

Can Sequential Human Embryo Culture Media be Used in Bovine *in vitro* Embryo Culture? [1]

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Summary

The aim of the study was to investigate potential use of sequential human embryo culture media in culture of bovine embryos. Bovine oocytes were matured in Tissue Culture Medium-199 (TCM-199) for 22 h at 38.5°C and fertilized in modified Tyrode-Albumine-Lactate-Pyruvate medium (mTALP). The putative zygotes were randomly allocated to two embryo culture media groups; (1) Synthetic Oviduct Fluid (SOF) supplemented with fatty-acid free bovine serum albumin (FAF-BSA) (8 mg/ml) and (2) sequential human embryo culture media [Quinn's Advantage Medium-(QAM)] supplemented with FAF-BSA (8 mg/ml). Zygotes were cultured in SOF and sequential QAM for 9 days (5% CO₂, 5% O₂, and 90% N₂) at 38.5°C. Cleavage (73.3% and 72.2%), morula (37.6% and 33.2%) and blastocysts rates (23.9% and 22.9%) were similar among groups ($P>0.05$), but the total blastocyst cell number were significantly higher in blastocysts developed in SOF (101.6±4.0) than those in sequential QAM (87.4±3.2) ($P<0.05$). QAM may be suggested to use in culture as an alternative media in terms of supporting embryo development, but low cell number in blastocysts produced in QAM may suggest a possible low pregnancy rate.

Keywords: Bovine, Embryo culture, SOF, QAM, Blastocyst quality

Ardışık İnsan Embriyo Kültür Medyumu Sığır Embriyolarının *in vitro* Kültüründe Kullanılabilir mi?

Özet

Bu çalışmanın amacı, ticari insan embriyo kültür medyumlarının sığır embriyolarının kültüründe kullanılabilirliğinin araştırılmasıdır. Sığır oositleri, TCM-199'da 22 saat süreyle 38,5°C'de maturasyona tabi tutulduktan sonra, mTALP medyumunda fertilize edildiler. Muhtemel zigotlar rastgele 2 gruba ayrıldı; (1) sığır serum albümmini (FAF-BSA) (8 mg/ml) ilaveli Sentetik Ovidukt Sıvısı (SOFaa) ve (2) sığır serum albümmini (FAF-BSA) (8 mg/ml) ilaveli ardışık Quinn's Advantage Medium (QAM). Muhtemel zigotlar SOFaa ve ardışık QAM medyumlarda 38,5°C'de 9 gün süre ile kültüre edildiler (%5 CO₂, %5 O₂ ve %90 N₂). Yapılan istatistiksel değerlendirme sonucunda; bölünme oranı (%73.3 ve %72.2), morula (%37.6 ve %33.2) ve blastosiste ulaşma oranları yönünden (%23.9 ve %22.9) deneme grupları arasında önemli bir farklılık gözlenmemiştir ($P>0.05$). Buna karşın total blastosist hücre sayıları yönünden yapılan değerlendirmede, SOFaa medyumu (101.6±4.0) ve QAM medyumunda (87.4±3.2) kültüre edilen blastosistlerin hücre sayıları önemli oranda farklılık göstermiştir ($P<0.05$). Sonuç olarak, ardışık QAM medyuminun sığır embriyolarının *in vitro* kültüründe alternatif bir medyum olarak kullanılabileceği, ancak bu medyumdan elde edilen blastosistlerin total hücre sayılarının düşük olması sebebiyle beklenilen düzeylerde gebelik oranlarının elde edilmesinde yetersizlik şekillenebileceği düşünülmektedir.

Anahtar sözcükler: Sığır, Embriyo kültürü, SOF, QAM, Blastosist kalitesi

INTRODUCTION

In vitro embryo technologies have enabled the production in large numbers of embryos of superior breeds in various livestock animals, including cattle, and

allows for embryo transfer at low costs. They have also enabled the production of embryos for scientific research purposes from slaughtered and/or live animals. Although

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several decades of research have gone into *in vitro* culture conditions that promote the maximal embryo yield are yet to be standardized [1,2]. *In vitro* embryo culture conditions depend on multiple parameters such as the composition of culture media and gases. An efficient culture media for *in vitro* embryo development should be formulated to protect from intracellular stress and include all demands of the embryo thereby embryo can maintain viability [1,3,4].

In vitro embryo production is widely used in the treatment of subfertility in human. Studies on the metabolism of the preimplantation cleaving human embryo enabled the formulation of culture media capable of sustaining normal development [3]. To satisfy all requirements of embryo, sequential culture media have been developed. Therefore, there are now commercially available, ready-to-use, and quality-controlled sequential commercial human embryo culture media [5-7]. These media were formulated specifically to prevent intracellular stress to the embryo. Additionally, these media take into account the changing carbohydrate and amino acid requirements of the embryo [1]. Due to these characteristics, these media are thought considered to support blastocyst development in culture of bovine embryos. Because, human and bovine are single ovulators and there are close similarities between human and bovine ovarian physiology, oocyte characteristics and reproductive functions. Moreover, bovine and human embryos are remarkably similar with respect to the micro-tubule timing of genome activation, metabolic requirements, interactions with the culture medium and duration of preimplantation development [2].

Synthetic oviductal fluid (SOF) is one of the medium commonly used for bovine embryo culture *in vitro*. Synthetic oviductal fluid has subsequently been modified by the addition amino acids [4,8,9]. Numerous studies have examined the ability of bovine embryos to develop *in vitro* using a wide variety of culture media. However, to our knowledge, no studies comparing the effects of commercially available human culture media on the development of bovine zygotes produced *in vitro* to the blastocyst stage have been reported. Therefore, the aim of the present study was to investigate potential use of sequential human embryo culture media (QAM) in bovine embryo culture and to compare the development of bovine zygotes to the blastocyst stage in SOF and sequential QAM culture media.

MATERIAL and METHODS

All chemicals and media used in this study were from Sigma-Aldrich Chemical Co. (Turkey) unless otherwise stated.

Collection and *in vitro* Maturation of Oocytes

Bovine ovaries at various stages of their oestrous cycle were collected from a local slaughterhouse and transported

to the laboratory at approximately 35°C in physiological saline solution (0.9% w/v NaCl) supplemented with 0.1 µl/ml gentamycin sulphate. Cumulus-oocyte complexes (COCs) were recovered from follicles 2-8 mm in diameter by aspiration, using an 18 gauge needle and 10 ml disposable syringe. The COCs were collected in 3-4 ml Hepes-buffered Medium 199 containing Earle's salts and supplemented with 1% v/v antibiotic-antimycotic solution (10.000 IU penicillin, 10 mg streptomycin and 25 µg amphotericin B per ml). The COCs were assessed morphologically before *in vitro* maturation and only oocytes with compact, non-atretic cumulus investment and evenly granulated cytoplasm were selected for maturation. All COCs were washed three times in Hepes-buffered Medium 199, and then twice in maturation medium. Maturation medium were prepared as reported by Cevik et al. [10]. Maturation medium was sodium bicarbonate-buffered Medium 199 containing Earle's salts and L-glutamine supplemented with 5.5 µg/ml sodium pyruvate, 1% v/v antibiotic-antimycotic solution, 10% v/v heat-inactivated fetal calf serum (FCS). The COCs were placed in 500 µl of maturation medium (approximately 25-35 COCs per well) covered with 300 µl mineral oil in four-well dishes (Nunc, Roskilde, Denmark) and matured for 22 h in a humidified atmosphere of 5% CO₂ in air at 38.5°C.

In vitro Fertilization

After *in vitro* maturation, COCs were washed twice in Hepes-buffered Medium 199 and then twice in fertilization medium. Fertilization medium was modified TALP supplemented with 6 mg/ml FAF-BSA, 10 µl/ml pyruvate (0.2 mM) and 0.5 µl/ml antibiotic-antimycotic solution (pH 7.4 and 280-300 mOsm/kg) [11]. After washing, COCs were then transferred into 44 µl drops (approximately 15 COCs per drop) of fertilization medium added 2 µL heparin (10 µg/mL), and 2 µL PHE mix (penillamine, 20 mM; hypotaurine, 10 mM; epinephrine 1 mM). Subsequently, 2-3 straws of frozen semen from tested bulls for IVF were thawed at 36°C for 1.0 min. The thawed semen was layered over 2 ml of both 45% and 90% Percoll discontinuous density gradient into a 15 ml conical tube and was centrifuged for 15 min at 1.200 g. After centrifugation, the supernatant above the sperm pellet was carefully removed. The pellet was resuspended with Sperm-TL medium (4 ml) and centrifuged for 5 min at 300 g. The pellet containing the motile sperm fraction was carefully collected from the bottom of the conical tube.

The sperm concentration was counted by hemocytometer using a phase-contrast microscope at a magnification of 400x. Sperm was then diluted to 50 × 10⁶/ml spermatozoa with fertilization medium [11]. The sperm motility was visually checked for acceptable motility (i.e. at least 80% progressively motile). The oocytes were fertilized with 2 µl diluted semen per fertilization drops for 22 h in a humidified atmosphere of 5% CO₂ in air at 38.5°C.

In vitro Culture

After fertilization, the putative zygotes were washed three times in Hepes-buffered Medium 199. They were then vortexed to remove cumulus cells. The putative zygotes were randomly allocated to two embryo culture media groups; (1) synthetic oviduct fluid (SOF) culture media supplemented with 8 mg/ml fatty-acid free BSA (FAF-BSA), 10 µl/ml BME (50×) essential amino acid solution, 20 µl/ml MEM (100×) non-essential amino acids solution (SOF); and (2) sequential commercial human embryo culture media (Quinn's Advantage Medium® (SAGE In-Vitro Fertilization, Inc Trumbull, C.T. USA) supplemented with 8 mg/ml FAF-BSA [6] (QAM).

The zygotes were washed twice and placed in 50 µl drops (approximately 15 zygotes per drop) of either SOF or sequential QAM media under mineral oil and cultured in a humidified atmosphere of 5% CO₂, 5% O₂ and 90% N₂ in air at 38.5°C. Following fertilization, embryos were cultured in Quinn's Advantage Cleavage Medium (QACM) supplemented with 8 mg/ml essentially fatty-acid free BSA for 72 h. Then developing embryos were cultured in Quinn's Advantage Blastocyst Medium (QABM) supplemented with 4 mg/ml essentially fatty-acid free BSA. The embryos in SOF media were cultured in the same drops until the end of the culture period (9 days). Cleavage, morula, and blastocyst development rates were evaluated from the zygotes (day 0 = *in vitro* fertilization) on days 4 and 9 using a stereomicroscope, and blastocyst rates on day 9 were present.

Determination of Total Cell Number of Blastocyst

The number of total cell of the blastocysts was determined as reported by Arat et al.[12]. Blastocysts in both groups were washed twice in Hepes-buffered Medium 199 and fixed in a solution of ethanol and acetic acid (volume ratio = 3:1) for 24 h. Subsequently, the blastocysts in both groups were stained with Hoechst 33342 for 15 min and the total number of cells in each blastocyst was determined by counting stained nuclei with the aid of a fluorescence microscope.

Statistical Analysis

Data were analyzed using by generalized linear models after appropriate transformation where necessary (proportion of cleaved zygotes, morula and blastocyst

yields, arcsine-transformation; total cell numbers of blastocyst, log₁₀ transformation) [13]. Significant differences between treatment means were tested using Student's *t*-test. The results are presented as untransformed mean ± SE values, and statistical significance was determined at the level of P<0.05.

RESULTS

In the present study, total number of 450 bovine cumulus-oocyte complexes (COCs) were used and matured in standard maturation medium. Approximately 85% of COCs matured following *in vitro* maturation (IVM). *In vitro* matured oocytes (382) were subjected to the *in vitro* fertilization (IVF) procedure. A total number of 164 and 218 bovine embryos were cultured in QAM and SOF media, respectively.

Development rates of bovine embryos cultured in SOF and sequential QAM media are presented in the Table 1. There were no significant differences between two culture media in terms of cleavage rates (73.3% vs 72.2%, P>0.05). Similarly no significant differences in morula (37.6% vs 33.2%) and blastocyst formation rates (23.9%) vs (22.9%) were observed on day 9 of between two culture media (P>0.05).

The mean total cell numbers of bovine blastocysts developed in SOF and sequential QAM culture media are presented in the Fig. 1. The mean total cell numbers were 101.6±4.0 and 87.4±3.2 for blastocysts cultured in SOF and sequential QAM culture media, respectively. There were significant reduction in total cell number of blastocysts developed in sequential QAM culture media compared to those in SOF culture media (P<0.05).

DISCUSSION

The results of the present study indicate that bovine embryos produced by *in vitro* fertilization and cultured in SOF and Quinn's Advantage sequential culture media (QAM) supplemented with BSA showed similar developmental competence *in vitro*. To allow reliable comparison between SOF and the sequential QAM human culture media systems, all embryos were cultured with the same oil overlay and drop volume, and in the same incubator,

Table 1. *In vitro* embryo development of bovine embryos cultured in SOF and sequential QAM culture media

Tablo 1. Ardişik QAM ve SOF medyumlarında kültüre edilmiş sığır embriyolarının *in vitro* gelişimi

Culture media	No. of oocytes	Cleavage (%)	<i>In Vitro</i> Embryo Development Rates (%)		
			Morula	Blastocyst	Blastocyst / Cleavage
SOF	218	73.3±6.3	37.6±3.3	23.9±2.0	34.4±5.5
QAM	164	72.2±4.4	33.2±1.2	22.9±0.9	32.3±3.4

Values (mean ± SE) within columns with different letters are insignificantly different (P>0.05)

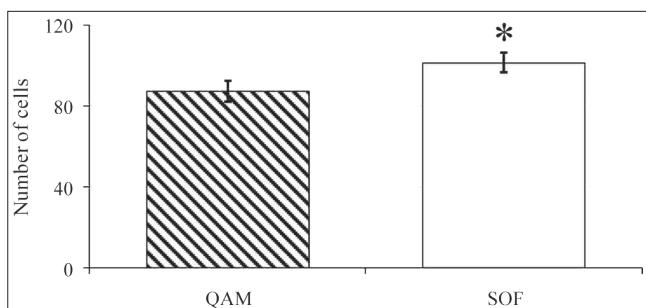


Fig 1. The mean total cell numbers of bovine blastocysts developed in SOF and sequential QAM culture media. Asterisk indicates significant differences between media groups ($P<0.05$)

Şekil 1. SOF ve ardışık QAM medyumlarda sığır blastosistlerinin total hücre sayıları

under the same oxygen conditions. However, sequential QAM culture media decreased embryo quality by reducing the total cell number of blastocysts, which is considered to be an indicator of viability and hence establishing a successful pregnancy, compared to blastocysts developed in SOF culture media.

Many studies have been performed to evaluate culture systems with respect to developmental competence, embryo quality and development rate in humans [6,7,14] and bovine [3,4,15,16]. Among these studies, mSOF culture medium containing BSA or FBS is widely used for producing bovine embryos [8,9,17,18]. However, comparisons between studies, even those evaluating the same culture system, remain problematic due to variations in culture parameters including type of overlay, oxygen tension, culture drop volume, serum supplement, combined procedures such as IVF/ICSI and many more [9,18-20].

In vivo, the developing embryo migrates from the oviduct to the uterine lumen where the fluid composition and gas atmosphere are likely to be different. Analysis of embryonic physiology and metabolism also shows that the requirements for exogenous substrates change with development. Therefore, sequential media may theoretically be more optimal for culture of developing embryos than single culture medium. Commercially available sequential media have been widely used for embryo culture in various species and have shown better competence to support development of IVF embryos both *in vitro* and *in vivo* than single culture media in some previous studies [1,3,8]. In addition, some studies showed that a medium cultured for long periods of time can rapidly deteriorate, resulting in its inability to support embryo development [1,4,16]. Sequential culture media formulated for *in vitro* development of mammalian embryos overcome this problem [8,16]. Currently, there are different sequential media available for culturing mammalian embryos such as G1/G2 [8,21], Quinn's Advantage sequential medium and Sydney IVF medium [5,7].

Problems with pregnancies following *in vitro* culture

have primarily been attributed to the presence of serum in the culture system [1,12,14,22]. These problems have included heavier birth weights (commonly referred to as the large lamb or calf syndrome, LOS), extended gestation periods, higher rates of abortion and increased rates of perinatal mortality [22]. Studies investigating culture of embryos with or without serum have determined that many of these problems with pregnancy and parturition are eliminated when serum is replaced in the medium with purified preparations of BSA [1,20]. Therefore, the need for a culture system that does not employ serum is essential for the extended application of *in vitro* culture procedures in a commercial setting.

As reported by Cooke et al. [6], one such serum-free culture system is commercially available the sequential QACM and QABM human culture media systems. These media were formulated specifically to prevent intracellular stress to the embryo thereby maintaining embryo viability. Additionally, these media take into account the changing carbohydrate and amino acid requirements of the embryo. As a result these media are able to support high rates of blastocyst development in culture of embryos from many species.

Perin et al. [23] reported that the percentage of blastocyst development by day 5 of culture of mouse zygotes was higher in the potassium-enriched simplex optimized culture medium (KSOM) compared with the G1/G2 sequential culture media. The notches of distributions of the total number of cells of the blastocysts produced in each culture condition overlapped, revealing no difference between the medians of these two distributions. Choi et al. [17] reported that the cleavage rates of equine zygotes cultured in either G1/G2 or Dulbecco modified Eagle medium (DMEM) both with and without BSA or 10% FBS were similar. In agreement with these observations, in our study, there was no significant difference in cleavage, morulae and blastocyst formation rates between culture media groups. However, cleavage and blastocysts formation rates of the bovine embryos in QAM media were reported to be lower than studies for human embryo culture [6,7].

Swain et al. [16] reported that the use of a single culture medium (NCSU23) and sequential G1.2/G2.2 media resulted in similar cleavage percentages for *in vitro* derived porcine embryos. In agreement with Swain et al. [16] in our results, the rates of cleavage and blastocyst formation in SOF and sequential QAM were similar, but total cell number of blastocysts developed in sequential QAM culture media was lower than those developed in SOF culture media. It is known that the total cell number and morphology of blastocysts are currently the best predictors available for assessing embryo quality [8,12]. Higher cell number has been associated with increased embryo viability after transfer into a surrogate [16]. The blastocyst cell numbers of embryos in the present study were similar to those reported by Arat et al. [12] and Wang et al. [8].

Althought not reported here, we have observed that bovine embryos cultured in SOF media showed faster developmental speed and higher developmental rate to form early blastocysts on day 7 of culture in compared to sequential QAM. The difference in speed of blastocyst formation between culture media studied in the present study may explain the differences in total cell number in blastocyst produced in these culture media. The late formation of blastocyst may yield a lower number of cells in each blastocyst.

In conclusion, results of present study showed that bovine blastocysts can develop in sequential human embryo culture medium and serum-free sequential human culture system can be used for *in vitro* bovine embryo production. However, the QAM sequential human embryo culture system supports lower quality of *in vitro* derived bovine embryos, based on the total cell number, compared to those cultured in SOF media. Therefore, further research is needed to examine substrate requirements such as BSA and amino acids of commercially available sequential human embryo media system for support a higher percentage of bovine blastocyst development and to determine how metabolism of these blastocysts compare to embryos developed in different culture medium *in vitro* or *in vivo*.

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