Original Article

Highly Efficient *In Vitro* Production of Bovine Blastocyst in Cell-Free Sequential Synthetic Oviductal Fluid vs. TCM199 Vero Cell Co-Culture System

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Abstract

Background: The aim of this study was to establish a cell-free sequential culture system that can support high levels of in vitro embryo development and blastocyst formation from bovine zygotes. To this end, this investigation was carried out to evaluate the effects of glucose, serum and EDTA on bovine zygote in vitro development.

Materials and Methods: Bovine presumptive zygotes were derived from oocytes matured, and fertilized in vitro and cultured in synthetic oviductal fluid sequential medium in a two-steps manner; SOF 1 for the first 3 days and SOF 2 for the second 5-6 days of in vitro embryo development. In order to evaluate the effect of different modifications of the basic medium on embryo development, glucose was added to the second phase (SOF A), serum was added to the first phase (SOF C) and EDTA alone (SOF D) or in combination with serum (SOF E) was added into the first phase of in vitro embryo culture. The results of each composition were compared with each other and with the results of embryo development in TCM199 vero cell co-culture system.

Results: Glucose addition to the second phase of embryo culture, improved the developmental competency; however, the differences were not significant. Serum addition to the first phase of embryo culture, significantly improved the developmental competency of embryos beyond the cleavage stage, compared to all the treatment and TCM199 co-culture groups. EDTA supplementation of culture medium, either alone or in combination with serum, significantly inhibits the embryo development beyond the morula stage.

Conclusion: The results indicated that culture of bovine presumptive zygotes in two steps cell-free culture system, can support embryo development, and addition of serum throughout the culture and glucose to the second step significantly increased overall developmental competency compared to TCM199 co-culture system.

Keywords: SOF, Co-Culture, Embryo Development, Bovine Zygote

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Introduction

The development of techniques for effective production of bovine preimplantation embryos from oocytes matured and fertilized in vitro is important not only for commercial programs of embryo transfer but also for basic scientific research (1). However, the success rate of the culture systems routinely applied for in vitro embryo production in terms of blastocyst yield remains modest and ranges between 30 and 40 percent (2) which is still lower than that obtained from in vivo- produced embryos (3). Furthermore, there are ample evidences showing that differences between in vivo and in vitro produced embryos still exist, which involves morphological and molecular aspects that impair the efficiency of in vitro embryo production (IVP) procedure (4). These differences are probably induced by several various factors including the quality of oocyte, protein source, somatic cells,

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culture media, oxygen tension, number of embryos per culture unit (embryo density), and energy substrate, may affect preimplantation embryo quality and it's competence to further development (5-7). During the past decade there have been substantial efforts to optimize culture conditions for the in vitro production of bovine embryos (8-11). In this regard, among the variety of techniques have been employed for culturing bovine embryos, the co-culture system with somatic cells such as oviduct epithelial cells, uterine fibroblasts, cumulus cells, vero cells and trophoblastic vesicles has been widely advocated in laboratory animal production and more importantly in human assisted reproduction (ART), till the establishment of sequential media (8-11), due to the inadequacy of simple media to support embryo development beyond the cleaving stage and implantation (9). Under these conditions, however, the proportion of embryos that develop to the morula or blastocyst stage is about 25-40% of inseminated oocytes (5-9). Furthermore, the use of animal cell lines for the co-culture of embryos renders it an undefined medium, which pose many problems such as the risk of infecting the embryos with pathogens (1). The risk of infection is very serious as certain pathogens cannot be removed by standard international embryo transfer society (IETS) washing procedures (9). To eliminate this disadvantage, a great number of studies have examined the ability of bovine oocytes and embryos to develop in vitro using a wide variety of culture media (9-11).
Synthetic oviductal fluid (SOF) is one medium commonly used for bovine embryo culture in vitro (12, 13). This medium was originally based upon the biochemical analysis of ovine oviductal fluid (12-14). Synthetic oviductal fluid has subsequently been modified by the addition of amino acids (13). Other modifications have included the addition of serum (15, 16), decreasing or the removal of glucose (17, 18), and the addition of EDTA for the initial 72h of the culture period (19). However, data about the effect of different ingredients are conflicting or inconsistent and there is not a general agreement toward a unique formulation of SOF culture medium even for a unique species. Therefore, the experiments of this study were undertaken to i) design a basic formulation of SOF based on the previous formulation, ii) investigate the effect of glucose, serum and EDTA, as more frequently discussed ingredients, on developmental competency of bovine IVP embryos and iii) compare the efficiency of different formulations of SOF medium designed in this study with TCM199-vero cell co-culture system as a common embryo culture system.

Materials and Methods

The experiments of this study were reviewed and approved by the regional committee of Royan Institute. Unless otherwise specified, chemicals and media were purchased from Sigma (St. Louis, MO, USA) and Gibco (Life Technologies, Rockville, MD, USA) companies, respectively.

Establishment of vero cells monolayer

Vero cells were obtained from Royan Institute (Tehran, Iran) as a cryopreserved cryotube and were used for co-culturing as described previously (4, 20). Briefly, after thawing and twice washing, vero cells were seeded at the concentration of 1×10^6/ml in 25 cm2 flasks (Falcon) containing TCM199 and cultured at 38.5°C, 5% CO_2 in humidified air. Upon confluency (3-4 days after culture), the medium and unattached cells were removed. After trypsinization with 0.25% Trypsin- EDTA, the harvested cells were seeded in maturation (100µl) and embryo culture (50 µl) droplets at a density of 2×10^4. Two hours before maturation or embryo culture, droplets media were refreshed with their related equilibrated culture media.

Table 1: Composition and concentration of SOF medium used in this study

Ingredients	Concentration (mM)
NaCl	107.70
KCl	7.16
KH_2PO_4	1.19
$CaCl_2.2H_2O$	1.78
$MgSO_4.7H_2O$	0.74
$NaHCO_3$	25.00
Na-Lactate	3.30
Na-Pyrovate	0.33
L-Glutamine	2.05
BME amino acids	2%
MEM amino acids	1%
BSA	8 mg/ml
Penicillin G	100 IU/ml
Streptomycin	100 µg/ml

In vitro maturation and fertilization

The culture procedures employed for in vitro maturation and fertilization of bovine follicular oocytes were as described previously (4, 20). Briefly, bovine ovaries were obtained from a local abattoir and cumulus-oocyte complexes (COCs) were aspirated from antral follicles (2-8 mm). The COCs were then washed with Hepes-buffered TCM199 (HTCM199) medium and then TCM199

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before being cultured upon the established monolayers of vero cells (approximately 1×10^5 cells/ml) in 100µl droplets of maturation medium (10 COCs per droplet), covered with mineral oil for 24 h at 38.5ºC, 5% CO_2 and humidified air (Labotect C200, Germany). The medium used for maturation was TCM199 supplemented with 10% fetal calf serum (FCS), 10 mg/ml ovine FSH (Ovagen; Immuno-Chemical Products), 1 mg/ml ovine LH (Immuno-Chemical Products), 1 mg/ml oestradiol and 100 mM cysteamine. Frozen-thawed and washed sperm from a single Holstein sire of proven in vitro fertility were used for fertilization after capacitation by the swim-up procedure (21). Spermatozoa (1×10^6/ml sperm) and matured COCs (40-45 COCs/200µl) were co-incubated in modified fert-TALP medium containing 0.01 mM heparin, 0.2 mM penicillamine and 0.1 mM hypotaurine for 18 h at 38.5°C under 5% CO_2 in humidified air (21).

Embryo Development

At 18-24 h post insemination (pi), inseminated oocytes were vortexed to remove cumulus cells and then presumptive zygotes were used to be cultured in sequential SOF and TCM119 co-culture systems as described in experimental design and Figure 1. For each experiment, embryos were developed in 50µl drops in 60-mm plates covered with 10 ml of paraffin oil. The concentration of salt and other ingredients of the development media (SOF and TCM199) were as described in Table 2. Embryos (10/drop) were first cultured for 72h (phase 1) when the ratios of cleavage, 8-16 and morula was recorded and then were refreshed into the same development droplets each 48h until Day 10 (phase 2) when the ratios of day 6-7-8 blastulation and day 9 and 10 hatching were compared. For embryos cultured in sequential SOF culture media, the composition of the medium was changed as described in experimental design (Fig 1) while the composition of TCM199 co-culture system was fixed throughout the culture period.

Basic SOF formulation of this study (Table 1) was adopted mainly from the formulation of Tervit et al., (13), containing 8 mg/ml BSA. When amino acids were added to SOF, osmolarity of the medium was maintained at 265–275 mOsmol by adjusting the concentration of sodium chloride and pH was sustained at 7.2- 7.4 by adding NaOH or HCl. All salts and glucose were of analar grade (BDH). Sodium lactate, glutamine, sodium pyruvate, and phenol red were embryo culture tested (Sigma). Betaine and antibiotics were from Sigma. Eagle's minimum essential medium (MEM) essential amino acids without glutamine and nonessential amino acids (22) (Table 2) were supplied by ICN Biomedical (Seven Hills, Australia). SOF modifications were performed regarding the numerous literatures available about different formulations of SOF. On the other hand, TCM199 were purchased (Sigma, ST, Louis, USA) and supplemented with 10% FCS, 20mM $NaHCO_3$ and mM Na-Pyruvate and vero cells were added to the complete culture medium as described previously (4).

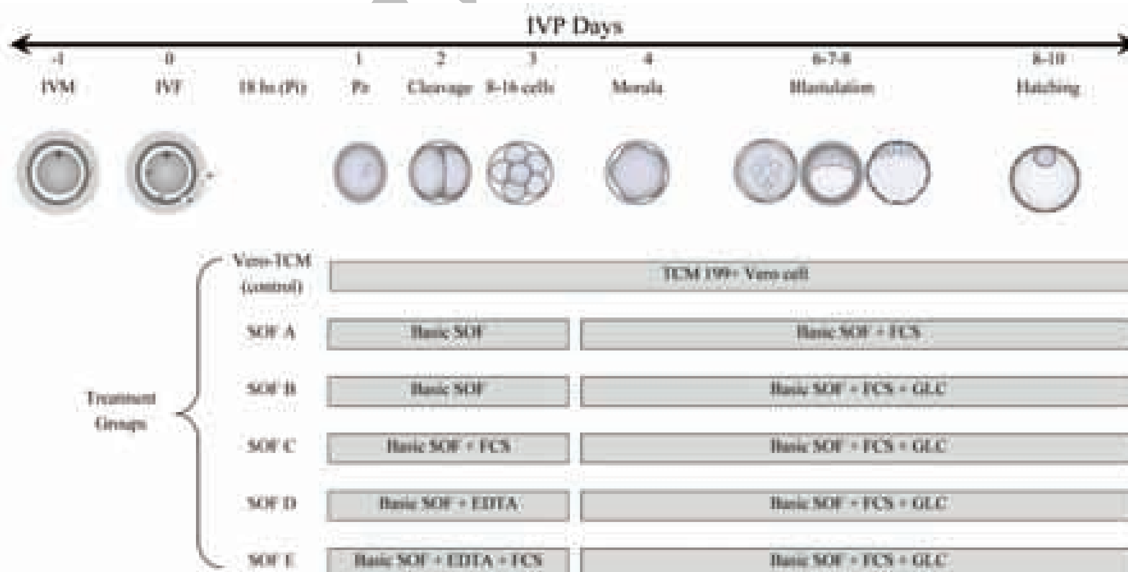

Fig 1: Experimental design: Embryos were derived from oocytes matured and fertilized in vitro. At 18-24h post insemination, presumptive zygotes were randomly allotted to 5 two step SOF and the effect of glucose addition to the second phase (SOF A), serum addition throughout the culture (SOF C), EDTA alone (SOF D) and EDTA combined with serum (SOF E) addition to the first phase of in vitro embryo culture were compared with together and with the results of embryo development in TCM199 vero cell co-culture system.

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Embryo development took place in a humidified incubator at 39°C, with an atmosphere of 5% CO_2 and 5% O2. Morphological examination was performed for blastocysts developed in each group.

Experimental design

This study was undertaken to design a basic formulation of SOF sequential culture media for in vitro development of bovine presumptive zygotes and to further evaluate the effect of glucose, serum and EDTA on developmental competency of embryos. Also, the efficiency of different formulations of SOF culture media was compared with the common culture system of TCM199 over vero cells monolayer (Fig 1). In this regard, presumptive zygotes were first cultured for the first 72h of embryo culture and then were refreshed every 48h until day 9 pi (phase 2 of embryo culture).

Experiment 1. Effect of glucose addition into SOF culture medium (SOF B vs. SOF A)

Presumptive zygotes were first cultured for 72h pi in basic SOF and then were randomly cultured in basic SOF + 10% FCS in either presence (SOF B) or absence (SOF A) of 1.5 mM glucose.

Experiment 2. Effect of serum addition into SOF culture medium (SOF C vs. SOF B)

Presumptive zygotes were first cultured in presence (SOF C) vs. absence (SOF B) of serum for 72h pi and then were cultured similarly in basic SOF + 10% FCS + 1.5 mM glucose.

Experiment 3. Effect of EDTA supplementation of SOF culture medium (SOF D vs. SOF B)

Presumptive zygotes were first cultured in basic SOF in presence or absence of EDTA for 72h and then were refreshed similarly into basic SOF plus 10% FCS and 1.5 mM glucose.

Experiment 4. Combined effect of serum and EDTA during the 1st phase of embryo culture (SOF E vs. SOF D and SOF E vs. SOF B)

To evaluate the combined effect of EDTA and serum addition during the 1st phase of embryo culture, presumptive zygotes were first cultured in basic SOF plus EDTA and serum for 72h and then were refreshed into basic SOF plus serum and 1.5 mM glucose.

Statistical analysis

Each experiment was repeated 3 times. The analysis of variance (ANOVA) and the t-test were used for comparisons of means. When the ANOVA test showed statistical differences, the Student-Neumann-Keuls test was used to discriminate between groups. Significance was defined at $p < 0.05$. All statistical evaluations were carried out using the Statistical Package for Social Sciences (SPSS).

Results

From 298 bovine ovaries obtained from abattoir, 1753 COC were cultured for IVM, 1610 were subjected to IVF and 1447 presumptive zygotes were finally cultured as described in experimental design (Fig 1). Table 2 compares developmental competency of presumptive zygotes cultured in 5 different formulation of SOF with TCM199 vero cell system and Figure 2 shows the developmental patterns of different groups.

Effect of glucose addition into SOF culture media (SOF B vs. SOF A)

As shown in Table 2, the presence of glucose during the second step of bovine embryo development did not significantly increase the developmental competency of bovine IVP embryos. However, the overall pattern of embryo development indicated a tendency for favor of glucose for IVP embryos.

Table 2: Developmental competency of bovine presumptive zygotes cultured in different modifications of sequential SOF medium compared with TCM199 vero cell co-culture system.

Treatments	Presumptive zygotes (N)	Proportion of embryos develop to *				
		Cleavage	Morula	D7 Bls	D8 Bls	Hatching
Vero-TCM	222	160(72.0) a	70(43.7) ab	25(15.6) b	33(20.6) b	14(42.4) bc
SOF A	335	197(58.8) ab	65(21.3) b	34(17.2) b	27(13.7) b	11(40.7) bc
SOF B	259	170(65.6) ab	36(33.0) b	42(24.7) b	29(17.0) bd	14(48.2) bc
SOF C	156	72(46.1) b	43(59.7) a	37(51.3) a	31(43.0) a	26(83.8) a
SOF D	291	203(69.7) ab	111(54.6) ab	15(7.3) c	13(6.4) c	5(38.4) bc
SOF E	184	106(56.5) ab	57(53.7) ab	7(6.6) c	11(10.3) cd	3(27.2) cd

**** Whithin each column, values with at least one comm***

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Effect of serum addition into SOF culture media (SOF C vs. SOF B)

The presence of serum during the first 72h of culture in SOF C resulted in to non-significantly decreased cleavage rate (46.1%) compared to SOF B (58.8%) in which serum was not added. However, the ratios of morula, D7 and D8 blastulation and hatching of embryos developed in SOF C group were 59.7%, 51.3%, 43.0% and 83.8% which were significantly greater than corresponding ratios of SOF B (21.3%, 24.7%, 17.0% and 48.2%, respectively).

Effect of EDTA addition into SOF culture media (SOF D vs. SOF B)

When EDTA was added during the first phase of embryo culture in SOF D group, the ratios of cleavage (69.7%) and morula (54.6%) production non-significantly increased. However, despite initial improvement of embryo development in presence of EDTA, the ratios of D7 and D8 blastulation rates were significantly decreased compared to SOF B group (7.3% and 6.4% vs. 24.7% and 17.0%).

Combined effect of EDTA and serum addition into SOF culture media-SOF E vs. SOF B:

The combined presence of serum and EDTA during the first 72h of embryo culture not only did not increase the cleavage and morula rate of embryos in SOF E vs. SOF B group (56.5% and 53.7% vs. 65.6% and 21.3%, respectively) but also significantly reduced the competency of D7 blastocyst production in SOF E vs. SOF B group (6.6% vs. 17.2%).

SOF E vs. SOF D:

Developmental pattern of embryos were similar between the two groups.

Developmental competency of presumptive zygotes cultured in different modification of sequential SOF culture medium; effect of glucose, EDTA and serum Table 2 compares the overall results of in vitro embryo development of bovine presumptive zygotes cultured in different modifications of sequential SOF medium. As shown in this table, although the cleavage rate of SOF C was non-significantly lower than the other groups, the ratios of morula, D7 and D8 blastulation and hatching were significantly higher than all the other groups. On the other hand, the presence of sole EDTA and/or EDTA combined with serum during the first 72h of embryo culture reduced the overall developmental competency of embryos into the lowest ratios compared to other groups. Accordingly, while SOF C composition promoted the highest embryo development, SOF D and SOF E groups provided the lowest rates of embryo development (Table 2 and Fig 2).

Developmental competency of presumptive zygotes cultured in different modification of sequential SOF vs. TCM199 vero cell co-culture system

Although the cleavage rate of embryos develooped in co-culture system (TCM199 over vero cells) was 72.0% which was significantly greater than SOF (46.1%) and non-significantly higher than all the other groups, the ratios of embryo development beyond the cleavage stage in co-culture group were significantly lower than SOF C (Table 2 and Fig 2). Moreover, developmental competency of embryos developed in TCM199 co- culture system was not significantly different with the sequential SOF A and SOF B. However, the ratios of D7 and blastocyst development of SOF D and SOF E were significantly lower than other sequential and also TCM199 co-culture groups.

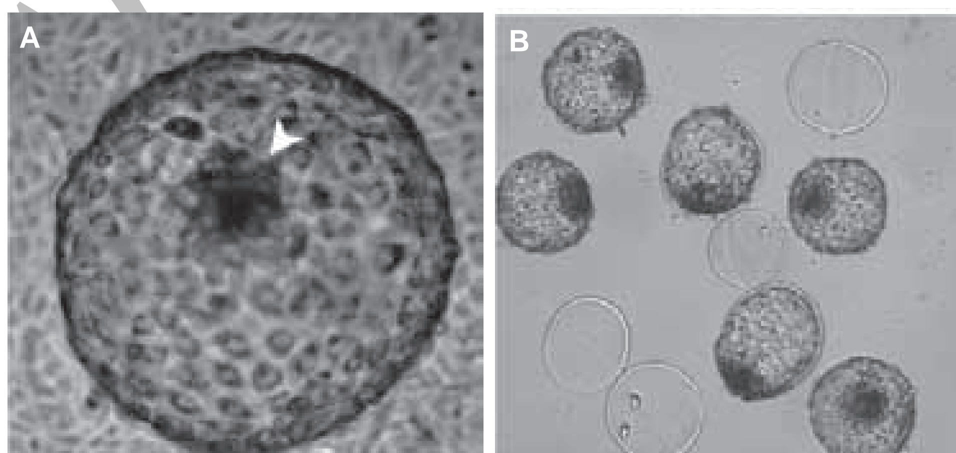

Fig 2: Hatched blastocysts developed in sequential TCM199 vero cell co-culture (A) vs. SOF (B) system. Arrow indicates the inner cell mass.

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Discussion

Co-culture has been routinely advocated in laboratory animal production and more importantly in human assisted reproduction (ART), till the establishment of sequential media (22, 23), due to the inadequacy of simple media to support embryo development beyond the cleaving stage and implantation (24-26). In this regard, numerous studies have demonstrated the beneficial effects of cellular monolayer of various somatic cell types on mammalian embryonic development in vitro (27-29). Suggested benefits of co-culture include secretion of trophic factors such as nutrients, substrates, growth factors, and cytokines and removal of potentially toxic substances from culture medium by co-cultured cells (30, 31).While these culture systems produce good developmental results as judged by the frequency of blastocyst formation, there are drawbacks because it is reported that co-culture may introduce toxic components or pathogens such as viruses. Furthermore, differences between cell types, lines, or even number of passages within a line often result in high variability in the culture system, making repetition of experiments within and between laboratories difficult. It is also noted that cell culture may change the substrates available for use by the embryo, altering the known concentrations in the original culture medium (8). Because of these constraints, there have been great deals of effort to develop new culture media independent of somatic cells. In this regard, although different formulations of embryo culture media have been developed and proposed, these formulations may mainly differ regarding the type and concentration of ingredients included. Therefore, many laboratories have tried to set up a private version of embryo culture medium based on available formulations.

This study developed a new formulation of SOF which can support high rates of bovine blastocyst development and hatching from zygotes cultured in vitro, implying that high rates of in vitro embryo production can be achieved with a home-made embryo culture medium. Furthermore, it was observed that while inclusion of EDTA significantly decreased developmental competency, the significant increase in embryo development of embryos cultured in SOF C vs. other SOF formulation and especially vs. TCM199 co-culture system can be attributed to both the inclusion of serum during the first 72h and glucose during the remaining part of culture period.

Most systems for producing mammalian embryos in vitro use glucose as an energy source in the media due to the beneficial qualities such as direct source of energy, energy reserve in the form of glycogen, and as a biosynthetic molecule. Glucose metabolized via the pentose phosphate pathway (PPP) can generate triacylglycerols; glycoproteins (Wales and Hunter). However, numerous investigations on the metabolism of mammalian early embryo have indicated that from the zygote to the 8- to 16-cell stage, pyruvate and lactate are the preferred energy sources, and from embryonic genome activation (8–16 cells) to blastocysts, embryos prefer glucose as the main source of energy for compaction and blastulation. It was also understood that during early stages of embryo development in vitro, there is a dynamic changes in embryo substrate requirements (16-18). This led to development of sequential media of differing composition. Similarly, we also observed inhibitory effect of glucose when added during first 72h (data not shown) and stimulatory effect when added during the second phase of embryo culture (Table1 and Fig 2).

Serum is the most commonly used macromolecule in embryo culture media. This is particularly true for co-culture systems where somatic cells are cultured in a tissue culture medium supplemented with serum. Numerous studies have indicated that serum has a stimulatory effect on embryo growth and accelerates blastocyst formation (32-34). This property is possible due to a complex and undefined mixture of proteins, growth factors, peptides present in the serum (32). However, factors leading to improved embryo development in culture systems using somatic cells and/or serum- proteins are not well understood, but include positive embryotrophic factors and inactivation of embryotoxic agents (e.g., free radicals, heavy metals and others,) (32-35). The results of the present study in agreement with the other reports indicated that inclusion of serum into bovine embryo culture media has dramatic effects on embryo development, blastocyst formation and hatching rate. Moreover, it was found that although the presence of serum during the whole period of in vitro embryo culture may decrease the early cleavage rate (as seen in cleavage rate of SOF C group compared to SOF A and B), to achieve the highest developmental competency, it should be included throughout the culture period. In agreement with our results, some believed that serum increases the blastocyst rate by a biphasic effect, inhibiting the first cell divisions and stimulating further embryo development.

Despite increasing blastocyst yield, serum also increases the accumulation of cytoplasmic lipids, reduces embryo survival after cryopreservation, thus, increases the male to female embryo ratio, and disturbs gene expression. Its use in culture

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media has been implicated in diverse phenotypic alterations observed during gestation and in bovine newborns such as placental defects and large offspring. In mice, it was shown that in vitro culture of embryos with serum also alters the gene expression, fetal development, and post-natal behavior (32-34). Therefore, a wide range of recent studies have tried to replace serum with other ingredient which can restore its beneficial effects while preclude the abovementioned detrimental effects (34).

Transition metals such as iron and copper have been demonstrated to inhibit embryo development in vitro as well as being implicated in the production of reactive oxygen species (19, 35). During in vivo embryo development, there exist numerous protective systems against oxidative agents and also against heavy ions which are produced during embryo catabolism and anabolism activities (35). Therefore, it is crucial to have an appropriate source of heavy ion chelators in the synthetic culture medium. Ethylenediaminetetraacetic acid (EDTA) is a chelator of divalent cations. In the mouse, EDTA has been demonstrated to be able to replace protein in the medium. Furthermore, development beyond the 2-cell block in the mouse embryo is facilitated by the inclusion of EDTA in the culture medium. Interestingly, the beneficial effects of EDTA have been found to be restricted to the cleavage-stage embryo. This increase in development beyond the 2-cell block in the mouse has been attributed to a decrease in the premature utilization of glycolysis and the concomitant maintenance of substrate oxidation. In contrast, the continual presence of EDTA during mouse blastocyst development in culture results in significantly reduced subsequent fetal development after transfer (19). In this study, although the presence of EDTA during the first 72h of culture reasonably supported cleavage rate (69.7%) and morula embryo production (54.6%), there seen a significant decrease in the D7 and 8 blastocyst development (7.3% and 10.3%, respectively) which were significantly lower than all the treatment groups. It is noteworthy that the combined presence of EDTA and serum during the first 72h of embryo culture in SOF E group did not improve the inhibitory effect of ETDA (Table 2). These results are in contrast with the other studies in which the transitional presence of EDTA during first 72h of embryo culture, but not beyond this period, improved the blastocyst production of embryos (19). Although the exact reason of this disparity was not understood, possible role of serum to act as a chelator of transition metals may describes why the combined presence of serum and EDTA did not improve the developmental competency of embryos developed in SOF E culture condition (19).

The result of this study also indicated that although culturing bovine embryos over the vero cell monolayer has a beneficial effect on cleavage (72%) and morula (43.7%) stage and also reasonably supports the embryo development beyond. Overall developmental competency of embryos cultured in cell free sequential SOF C would be significantly greater. By considering the elaborative, time consuming and cost-demanding procedures of cell culturing along with the possible hazard potential of embryo culturing over monolayers, one may concluded that using cell free sequential systems have a great beneficial over traditional co-culturing systems.

Coclusion

A two steps culture system has been developed that is capable of supporting high levels of blastocyst formation and cleavage in absence of somatic cells and in presence of serum during the whole culture period and glucose during the second phase (72h -144h) of embryo culture. The development of such a culture system will have a significant impact on the introduction of new reproductive technologies in domestic animal breeding.

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