resveratrol and superoxide dismutase (SOD) were used to protect in vitro-produced bovine embryos against oxidative stress (4, 15, 32).

Essential amino acids, especially those that cannot be synthesized by the body, play a crucial role in embryo development (12, 33). However, although it has been reported that protein supplements used in bovine in vitro embryo production such as L-glutamine, fetal calf serum, fetal bovine serum, and bovine serum albumin were useful in the embryonic implantation stage (16, 19). In addition, some scientists have also stated that adding serums into in vitro culture medium resulted in some changes in the metabolism of embryos, which lead to accumulation of fatty acids (23) and increased the number of cytoplasmic lipid droplets (1) and eventually caused a decrease in cryotolerance of the cells (18). In particular, ammonia released by the metabolism of glutamine has some negative effects on the embryo (11, 24). Most importantly, ammonia is one of the main factors causing “large calf syndrome” (17). Therefore, serum-free media have been preferred in recent studies.

The ovarian tissues or cells are basically frozen by two methods. The first one is the traditional slow-freezing method, and the second one is the rapid freezing (vitrification) method. The vitrification method includes flash-freezing of the intracellular and extracellular water, forming a glass-like structure. The major advantage of this method is that it forms smaller ice crystals due to rapid and flash freezing, unlike the traditional slow freezing method (27).

There are many difficulties in genital cell freezing studies as known. It is up to new scientific studies to overcome all previously mentioned positive or negative situations. In our study bovine species preferred for the material, especially considering the contribution to the country livestock. Based on this information, we decided to investigate the effect of cysteamine and fetal calf serum on the production and freezability of in vitro bovine embryos.

Material and Methods

All chemicals are obtained from Sigma-Aldrich (St. Louis, Mo, USA).

Oocyte collection and selection: Bovine ovaries were obtained from a local slaughterhouse and transported to the laboratory within 2-3 h in dulbecco's phosphate buffered saline (D-PBS / FTTS) at 30-35 °C. Oocytes collected by slicing method were assessed and only those with smooth cell walls, homogenous cytoplasm, undamaged zona pellucida and those with at least three or more layers of cumulus oophorus cells were selected.

Oocyte maturation: After 3 passages in HEPES-buffered TCM-199 (M5017) medium, with and without 100 µM cysteamine (M9768), selected oocytes were washed in maturation medium (IVM-5% FCS (F0804)) and transferred to 700 µl IVM medium (20-30 oocytes/well). The cells were allowed to mature at 38.8 °C in a 5% CO_2 incubator for 24 h.

Fertilization of maturated oocytes: The oocytes with scattered cumulus oophorus cells around them were classified as mature. They were transferred to in vitro fertilization (IVF-TALP) medium and allowed to adapt to the new environment. The frozen straws of 2 Holstein bulls were thawed at 37 °C for 30 sec. The sperm motility was examined and those of at least 45-60% motility were detected. Swim down method was preferred for sperm preparation. Washed spermatozoons were added on top of the oocytes (400-600x10^3 spermatozoon/well). The motility of oocytes and spermatozoon was then checked and allowed to fertilize for 18-22 h in 5% CO_2 at 38.8 °C.

Transfer of fertilized oocytes into the culture medium: The fertilized oocytes were transferred into 1 ml of washing medium at 38.8°C (HEPES-buffered TCM-199). After 1-minute mixing, fertilized zygotes were then cultured at 38.8 °C in 5% CO_2, 5% N_2 and 90% humidity for 7-9 days. 8 groups were formed such as SOF+FCS+Cys, SOF+Cys, SOF+FCS, SOF, CR1aa+FCS+Cys, CR1aa+Cys, CR1aa+FCS, and CR1aa. 5% FCS was used in the serum groups.

Freezing mature embryos: At the end of the culture period, the healthy embryos reaching the blastocyst and expanded blastocyst stage in all groups in the presence of

SOF and CR1 medium were selected and frozen using the vitrification method in straws. Equilibration solution (VS1) was 1.5 M ethylene glycol (EG-E9129) and 1 M dimethylsulfoxide (DMSO-D2650), and the vitrification solution (VS2) was 2.5 M EG, 2 M DMSO and 0.5 M sucrose (S1888). For adaptation, selected embryos were kept in PBS droplets at room temperature. Firstly, the embryos were passaged in VS1 solution prepared as 3 separate 70 µl drops for 3 min. Then, they were placed in VS2 solution using the same volume and repetition count for 40 sec. 1 to 3 embryos were placed in pre-prepared embryo straws for freezing and sealed with polyvinyl alcohol (PVA). After the straws were sealed, they were immediately immersed into liquid nitrogen (-196 °C) and frozen.

Thawing of frozen embryos: Frozen embryo straws were pulled out of the liquid nitrogen after at least 1 week. After being kept at room temperature for 10 sec, they were directly thawed in the 37 °C water-bath in 30 sec. The embryos were stored in a petri of 1 M sucrose solution prepared with PBS containing 20% FCS for 3 min. They were then taken into a 0.5 M sucrose solution and allowed to stand for 1 minute there. Subsequently, they were transferred into HEPES-buffered TCM-199 for the removal of cryoprotectants.

The culture of thawed embryos: Thawed embryos were washed 3 times in HEPES-buffered TCM-199 medium and then cultured in SOF and CR1aa culture media of their groups. At the 12^{th} and 24^{th}, the embryos were monitored to see whether they continued to develop after thawing.

Statistical analysis: Chi-square test in SPSS 13.0 package software was used for statistical analysis. P-value < 0.05 was accepted statistically significant.

Results

The number of oocytes undergoing maturation during the study period was recorded as 1247. A total of 1019 cells proceed to in vitro culture immediately after in vitro fertilization. The mean cleavage rate of all groups was found to be 62.5%. At the end of 7-9 days, the average rate of blastocyst stage was 20.2%. The evaluation of the study results reveals that the highest cleavage rate belonged to the CR1aa+Cys group with 69.9%, which was followed by the SOF+Cys group with 67.8% The group with the lowest rate was CR1aa+FCS with 58.2%. Similarly, the SOF+FCS group showed a low cleavage rate of 57.8% (Table 1). The evaluation of other experimental groups revealed no statistical difference although there was a percentage difference between the rates of cleavage. Although there was no statistical difference between the groups in terms of division rates, in the blastocyst stage, the effects of FCS and cysteamine appeared differently in both cultures. While the highest cleavage rate of CR1aa medium with cysteamine (69.9%) was achieved in the cleavage stage, the rate of reaching blastocysts decreased to 2.3%. The lowest cleavage rate in the CR1aa medium with FCS was 58.2% and increased to

Table 1. Development of bovine embryos from cleavage to blastocyst stages after vitrification and IVC

	In Vitro Culture n (%)					Cryopreservation n		Viability n (%)	
Groups	Cleavaged /cultured cell	Compact morula/ cleavaged	Early blastocyst/ cleavaged	Blastocyst/ cleavaged	Expanded blastocyst/ cleavaged	Vitrified embryos	Thawed* embryos	12h/ vitrified	24h/ vitrified
SOF	78/120 (65.0)	4^{b}/78 (5.1)	11/78 (14.1)	5^{b}/78 (6.4)	0 (0)	16	15	7/16 (43.7)	0/16 (0)
SOF+FCS	67/116 (57.8)	8^{ab}/67 (11.9)	4/67 (6.0)	12^{a}/67 (17.9)	1/67 (1.5)	17	15	8/17 (47.0)	1/17 (5.8)
SOF+Cys	78/115 (67.8)	10^{ab}/78 (12.8)	3/78 (3.8)	10^{ab}/78 (12.8)	1/78 (1.3)	14	13	5/14 (35.7)	1/14 (7.1)
SOF+FCS+Cys	90/144 (62.5)	18^{a}/90 (20.0)	12/90 (13.3)	12^{ab}/90 (13.3)	2/90 (2.2)	26	23	11/26 (42.3)	2/26 (7.6)
CR1aa	67/115 (58.3)	4^{bc}/67 (6.0)	4/67 (6.0)	3^{b}/67 (4.5)	0/67 (0)	7	7	2/7 (28.5)	0/7 (0)
CR1aa+FCS	78/134 (58.2)	11^{ab}/78 (14.1)	8/78 (10.3)	12^{a}/78 (15.4)	0/78 (0)	20	18	8/20 (40.0)	1/20 (5.0)
CR1aa+Cys	86/123 (69.9)	3^{c}/86 (3.5)	10/86 (11.6)	2^{b}/86 (2.3)	0/86 (0)	12	11	3/12 (25.0)	0/12 (0)
CR1aa+FCS+Cys	93/152 (61.2)	18^{a}/93 (19.4)	7/93 (7.5)	8^{b}/93 (8.6)	3/93 (3.2)	18	14	6/18 (33.3)	1/18 (5.5)

*Number of cultured embryos after thawing

a,b,c There is a significant difference between the values in the same column with no common letters (P < 0.05)

15.4% when reaching blastocyst. In the same perspective, the low rate of cleavage in the SOF medium with FCS (57.8%) and increased to 17.9% when reaching blastocyst stage. This suggests that the use of cysteamine and fetal calf serum in different culture media have no effects on the cleavage or blastocyst stages. A total of 130 embryos were obtained, which were frozen using vitrification and found to be at different developmental stages at the end of in vitro culture after the thawing process. The viability assessment of embryos was performed in 12 h and 24 h after the thawing. From the 12 h onwards, degenerations were observed. By the end of the 24 h, embryonic development stopped in all groups. The rates of reaching all of embryonic stages and viability rates between groups are given in Table 1.

Discussion and Conclusion

In a study, Sovernigo et al. (28) investigated the effects of different antioxidant supplements on the development of embryos and found that quercetin, vitamin C, resveratrol, and carnitine did not affect the rate of cleavage and reaching the blastocyst stage. These results are in parallel with the results of the research conducted by Sandal and Ozdas (26), Enginler et al. (7), Anchordoquy et al. (3) and Kharche et al. (13) also reported that the supplementation of 100 µM cysteamine did not result in a statistically significant in cleavage rates. Although many studies have reported that FCS has a positive effect on embryo development, it is known that the incidence of large calf syndrome is high among offsprings born as a result of the transfer of embryos, which were developed in a high-protein-containing medium (25). Therefore, serum-free media have been preferred in recent studies (8, 10, 30). Some researchers argue that the use of FCS in the in vitro culture stage leads to high lipid accumulation in blastocysts, and deteriorates embryo quality (5). According to the results obtained in the study, the low rates of cleavage in the serum and cysteamine-free groups (SOF+FCS and CR1aa+FCS), regardless of the culture medium used, seems to support this argument (11, 17). In a study, Murillo et al. (20) developed bovine embryos both (5%) in and without FCS and did not detect a statistical difference in their development stages up to morula, however, they found a statistical difference in the early blastocyst stage ($P<0.005$). In this study, a statistical difference was found in the compact morula and blastocyst stages in favor of the groups that used FCS compared to the other groups. Gomez et al. (8) in their study, they preferred FCS (5%) and BSA (20 g L^{1}) in SOF medium of bovine embryo culture. The researchers achieved the best blastocyst development rate in the FCS group (21.2%) and found a statistical difference compared to other groups ($P<0.05$). It is seen that this result and our result (17.9%) are parallel to each other. Duque et al. (6) reported the rate of bovine embryos that they produced in vitro in the SOF medium containing 5% FCS as 21.1% and found a statistical difference between the blastocyst rate (6.6%) found in the culture medium they did not add serum. In this study, the rate of reaching blastocyst in the SOF group (6.4%) and the rate of blastocyst in the SOF+FCS group (17.9%) is similar to Duque et al. (6)'s results. When the SOF+FCS, SOF+Cys and SOF+FCS+Cys groups of the study were examined, there was no statistical difference even in the percentage of achieving the blastocyst and compact morula stages. This result suggests that the combination of FCS and Cys may have an antagonistic effect on embryo development. Considering the CR1aa+FCS (15.4%), CR1aa+Cys (2.3%) and CR1aa+FCS+Cys (8.6%) groups of the study, it shows similar results in terms of the rate of reaching blastocyst compared to the same SOF groups. This situation gives us the positive effects of FCS and Cys on embryo development when used separately; it suggests that when used in combine, the effect turns into either a negative or antagonist type. The presence of antagonist effect is evident from the statistical difference between CR1aa+FCS and CR1aa+ FCS+Cys groups ($P<0.05$). These results show that FCS has a positive effect on the development of embryos even in the absence of antioxidants in the blastocyst stage. This appears to be consistent with the study carried out by Murillo et al. (20), which argued that the use of low concentration of FCS has a positive effect on the development of embryos. To make a comparison in terms of culture media, which are frequently preferred in bovine in vitro embryo culture; CR1aa appears to need more serum or protein addition than SOF (30). The fact that the blastocyst rate obtained in a study of Wan et al. (30) was higher in the CR1aa+BSA+FCS group compared to other groups and statistically different from other groups (25.5%) is an indicator of this. Likewise, in this study the blastocyst ratios of embryos developed in CR1aa medium are lower than the SOF medium supports that SOF is more preferable in bovine in vitro embryo culture.

As a result of the study, it can be concluded that SOF+FCS can support bovine IVC embryos developing to blastocyst with the same efficiency as SOF+FCS+Cys or SOF+Cys, but combination of 5% FCS and 100 µM cysteamine in CR1aa medium show an antagonist effect and affect the development of bovine embryos negatively.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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