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Replacement of Fetal Calf Serum With Two Different Synthetic Sera in in Vitro Maturation Medium: Effects On Maturation, Fertilization and Subsequent Development of Cattle Oocytes in Vitro

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Abstract: The aim of this study was to investigate the effect of using synthetic sera (synthetic serum substitute, SSS and serum replacement 1, SR1) instead of fetal calf serum (FCS) in in vitro maturation medium on in vitro maturation (IVM), fertilization (IVF) and subsequent development of bovine oocytes. Selected oocytes collected from ovaries obtained from a local slaughterhouse were matured in tissue culture medium 199 (TCM-199) supplemented with 2 mM glutamine, 0.25 mM Na-pyruvate, 0.5 μg/ml FSH, 5 μg/ml LH, 100 U/ml penicillin, 100 μg/ml streptomycin and 10% FCS, SSS or SR1 (FCS, SSS and SR1 groups, respectively) for 22 hours. Matured oocytes were in vitro fertilized using frozen bull sperm. Fertilization day was considered as day 0 in the present study. Eighteen hours after IVF, fertilized oocytes were transferred into synthetic oviduct fluid (SOF) culture medium for in vitro culture. Two cell, 8-cell, morulae and blastocyst numbers were recorded. A total of 238, 243 and 200 oocytes were used for FCS, SSS and SR1 groups, respectively. In FCS, SSS and SR1 groups, 179 (75.21%), 177 (72.84%) and 80 (40.00%); 121 (50.84%), 114 (46.91%) and 38 (19.00%); 97 (40.76%), 98 (40.03%) and 21 (10.50%); and 71 (29.83%), 69 (28.40%) and 10 (5.00%) oocytes developed to 2-cell, 8-cell, morulae and blastocyst stage embryos, respectively. The development rates of SR1 group at all recorded stages were significantly lower than those of FCS and SSS groups. There was no significant difference between FCS and SSS groups in terms of the developmental rates at any stage recorded. As a result, SSS is a good alternative for FCS replacement in in vitro maturation of bovine oocytes, however, SR1 is not a good alternative for FCS replacement in in vitro maturation medium.

Key Words: in vitro maturation, cattle, oocyte, FCS, SSS, SR1, embryo.

İn Vitro Olgunlaştırma Medyumunda Fötal Buzağı Serumu Yerine İki Farklı Sentetik Serumun Kullanılması: Sığır Oositlerinin İn Vitro Olgunlaştırma, Fertilizasyon Ve Sonraki Gelişimleri Üzerindeki Etkileri

Özet: Bu çalışmanın amacı sığır oositlerinin in vitro olgunlaştırılması, fertilizasyonu ve sonraki gelişimleri üzerinde olgunlaştırma medyumunda fötal buzağı serumu (FCS) yerine sentetik serumların (synthetic serum substitute, SSS and serum replacement 1, SR1) kullanılmasının etkilerini incelemektir. Yerel bir mezbahadan elde edilen ovaryumlardan kazanılan seçilmiş oositler 2 mM glutamin, 0,25 mM Na-piruvat, 0,5 μg/ml FSH, 5 μg/ml LH, 100 U/ml penisilin, 100 μg/ml streptomisin ve %10 FCS, SSS ya da SR1 (sırasıyla FCS, SSS ve SR1 grupları) ile takviye edilmiş doku kültür medyumu (TCM-199) içerisinde 22 saat süreyle olgunlaştırılmıştır. Olgunlaşan oositler dondurulmuş boğa spermiyle in vitro fertilize edilmiştir. Fertilizasyon günü bu çalışmada 0. gün olarak kabul edilmiştir. İn vitro fertilizasyondan 18 saat sonra, fertilize edilmiş oositler in vitro kültür için sentetik ovidukt sıvısı (SOF) kültür medyumuna transfer edilmiştir. İki hücre, 8-hücre, morula ve blastosist sayıları kaydedilmiştir. FCS, SSS ve SR1 grupları için sırasıyla toplam 238, 243 ve 200 oosit kullanılmıştır.

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FCS, SSS ve SR1 gruplarında sırasıyla, 179 (%75,21), 177 (%72,84) ve 80 (%40,00); 121 (%50,84), 114 (%46,91) ve 38 (%19,00); 97 (%40,76), 98 (%40,03) ve 21 (%10,50); ve 71 (%29,83), 69 (%28,40) ve 10 (%5,00) oosit 2-hücre, 8-hücre, morula ve blastocyst aşamalarına gelişmiştir. SR1 grubunun kaydedilen tüm gelişim aşamalarındaki gelişim oranları FCS ve SSS gruplarının kaydedilen tüm gelişim oranlarından önemli düzeyde düşük bulunmuştur. FCS ve SSS grupları arasında gelişim oranları bakımından gelişim aşamalarının hiçbirinde fark bulunmamıştır. Sonuç olarak, SSS sığır oositlerinin in vitro maturasyonunda FCS'nin yerine kullanılabilen bir alternatifken, SR1 maturasyon medyumunda FCS'nin yerine kullanılabilecek iyi bir alternatif değildir.

Anahtar Kelimeler: İn vitro olgunlaştırma, sığır, oosit, FCS, SSS, SR1, embriyo.

Introduction

In-vitro embryo production (IVP) using the slaughtered animals as a source of oocytes has a great importance not only for mass production of cattle embryos and the improvement of cattle populations but also for research purposes. A reliable high rate of blastocyst development in IVP is very critical for laboratories and requires great cautions and standards at each step of IVP in terms of every aspect, such as the collection of oocytes, chemicals, hormones, water used for the preparation of media, sperm used for fertilization, and culture conditions, etc. Even though the same technique and media are used by different laboratories, the rate of development to the blastocyst stage obtained from the same IVP application differs from one laboratory to another[10,18].

In vitro maturation (IVM) of immature oocytes obtained from follicles in 2-8 mm diameter on ovaries is one of the most important steps in cattle IVP system. Tissue culture medium 199 (TCM-199) is the most common medium used for IVM of cattle oocytes. In mammalian animals, maturation of oocytes starting during fetal development progresses until the first meiotic arrest at the dictyate stage of prophase just before or immediately after the birth. Resumption of meiosis is stimulated after either hormonal stimulation in situ or spontaneously after the release of a cumulus-oocyte complex from the follicle in vitro. Developmental competence of bovine oocytes is generally low under common IVM conditions. Improvement of the developmental capability of cattle oocytes by supplementing IVM media with different additives has been the subject of many studies. Most common used additives for IVM of oocytes in TCM-199 are FSH, LH, fetal calf serum (FCS) and some growth factors, such as insulin-like growth factor-I (IGF-I) and epidermal growth factor (EGF). Supplementation of IVM media with gonadotropins (FSH and LH) is important for the acquisition of developmental capacity of oocytes in cattle[5,7,9]. It was shown that the supplementation of IVM media with bovine serum albumin (BSA) or FCS is useful for obtaining high in vitro maturation, fertilization and subsequent development rates in cattle IVP[15,17].

It is known that the effectiveness of FCS in IVM and IVF might change significantly from one batch to another[16]. Non-defined ingredients, such as amino acids, hormones, growth factors, cytokines, vitamins and many other substances forming FCS shows enormous differences among FCS samples[12]. These differences lead to various results in IVM, IVF and subsequent development rates. Although factors improving embryo development rates in media supplemented with FCS are not completely understood, FCS includes some positive embryotrophic factors and plays important roles in the inactivation of embryotoxic agents such as free radicals, heavy metals etc. Recently, bovine-derived sera or proteins are notably avoided in human IVP system because of the appearance of bovine spongioform encephalopathy (BSE) and viral or prion contamination risk. In addition, more defined conditions are useful to understand the effects of some factors affecting the success of IVP individually. Therefore, defined culture conditions supporting high developmental rate are important to obtain reliable results. For this reason, there is a trend to use more defined proteins in recent years, such as human serum albumin (HSA), BSA and synthetic serum preparations instead of poorly known natural serum preparations like FCS and oestrus cow serum (OCS)[6].

The aim of this study was to investigate the effect of using synthetic sera (synthetic serum substitute, SSS, and serum replacement 1, SR1) instead of FCS in in vitro maturation medium on the maturation, fertilization and subsequent development of cattle oocytes in vitro.

Materials and Methods

All chemicals used in the present study were purchased from Sigma Chemicals Co., St. Louis, MO USA unless otherwise indicated.

Oocyte Collection

Bovine ovaries obtained from a local slaughterhouse were transported to the laboratory in a thermos filled with physiological saline (0.9% w/v NaCl) within 4 to 6 h. Temperatures of thermoses on arrival were approximately 32 ± 2°C. Ovaries were rinsed at least 3 times with warm tap water (approximately 35°C) before starting aspiration. Oocytes were aspirated from 2 to 8 mm antral follicles using an 18-gauge needle attached to a vacuum system. Aspirated follicular fluids were collected in a 50 ml centrifuge tube and then the sediment was transferred into a 100 mm plate by Pasteur pipette. Oocytes were searched under a stereomicroscope in a warm culture room and oocytes with homogeneously granulated cytoplasm and at least 3 layers of compact cumulus cells were used in the experiment. Oocytes were washed three times with TL-HEPES medium[2] containing 0.25 mM Na-pyruvate, 3 mg/ml BSA, 100 U/ml penicillin and 100 µg/ml streptomycin.

In Vitro Maturation

The main medium used for IVM was tissue culture medium (TCM-199, Gibco/Invitrogen, Grand Island, NY) supplemented with 0.2 mM pyruvate, 0.5 µg/ml of FSH (Sioux Biochemicals, Sioux City, IA), 5 µg/ml LH (Sioux Biochemicals, Sioux City, IA), 100 U/ml penicillin and 100 µg/ml streptomycin (Gibco/Invitrogen, Grand Island, NY). Additionally, maturation medium was also supplemented with 10% FCS (FCS group), 10% SSS (Irvine Scientific, Santa Ana, CA) (SSS group) or 10% SR1 (S-0638) (SR1 group). SSS contains HSA, alpha and beta globulins. SR1 is composed of heat treated BSA, heat treated bovine transferrin and bovine insulin. Approximately 10^{8-12} oocytes were cultured in a 50 µl drop of maturation medium supplemented with FCS, SSS or SR1 under 9 to 10 ml mineral oil in 60 mm petri dishes for about 22 h at 39°C in 5% CO_2 in highly humidified atmosphere air[4,26,27]. The same culture condition was also used through all IVF and IVC protocols. After IVM period, maturation of oocytes was roughly evaluated by checking cumulus cells expansion.

In Vitro Fertilization

After 22 h maturation period, oocytes were washed twice in TL-HEPES and then approximately 10 oocytes were transferred to the 44 µl fertilization drops under mineral oil. The fertilization medium was glucose-free TALP supplemented with 6 mg/ml fatty acid free BSA (BSA-FAF), 0.2 mM pyruvate, 100 U/ml penicillin and 100 µg/ml streptomycin. Percoll density gradient system was used for the separation of motile fraction of frozen-thawed semen from a single bull[23]. Half ml of 90% Percoll was pipetted to the bottom of a 1.5 ml eppendorf centrifuge tube, and 0.5 ml of 45% Percoll was placed on top with a great caution. Frozen sperm was first thawed at 35°C for 1 minute, and then carefully layered onto the Percoll gradient. The tube containing sperm sample and Percoll layers was then centrifuged at 700xg for 10-15 minutes at room temperature. The supernatant was carefully removed without any disruption of the pellet containing live sperm cells. After the pellet resuspended, sperm concentration was determined using a hemocytometer. Sperm was then diluted to $50x10^6$ sperm cells/ml in TL-HEPES to get $2x10^6$ spermatozoa/ml final concentration. Fertilization of in vitro matured oocytes was completed by adding 2 µl of diluted sperm, 2 µl of 5 µg/ml heparin and 2 µl of PHE solution (20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine in final concentration) into the 44 µl fertilization drops, respectively. Oocytes and sperm were co-cultured for about 18 hours in the incubator. Fertilization time was considered as 0 h in the present study[22,23,27,28,30].

In Vitro Culture

Eighteen hour after IVF, the cumulus cells were removed by vortexing the embryos in a 1.5 ml Eppendorf tube at the highest speed for 3 minutes. Fertilized oocytes were washed three times in TL-HEPES and transferred into 50 µl drops of embryo culture medium known as synthetic oviduct fluid, SOF (Specialty Media, NJ), under mineral oil (25 to 30 fertilized oocytes per each 50 µl drop). On the day of use, SOF medium was supplemented with 8 mg/ml BSA-FAF, 0.4 mM pyruvate, 20 µl/ml 100xMEM, 10 µl/ml 50xBME, 100 U/ml penicillin and 100 µg/ml streptomycin. In addition, all drops of FCS, SSS and SR1 groups were supplemented with 10% FCS, SSS and SR1 on day 4 of IVC, respectively. Developmental data were recorded for 2-cell, 8-cell, morulae and blastocyst stage

embryos after 48, 96, 120 and 192 h culture periods, respectively[27,28].

Statistical Analysis

Developmental rates to the different stages were calculated from the number of oocytes used for IVF. Significance of difference was determined by one-way ANOVA of SPSS program. Difference at $P<0.05$ was considered significantly important.

Results

In the present study, 238, 243 and 200 oocytes were used for FCS, SSS and SR1 groups, respectively. In FCS, SSS and SR1 groups, 179 (75.21%), 177 (72.84%) and 80 (40.00%); 121 (50.84%), 114 (46.91%) and 38 (19.00%); 97 (40.76%), 98 (40.03%) and 21 (10.50%); and 71 (29.83%), 69 (28.40%) and 10 (5.00%) oocytes developed to 2-cell, 8-cell, morulae and blastocyst stage embryos, respectively (Table 1).

The development rates of SR1 group to the 2-cell, 8-cell, morulae and blastocyst stage were significantly lower than those of FCS and SSS groups ($P<0.01$). There was no significant difference between FCS and SSS groups at any developmental stage investigated.

Table 1. Developmental data of embryos matured in three different maturation conditions

Tablo 1. Üç farklı olgunlaştırma şartlarında olgunlaştırılan embriyoların gelişim verileri

Groups	number of fertilized oocytes	number of 2-cell embryos (%)	number of 8-cell embryos (%)	number of morulae (%)	number of blastocyst (%)
FCS	238	179(75.21)[a]	121(50.84)[a]	97(40.76)[a]	71(29.83)[a]
SSS	243	177(72.84)[a]	114(46.91)[a]	98(40.03)[a]	69(28.40)[a]
SR1	200	80(40.00)[b]	38(19.00)[b]	21(10.50)[b]	10(5.00)[b]

[a,b] Different superscripts within the same columns are significantly important.

Discussion

In the present study, the effects of the FCS replacement in the maturation medium with SSS or SR1 on the development of bovine oocytes were investigated. In vitro maturation of immature bovine oocytes is one of the most important steps affecting the success of in vitro fertilization and subsequent development[6]. In in vitro maturation media, different sera and BSA are the most common used protein sources[24,29]. Scientifically the beneficial effect of serum supplementation has not been clearly understood. However, it is generally believed that one of the major biological effects of serum is to provide whatever necessary elements are absent from the medium by profiting as a reservoir for many useful components, such as energy substrates, vitamins, fatty acids, steroids, amino acids and growth factors. In addition, serum also serves as a protective conjugate by chelating metal ions and toxic materials produced by developing embryos. However, it is well known that serum has a biphasic effect during early embryonic development; in the presence of serum, early cleavage divisions could be blocked, while the development could be improved at later stages of development[13,19]. In addition, FCS causes some abnormalities such as, large offspring syndrome, increased placental and fetal weights, abnormal development of fetal skeletal muscle and placental blood vessels, and altered metabolism[21]. Because of these concerns and other safety requirements such as BSE and virus or prion contaminations, the use of FCS is restricted especially in human IVF system. Consequently, synthetic serum substitutes are commercially produced and using these substitutes in media has allowed the researchers to investigate the effects of individual ingredients of FCS and other agents on the development of embryos and to develop well-defined culture media for in vitro embryo production to obtain reliable and constant results[11,21]. Most of the studies about the replacement of serum in media have involved the in vitro culture period[3,8,13,19,20,27,31,32,33]; however, few of them involved the in vitro maturation period[1,6,14,20,25,27,29]. In the present study, we aimed to investigate the effect of using synthetic sera (synthetic serum substitute, SSS and serum replacement 1, SR1) instead of FCS in in vitro maturation medium on the maturation, fertilization and subsequent development of cattle oocytes in vitro. According to the groups (FCS, SSS and SR1), developing embryos were respectively supplemented with 10% FCS, SSS and SR1 on day 4 of in vitro culture to follow the procedure as applied in routine[26,28].

Development rate to 2-cell, 8-cell, morulae and blastocyst stage of SR1 group was found significantly lower than those of FCS and SSS groups in this study and there was no significant difference between FCS and SSS groups. This was contradictory to the result of a previous study reporting 30% cleavage and no blastocyst

development in SSS group[29]. In that study, CR1aa culture medium was used for the culture of developing embryos. In another study in which the same culture medium (SOF) was used for the culture of developing embryos, results were similar to our present results, and they could not find any significant difference when they used SSS instead of FCS in in vitro maturation[27]. Similarly, Dukue et al.[8] reported that serum replacement CPSR-3 can supplement bovine embryo culture with blastocyst rates and quality similar to those for serum. In the same study, another serum replacement Ultroser-G, however, was not found as a good replacement of FCS for bovine embryo culture in vitro. Moore et al.[20] reported that fertilization rates did not differ significantly for oocytes matured in either FCS or serum replacer (SR, Knockout SR), which is contradictory to our results for FCS and SR1 groups In our study, development rate to 2-cell stage in SR1 group was significantly lower than the development rate to 2-cell stage in FCS group. However, result of them in terms of blastocyst development was similar to our results for FCS and SR1 groups and oocytes matured in FCS had a significantly higher development rate to the blastocyst stage when compared with the development rate to the blastocyst stage of oocytes matured in SR[20].

SSS was used for the IVM of immature human oocytes[25]. Moreover, Chanson et al.[6] compared SSS with FCS for IVM of immature bovine oocytes. In their study the cleavage rate of the control group supplemented with FCS and hormones was reported 31.5% and development rate to the blastocyst stage were found 7.4%. Furthermore, when FCS was replaced with SSS, the cleavage rate and development rate to the blastocyst stage were reported 33.1% and 11.0%, respectively. In this study, the cleavage rate and development rate to the blastocyst stage in FCS group (75.21% and 29.83%, respectively) were found higher than the result of the control group of Chanson et al.[6]. While the cleavage rate of the present study in SSS group was found higher than the result of Chanson et al.[6] but closer in SR1 group, development rate to the blastocyst stage of this study in SSS group was higher but lower in SR1 group.

The explanation of the differences between SSS and SR1 groups could be related to the ingredients of these two different synthetic sera. As mentioned above while SSS contains HSA, alpha and beta globulins, SR1 is composed of heat treated BSA, heat treated bovine transferrin and bovine insulin. Replacement of natural serum with synthetic sera would be very useful to get constant results because of well-defined ingredients of synthetic serum substitutes and some different synthetic serum substitutes have already been available for use in routine.

In conclusion, according to our present results SSS is a good FCS replacement for in vitro maturation of bovine oocytes and SR1 is not a good choice for FCS replacement in in vitro maturation medium. In addition, this study shows that SSS could be used to investigate the effect of some individual factors such as growth factors, antioxidants, chemicals and other substances affecting IVP results.

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