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Research Article

Effects of Sperm from Different Bulls on Developmental Competence, Blastosist Quality and Cell Number of Bovine Embryos *In Vitro*

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Abstract

The aim of this study was to investigate the effects of sperm from different bulls on the developmental competence, blastosist quality and cell number of bovine embryos *in vitro*. *In vitro* matured bovine oocytes were fertilized with frozen-thawed sperm from five different bulls of Austrian Simmental Fleckvieh and then cultured in Synthetic Oviduct Fluid (SOF) medium at 38.5°C, 5% CO_2, 5% O_2 and 90% N_2 atmosphere with high humidity for 8 days. In the present study, there was no significant effect of bull variations on cleavage, morula, morula/cleavage, blastocyst and blastocyst/cleavage rates of embryos. Additionally, ICM cell numbers and ICM/total cells ratio of blastocysts obtained from *in vitro* fertilized oocytes with five bull's sperm were similar. However trophectoderm and total cell numbers of blastocysts obtained from *in vitro* fertilized oocytes with bull 5 sperm were lower than other bulls ($P<0.05$). In the present study, blastocysts quality was affected by bull variations and the percentage of excellent or good quality blastosist (Grade I) were lower ($P<0.05$) in embryos from fertilized oocytes with bull 5 sperm than those of other bulls, but the percentage of low quality blastosist (Grade III) were higher ($P<0.05$). There was a positive correlation between quality and total cell numbers of blastocysts in experimental groups. Result of present study showed that developmental competence of embryos *in vitro* were not affected by bulls variations, but may be influence blastocyst quality and cell numbers of blastocyst.

Keywords: *Bovine, Bull, in vitro fertilization, Embryo development, Blastocyst quality*

Farklı Boğa Spermalarının Sığır Embriyolarının *İn Vitro* Gelişim Yetkinliği, Blastosist Kalitesi ve Hücre Sayısı Üzerine Etkileri

Özet

Bu çalışma farklı boğa spermalarının sığır embriyolarının *in vitro* gelişim yetkinliği, blastosist kalitesi ve hücre sayısı üzerine etkilerini belirlemek amacıyla yapılmıştır. İn vitro olgunlaştırılan sığır oositleri, 5 farklı Avusturyan Simental Fleckvieh boğasına ait dondurulmuş-çözdürülmüş boğa sperması ile fertilize edilmiş ve sonra sentetik ovidukt sıvısı medyumu içerisinde yüksek oranda nemlendirilmiş 38,5°C, %5 CO_2, %5 O_2 ve %90 N_2 içeren atmosferde 8 gün boyunca kültüre alınmıştır. Sunulan çalışmada, embriyoların bölünme, morula, morula/bölünme, blastosist ve blastosist/bölünme oranları üzerine boğa çeşitliliğinin etkisi tespit edilmemiştir. Ayrıca beş farklı boğa sperması ile *in vitro* fertilize edilmiş oositlerden elde edilen blastosistlerin İHK hücre sayısı ve İHK/toplam hücre oranı benzer bulunmuştur. Ancak boğa 5 sperması ile *in vitro* fertilize edilmiş oositlerden elde edilen blastosistlerin trofektoderm ve toplam hücre sayısı diğer boğalardan daha düşük olduğu belirlenmiştir ($P<0.05$). Sunulan çalışmada blastosist kalitesi boğa çeşitliliği tarafından etkilenmiş olup mükemmel ve iyi kaliteli (Grade I) blastosistlerin yüzdesi boğa 5 sperması ile fertilize edilmiş oositlerden elde edilen embriyolarda diğer boğalarınkilerden daha düşük ($P<0,05$), fakat düşük kalitedeki (Grade III) blastosistlerin oranı ise daha yüksek olduğu belirlenmiştir ($P<0.05$). Bütün deneme gruplarında blastosist kalitesi ile toplam hücre sayısı arasında pozitif bir korelasyon saptanmıştır ($P<0.05$). Mevcut çalışmanın sonuçları boğa çeşitliliğinin *in vitro* embriyo gelişim yetkinliğini etkilemediği, ancak blastosist kalitesinin ve blastosistlerin hücre sayılarını etkileyebileceğini göstermiştir.

Anahtar sözcükler: *Sığır, Boğa, in vitro fertilizasyon, Embriyo gelişimi, Blastosist kalitesi*

INTRODUCTION

In vitro embryo production is one of reproductive biotechnology applications and increases the speed of genetic improvement in farm animals [1]. Moreover this reproductive biotechnology has enabled large amounts embryo production of superior breeds in various domestic animals and the production of large numbers of embryos for scientific research purposes from slaughtered and/or live animals [2,3]. Production of embryos *in vitro* also

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has potential for revolutionizing in cattle breeding and husbandry around the globe. Although several decades of research have gone into *in vitro* embryo production, the process involve *in vitro* embryo production is yet to be standardized and ratio of *in vitro* fertilized oocytes reaching the blastocyst stage is still low, approximately 30-40% [1]. Probably, the reason of this situation is *in vitro* embryo production practices cannot mimic *in vivo* conditions, resulting in alterations of development, morphology and gene expression in embryos [4].

Many factors can impact efficiency of *in vitro* embryo production [5,6] and one of these factors is use of sperm from different bulls [7]. Fertility ability of sperm is one of the main factors for success of *in vitro* fertilization, and previous studies reported that there are differences in *in vitro* embryo production rates among bulls [8-10]. When success rate of bulls in in-vitro fertilization is determined, *in vitro* embryo production may be standardized and decrease the differences in rate of embryos reach to blastocyst stage among bulls. Additionally genetic potential of sperm from different bull should be considered due to genetic information transmitted by sperm to the embryo. Moreover, embryo quality and success of *in vitro* embryo production applications can be influenced by genetic heritage of sperm [11]. The aim of the study was therefore to investigate the effects of sperm from different bulls on the developmental competence, blastosist quality and cell number of bovine embryos *in vitro*

MATERIAL and METHODS

All chemicals and media used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA), except where otherwise indicated.

Collection and In Vitro Maturation of Oocytes

Cumulus-oocyte complexes (COCs) were obtained by aspirating antral follicles (2 to 5 mm in diameter) of bovine ovaries obtained from a local slaughterhouse. 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). COCs were assessed morphologically and only oocytes with compact, intact cumulus cells around and homogeneous cytoplasm were selected for maturation. 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.[12]. Maturation medium was sodium bicarbonate-buffered Medium-199 containing Earle's salts and L-glutamine supplemented with sodium pyruvate (5.5 µg/ml), antibiotic-antimycotic solution (1% v/v), heat-inactivated FCS (10% v/v), LH (5.0 µg/ml), FSH (0.5 µg/ml) and EGF (10 ng/ml). The COCs were placed in 500 µl of maturation medium (approximately 30-35 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% CO_2 in air at 38.5°C.

Sperm Preparation and In Vitro Fertilization

Cumulus expansion degree of COCs was assessed under a stereomicroscope at the end of the *in vitro* maturation period. COCs with full cumulus expansion considered as matured oocytes and immature COCs were discarded from experiment. The matured COCs were washed twice in Hepes-buffered Medium-199 and twice in fertilization medium. The fertilization medium was glucose-free modified TALP supplemented with 25 mM sodium bicarbonate, 22 mM Na-lactate, 1 mM Na-pyruvate, 6 mg/ml fatty acid-free BSA, 10 mg/ml heparin-sodium salt and 0.5 µl/ml antibiotic-antimycotic solution (pH 7.4 and 280-300 mOsm/kg). The matured COCs were then randomly separated and transferred into 45 µl fertilization drops (approximately 15 COCs per drop) covered with mineral oil. Frozen thawed semen from five different bulls (named; Bull 1, Bull 2, Bull 3, Bull 4 and Bull 5) of Austrian Simmental Fleckvieh was used for the *in vitro* fertilization in this study (Genovet L.T.D., S.T.I, Samsun, Turkey). Before *in vitro* fertilization frozen–thawed semen from five different bulls were separated by Percoll density gradient technique [13]. The percentage of sperm motility was visually evaluated using a phase-contrast microscope at a magnification of 400× (at least 80% progressively motile). The sperm concentration was determined by hemocytometer using a Thoma counting chamber. Sperm was then diluted to 2×10^6 sperm/ml with fertilization medium. COCs were fertilized randomly with 5 µl diluted sperm (approximately 13×10^3 sperm for per oocytes) from five bulls for 22 h in a humidified atmosphere of 5% CO_2 in air at 38.5°C.

In Vitro Culture

After *in vitro* fertilization, the putative zygotes were washed three times in Hepes-buffered Medium 199 and vortexing for approximately 5 min, to remove the cumulus cells. The naked putative zygotes were then washed twice in Hepes-buffered Medium 199 and twice in synthetic oviduct fluid (SOF) embryo culture media. The SOF embryo culture media was supplemented with 20 µl/ml pyruvate, 8 mg/ml fatty acid-free BSA, 20 µl/ml MEM non-essential amino acids solution 100×, 10 µl/ml BME Amino Acids Solution 50× and 0.5 µl/ml antibiotic-antimycotic solution on the day of use. The naked putative zygotes were placed in 50 µl drops (approximately 15 zygotes per drop) of SOFaa media under mineral oil and cultured in a humidified atmosphere of 5% CO_2, 5% O_2 and 90% N_2 in air at 38.5°C. *In vitro* fertilization was considered as 0 day. On day 3 of development the proportion of zygotes cleaved was recorded. Morula and blastocyst development the proportion of zygotes were evaluated

on days 5 and 8, respectively. Experiments were repeated 5 times for each group.

Determination of Blastocyst Quality

The quality grading of the recovered blastocysts was done according to the morphological criteria of quality determined by International Embryo Transfer Society [14]. Blastocysts were classified as Grade I; excellent or good quality blastocysts with blastocoele filling the entire blastocyst, oval shaped and compact inner cell mass (ICM) and multicellular cohesive trophectoderm (TE), Grade II; moderate quality blastocysts with normal ICM, but non-optimal (fragmented or necrotic) TE, and Grade III poor quality blastocysts with very few cells or without ICM and with large vacuole instead of blastocoel.

Differential Staining of ICM and TE Cells

After determination of blastocyst quality, blastocysts were differential stained as described by Van Soom et al.[15] with some modifications. Briefly, the blastocysts were washed in PBS supplemented with 0.1 mg/ml polyvinylalcohol and then incubated with picrylsulphonic acid diluted to with Ca^{2+}-free PBS (10 mM) for 5 min in the refrigerator (4°C). The blastocysts were then were washed in PBS supplemented with 0.1 mg/ml polyvinylalcohol and incubated for 30 min at 38.5°C in anti-dinitrophenyl antibody diluted to 30% (v/v) with Ca^{2+}-free PBS. The blastocysts were repeat washed in PBS supplemented with 0.1 mg/ml polyvinylalcohol and transferred into guinea pig complement (55852, ICN biochemicals, Irvine, CA, USA) diluted to 20% (v/v) with Ca^{2+}-free PBS supplemented with 50 µg/ml propidium iodide and 12.5 µg/ml bisbenzemide for 30 min at 38.5°C. Finally, blastocysts were fixed in 2% paraformaldehyde for 1-2 min at room temperature. Before mounting on slides with a 10 µl drop of 0.2 M 1.4 diazabicyclo-octanes in glycerol (50%, v/v) in Ca^{2+}-free PBS as an anti-fading solution. Blastocysts were examined under a fluorescence microscope (Nikon Invert Microscope Eclipse Ti-FL, 340-380 nm excitation and 430 nm suppression). ICM nuclei labelled with bisbenzimide appeared blue and TE nuclei labelled with both bisbenzimide and propidium iodide appeared pink to red *(Fig. 1)*. The numbers of ICM and TE nuclei were counted directly under the fluorescence microscope using a 345 nm ultraviolet light filter.

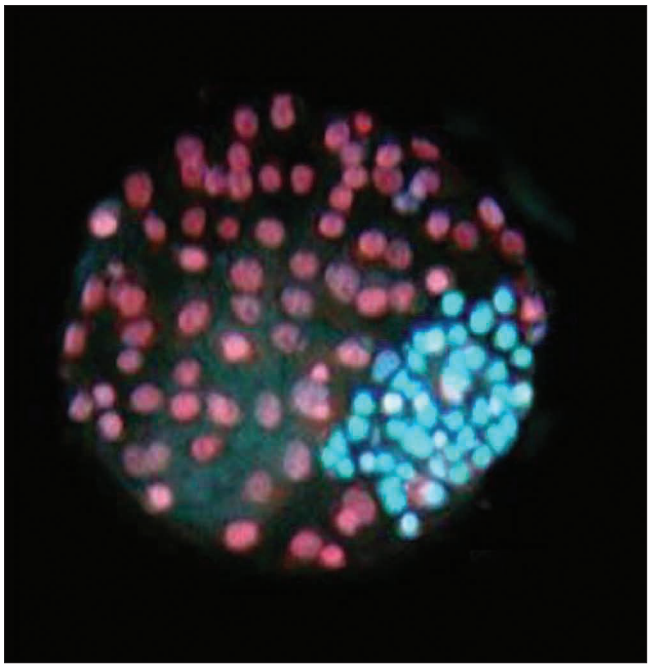

Fig 1. The picture of a blastocyst after differential staining (400× magnification)

Şekil 1. Diferansiyel boyama sonrası bir blastosist resmi (400× büyütme)

Statistical Analysis

Data were analyzed by one-way ANOVA after appropriate transformation where necessary (percentage of cleaved zygotes, morula and blastocyst yields, arcsine-transformation; cell numbers of blastocyst, $\log_{10}$ transformation). Data involved in blastocyst quality were analyzed by chi-square (χ^2) test. Relationships between quality and total cell numbers of blastocysts were determined with a Pearson correlation analysis at the 95% confidence interval. Significant differences between means were tested using Duncan's test with SPSS 20.0. The results are presented as untransformed mean ± SE values, and statistical significance was determined at the level of 0.05.

RESULTS

In the present study, total number of 2389 bovine cumulus-oocyte complexes (COCs) were used and maturated in standard maturation medium. Approximately 92% of COCs matured following *in vitro* maturation. *In vitro* matured oocytes were subjected to the *in vitro* fertilization procedure with sperm from various bulls and total numbers of 2198 bovine embryos were cultured in SOF media.

Developmental characteristics of *in vitro* produced bovine embryos obtained from *in vitro* fertilized oocytes with different bulls' sperm are presented in the *Table 1*. There were no significant differences between bulls in terms of cleavage, morula and blastocyst formation rates. Similarly, there was no significant effect of bull variations on morula/cleavage and blastocyst/cleavage rates of embryos.

Inner cell mass, trophectoderm and total cell numbers of *in vitro* produced bovine blastocysts obtained from *in vitro* fertilized oocytes with different bulls' sperm are presented in the *Table 2*. ICM cell numbers of blastocysts obtained from *in vitro* fertilized oocytes with five bull's sperm were similar, but trophectoderm and total cell

Table 1. *Developmental characteristics of in vitro produced bovine embryos obtained from in vitro fertilized oocytes with different bulls' sperm. Results are presented as untransformed mean values (± SEM) per groups for 5 replicates*

Tablo 1. *Farklı boğaların sperması ile in vitro fertilize edilen oositlerden elde edilen in vitro üretilmiş sığır embriyolarının gelişim özellikleri. Sonuçlar 5 tekrarlanmış grup başına dönüştürülmemiş ortalama değerler (± SEM) olarak sunulmuştur*

Bulls	Oocytes Fertilized (n)	Developmental Competence of Bovine Embryos (%)				
		Cleavage	Morula	Morula/Cleavage	Blastocyt	Blastocyst/Cleavage
1	420	73.8±4.03	34.2±4.23	46.3±4.03	25.37±3.79	34.02±4.71
2	498	69.5±2.08	44.6±2.84	64.7±5.54	29.23±1.73	42.34±3.03
3	476	73.9±1.69	39.3±4.18	52.5±5.23	29.26±4.63	39.48±6.02
4	424	74.8±4.11	34.1±3.47	45.4±3.17	26.76±3.95	34.93±3.86
5	380	73.1±2.86	37.4±1.63	51.8±3.87	25.38±0.91	34.92±1.62

Table 2. *Inner cell mass, trophectoderm and total cell numbers of in vitro produced bovine blastocysts obtained from in vitro fertilized oocytes with different bulls' sperm. Results are presented as mean values (± SEM) per groups for 5 replicates*

Tablo 2. *Farklı boğaların sperması ile in vitro fertilize edilen oositlerden elde edilen in vitro üretilmiş sığır blastosistlerinin iç hücre kitlesi, trofektoderm ve toplam hücre sayıları. Sonuçlar 5 tekrarlanmış grup başına ortalama değerler (± SEM) olarak sunulmuştur*

Bulls	Cell Numbers		
	Inner Cell Mass	Trophectoderm	Total
1	37.0±1.70	67.4±1.91[a]	104.4±2.54[a]
2	34.7±1.15	62.2±1.71[a]	96.9±2.26[a]
3	34.4±1.18	63.7±2.48[a]	98.1±3.06[a]
4	35.5±0.94	62.5±1.46[a]	98.0±2.03[a]
5	34.8±1.53	51.0±2.40[b]	85.8±2.74[b]

[a,b] Means in column with different superscripts are significantly different at P<0.05

Table 3. *The percentages of blastocyst quality of in vitro produced bovine blastocyst obtained from in vitro fertilized oocytes with different bulls' sperm. Results are presented as mean values per (± SEM) groups for 5 replicates*

Tablo 3. *Farklı boğaların sperması ile in vitro fertilize edilen oositlerden elde edilen in vitro üretilmiş sığır blastosistlerinin blastosist kalite oranları. Sonuçlar 5 tekrarlanmış grup başına ortalama değerler (± SEM) olarak sunulmuştur*

Bulls	Blastocyst Quality		
	Grade I	Grade II	Grade III
1	36.5[a]	60.4[b]	3.1[c]
2	24.4[b]	73.5[a]	2.1[c]
3	21.7[b]	72.4[a]	6.9[b]
4	26.9[b]	71.4[a]	1.7[c]
5	12.5[c]	78.3[a]	9.2[a]

[a,b] Means in column with different superscripts are significantly different at P<0.05

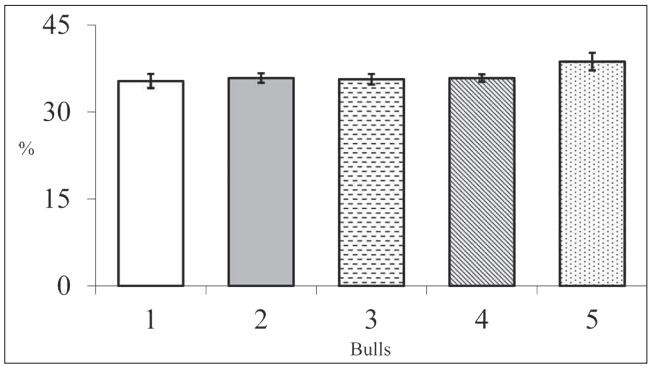

Fig 2. ICM/total cells number ratio of *in vitro* produced bovine blastocyst obtained from *in vitro* fertilized oocytes with different bulls' sperm

Şekil 2. Farklı boğaların sperması ile *in vitro* fertilize edilen oositlerden elde edilen *in vitro* üretilmiş sığır blastosistlerinin İHK/toplam hücre sayısı oranlar

Table 4. *Pearson correlation coefficients (95% confidence intervals) between quality and total cell numbers of in vitro produced bovine blastocyst obtained from in vitro fertilized oocytes with different bulls' sperm*

Tablo 4. *Farklı boğaların sperması ile in vitro fertilize edilen oositlerden elde edilen in vitro üretilmiş sığır blastosistlerinin kalitesi ve toplam hücre sayısı arasındaki Pearson korelasyon katsayısı*

Bulls	Correlation Coefficients
1	.521*
2	.493*
3	.624*
4	.454*
5	.620*

** P<0.05*

numbers of blastocysts obtained from *in vitro* fertilized oocytes with bull 1 sperm were lower than those of other bulls (P<0.05). ICM/total cells number ratio of *in vitro* produced bovine blastocyst obtained from *in vitro* fertilized oocytes with different bulls' sperm are presented in the *Fig. 2*. There were no significant differences between bulls in terms of ICM/total cells ratio of blastocysts.

The percentages of blastocyst quality of *in vitro* produced bovine blastocyst obtained from *in vitro* fertilized oocytes with different bulls' sperm are presented in the *Table 3*. Blastocyst quality was dependent on bull variations. The percentage of excellent or good quality blastocyst (Grade I) were higher in embryos from fertilized oocytes with sperm of bull 1 than those of other bulls (χ^2 = 0.39; P<0.05), but moderate quality blastocyst

(Grade II) were lower in same bull than those of other bulls ($\chi^2 = 0.37$; $P<0.05$). The percentage of poor quality blastocyst (Grade III) were higher in embryos from fertilized oocytes with sperm of bull 3 and 5 than those of bull 1, 2 and 4 ($\chi^2 = 0.32$; $P<0.05$).

Pearson correlation coefficients (95% confidence intervals) between quality and total cell numbers of *in vitro* produced bovine blastocyst obtained from *in vitro* fertilized oocytes with different bulls' sperm are presented in the *Table 4*. There were positive correlations between quality and total cell numbers of blastocysts from *in vitro* produced bovine embryos obtained from *in vitro* fertilized oocytes with five bulls sperm ($P<0.05$).

DISCUSSION

The present study demonstrates that the use of semen from different bull for *in-vitro* fertilization did not affect developmental competence of embryos, but bull variations influenced trophectoderm and total cell numbers and quality of blastocyst derived *in vitro*. Additionally, excellent or good quality blastocysts had more total cell number than moderate or low quality blastocyst in the experimental groups.

The use of spermatozoa from different bulls during *in vitro* fertilization results in variable fertility rates [16]. Saeki et al.[17], Marquant-Le Guienne et al.[18] and Thara and Nair [19] showed that spermatozoa from different bulls have different rates of fertilization on *in vitro* matured bovine oocytes. Similarly, Totey et al.[20] and Jamil et al.[21] reported that fertilization rate was significantly different in buffalo oocytes inseminated with sperm from different buffalo bulls. Unfortunately, fertilization rates of *in vitro* fertilized oocytes with sperm from various bulls were not investigated in the present study, but Ciray et al.[22] indicated that early cleavage is a biological indicator of fertilization. Thus, it may be mentioned that the fertilization rates were not affected by sperm from various bulls in the present study.

Shamsuddin and Larsson [23], Zhang et al.[9] and Sudano et al.[16] reported that the use of sperm from various bulls or bulls with different fertility levels during *in vitro* fertilization leads to different cleavage rates of bovine embryos. Additionally, Al Naib et al.[10] found that *in vitro* fertilized oocytes with sperm from high fertility Holstein Friesian bulls had a higher rate of early cleavage compared with oocytes fertilized by sperm from low fertility Holstein Friesian bulls. Contrary to these studies, in the present study the cleavage rates were not affected by bull variations and average cleavage rate were 73% in the experimental groups. These finding is in agreement with the arguments of Schneider et al.[8] who reported that mean cleavage rates were similar in oocytes inseminated with semen from different bulls with different fertility levels. Also Galli and Lazzari [24] have compared cleavage rates of bulls and they have not seen a difference in the rates of cleavage between bulls. The differences in effects of bull variations on cleavage or fertilization rates with the above-mentioned studies may possibly be due to differences in age and breeds of bull, sufficient capabilities in *in-vitro* fertilization and metabolic characteristics of sperm cells.

Generally blastocyst yield is approximately 25-30% in *in-vitro* embryo production depending on embryo culture media and protocol [25]. In the present study, blastocyst development rates were approximately 27% in the experimental groups. Additionally developmental competence of embryos, obtained from *in vitro* fertilized oocytes with five bull's sperm, until the morula and blastocyst stage were similar in the present study. Moreover morula/cleavage and blastocyst/cleavage rates were similar among bulls. These results agree with the work of Shamsuddin and Larsson [23] who have demonstrated that the use of different bulls during *in vitro* fertilization have similar effect on embryo development until the morula and blastocyst stage. Similarly Sudano et al.[16] reported that although the cleavage rates observed between different bulls changed, differences were not observed among bulls in terms of blastocyst formation rates. On the contrary Akyol et al.[7] show that blastocyst development rate had a wide range among bulls. These differences may be explained with the variation at phenotypic traits and breed type of bulls.

Morphological observations are most widely used as indicator of blastocyst quality [26]. However the cell number of the blastocyst is a valid indicator of the viability of preimplantation embryos, while morphological criteria alone are poor indicators [27,28]. Additionally, Jiang et al.[29] reported that the cell number of *in vitro* derived blastocyst decreased with decreasing quality of the blastocyst. The analysis of distribution of ICM and trophectoderm cells by differential staining has been used as a technique to evaluate embryo quality in several species. Additionally ICM cells/total cell numbers ratio is an indicator of quality of blastocysts. In bovine embryos, poor quality is associated with blastocysts with low numbers of ICM or total cells [25,27,30]. In the present study blastocysts obtained from *in vitro* fertilized oocytes with five bulls's sperm had same cell numbers in ICM and the ICM/total cells ratios. However, trophectoderm and total cell numbers of blastocysts were different between bulls. Moreover quality of blastocysts obtained from *in vitro* fertilized oocytes with five bull's sperm quality of blastocysts varied between bulls. Generally, excellent or good quality blastocysts had more total cell number than moderate or low quality blastocyst in all bulls. Additionally the positive correlations observed between blastocysts quality and total cell number of blastocysts in all bulls. This result supports the view that total cell number influence blastocysts quality

and better quality blastocysts have more cell number than poor quality blastocysts.

The results of this study indicate that the use of sperm from various bulls in *in-vitro* fertilization does not have any effect on embryonic development. However, use of different bulls can influence the quality and cell numbers of the embryos reached the blastocyst stage in similar culture conditions. These results show that effects of bull have to be determined with preliminary studies in *in-vitro* researches and successful bulls should be selected for *in vitro* fertilization. Thus, the effects of bull on the success of *in vitro* embryo production may be eliminated. Moreover, determining the success rate after transfer of embryos obtained from *in vitro* fertilized oocytes with different bulls' sperm will help to identify the bull effect on *in vitro* embryo production more clearly.

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