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RESEARCH ARTICLE

Investigation of Bull Effect on *in vitro* Embryo Production

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Summary

The aim of the study was to show whether there were some differences among 9 Holstein bulls and within their replications on their ability of *in vitro* fertilization for *in vitro* embryo production as cleavage and coming into the blastocyst stage. Semen collected and frozen from nine Holstein bulls with satisfactory in vivo fertilization capabilities for artificial insemination was used for *in vitro* fertilization. Direct washing method by Brackett and Oliphant medium and 5 or 6 h incubation period were used for *in vitro* fertilization. Charles Rosenkrans medium was used for *in vitro* embryo culture. An atmosphere with a higher than 95% relative humidity, 39°C, 5% CO_2 and was used for all *in vitro* embryo production processes. Totally 2519 A and B quality oocytes were treated for *in vitro* embryo production. As a result statistically significant ($P<0.05$) variation was found for cleavage and blastocyst development among bulls. However there was no significant difference ($P>0.05$) between the replications for each bull as cleavage and coming into blastocyst stage. The results showed varied capabilities of bulls for *in vitro* fertilization and embryo production and male factor can affect success of *in vitro* embryo production.

Keywords: *Bull, In vitro, Embryo, Cleavage, Blastocyst*

In vitro Embriyo Üretimine Boğa Etkisinin Araştırılması

Özet

Dokuz farklı Holştayn boğaya ait spermanın kullanıldığı bu *in vitro* embriyo üretim çalışmasının amacı, hem boğalar arasında hem de boğaların kendi tekrarları arasında yarıklanma ve blastosiste ulaşma oranları bakımından fark olup olmadığının gösterilmesi olmuştur. Suni tohumlama boğası olarak kullanılan ve fertilite sorunu olmayan ve tatminkar düzeyde fertilite oranlarına sahip dokuz Holştayn boğadan alınan ve dondurulan spermalar *in vitro* fertilizasyon amacıyla kullanılmıştır. İn vitro fertilizasyon için Brackett ve Oliphant mediumu ile Direkt yıkama metodu ve 5-6 saat inkubasyon periyodu; *in vitro* embriyo kültürü için de Charles Rosenkrans mediumu kullanılmıştır. Kültür periyotlarında %95'in üzerinde bağıl nem, 39°C, %5 CO_2 içeren bir kültür ortamı sağlanmıştır. Toplam 2519 A ve B kalite oosit *in vitro* embriyo elde etme sürecine alınmıştır. Sonuç olarak yarıklanma ve blastosiste ulaşma oranları bakımından boğalar arasında önemli düzeyde istatistiki farklılık bulunmuştur ($P<0.05$). Fakat her bir boğanın kendi içindeki tekrarları arasında yarıklanma ve blastosiste ulaşma oranları bakımından önemli düzeyde fark görülmemiştir ($P>0.05$). Bu sonuçlar boğaların değişik düzeyde *in vitro* fertilizasyon ve embriyo üretim yeteneklerine sahip olduklarını ve *in vitro* embriyo üretim başarısı için erkek faktörünün belirleyici olduğunu göstermektedir.

Anahtar sözcükler: *Boğa, İn vitro, Embriyo, Yarıklanma, Blastosist*

INTRODUCTION

There are many factors affecting of success for *in vitro* embryo production (IVEP) such as oocyte quality, sperm preparation methods and media for *in vitro* maturation, fertilization and embryo culture, duration of fertilization. *In vitro* fertilization ability of bull semen and skills of fertility of the semen are the difference between them for bulls using for artificial insemination (AI) [1]. Semen used for IVEP, cleavage and blastocyst development rates vary depending on the bull fertilization capabilities [2-4]. However *in vitro* fertilization (IVF) capabilities of bulls can be used to predict the bulls' *in vivo* fertility capacity, in order to determine the relationship between the levels of fertilization *in vitro* and *in vivo* is unclear [5,6]. Bulls have a significant correlation in the ability of *in vivo* and *in vitro* fertilization [7]. There are wide variety of methods of IVF, sperm penetration and capacitation that are quite

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satisfactory but differences emerge between bulls in terms of IVEP success [8].

The aim of the study was to determine the variation among Holstein bulls on the individual and general yield of IVEP as cleavage and coming into the blastocyst stage rates.

MATERIAL and METHODS

Cow ovaries were obtained from the local slaughter-house and were transported to the laboratory in transport medium, consisting of physiological saline (0.9%) without antibiotics, at 25°C within 2-3 h. Cumulus oocyte complexes (COCs) (n = 2519) were provided from antral follicles (2-8 mm) by using both of aspiration and also slicing methods. *In vitro* maturation and fertilization were performed according to Kanagawa et al.[9].

A and B quality oocytes were used mixed for *in vitro* maturation (IVM) procedure. All of the A and B quality oocytes were cultured in groups of 20 oocytes per 100 µl TCM-199 (M7528/Sigma-Aldrich Co.) modified by 0.1 mg/ml L - Glutamin, 25 mM HEPES, 5% Fetal calf serum (FCS) and 2 µg/ml FSH (Folltropin-V, Bioniche, Ireland) for 22 h in petri dishes (Falcon 1008/Dickinson).

Semen collected from nine different Holstein bulls was used. One of the bull had most of capability of *in vitro* fertilization (IVF) was used as a control group. The comparisons of bull semen of *in vivo* and *in vitro* fertilization capabilities in all groups were done using this bull has high performance in *in vitro* fertilization.

Semen collected and frozen from nine Holstein bulls with satisfactory *in vivo* fertilization capabilities for artificial insemination was used for *in vitro* fertilization. All the semen was obtained from the bulls at once ejaculated in one week at autumn season and was frozen in Laciphos-477 (IVM, France) and packaged in 0.25 ml straws (IVM, France). In all cases the semen of each bull had about 50-60% motility after thawing was used.

For the purpose of fertilization BO medium modified by 5 IU/ml heparin (H3149, Sigma-Aldrich Co.) and 2 mM Caffeine (C4144, Sigma - Aldrich Co.) was prepared. Optimized amount of heparin already preferred for the laboratory routine was used. Nearly 30.000 spermatozoa were put in insemination droplets per oocyte and motile spermatozoa were at least 15.000 spermatozoa per oocyte after direct washing method of fertilization process for *in vitro* fertilization. Final concentration was adjusted as 6.25 x 10^6 spermatozoa/ml in 100 µL Brackett and Oliphant (BO) medium and covered with 400 µL of paraffin oil (Sigma - Aldrich Co.). Approximately 20 oocytes were added each one of fertilization droplets and petri dishes placed for 5 - 6 h incubation environment.

After fertilization period of oocytes, presumptive zygotes transferred droplets of Charles Rosenkrans medium (CR - 1aa) [10] and cumulus cells were removed from by pipetting with appropriate sized pipette in a few minutes. Denuded oocytes were transferred in groups of 20 in droplets of CR - 1aa. Culture medium was prepared as 100 µL and covered paraffin oil in 35 mm petri dishes (Falcon 3001/Dickinson) and was kept in same incubation environment at least 3 h earlier than culture onset. Embryos had two or more cells were transferred a new culture medium and incubated 5 days more. Culture medium was not exchanged and renewed in this culture period. Evaluation was done on hours 48 and at days 5 after cleavage (two or more cells).

Data were analyzed using general linear model (GLM) procedure of SAS (SAS Inst. Inc., Cary, NC, USA). Means were separated by Duncan test ($P<0.05$). All data were expressed as means ± standard deviation.

RESULTS

Comparison of cleavage and coming into the blastocyst stage for different bull sperm was given in *Table 1*. The highest cleavage and blastocyst development rate were 69.23±13.62% and 28.54±2.96% in control group respectively, the lowest cleavage and blastocyst development rate were 16.85±7.96% and 5.57±0.44% for "F" coded bull in experimental group *(Table 1)*. There was statistically significant difference among Bulls in terms of blastocyst rates of cleavage ($P<0.05$). Cleavage and blastocyst development rate had a wide range among bulls and 4 different groups were emerged in terms of cleavage and blastocyst development among bulls. However, among of repetitions within a bull for each bull, there was no significant difference both in terms of rates of cleavage as well as reaching the blastocyst stage ($P>0.05$).

DISCUSSION

In this study, the cleavage and blastocyst development were determined from semen collected and frozen from nine different Holstein bulls after IVF procedure. Then, they were compared for their achievement rates among bulls and their replications for each bull. A significant level of cleavage and blastocysts development rates were detected among bulls ($P<0.05$), although there was no significant difference among bulls within their own replications ($P>0.05$).

Schneider et al.[6] have found that mean cleavage rate was 57% with different levels of bull semen fertility in their IVF studies. Niwa and Ohgoda [11] identified a 68% penetration rate of spermatozoa for IVF. Brackett and Zuelke [12] were reported a range from 75% to 41% *in vitro* fertilization rates by variable methods. In this study,

Table1. *In vitro embryo production with semen of nine bulls*
Tablo1. *Dokuz boğaya ait sperma ile in vitro embriyo üretim sonuçları*

Bull	Number of Oocyte (n)	Replication of IVEP (n)	Cleavage (%)	Blastocyst (%)
Control	650	34	69.23 ± 13.62^{a}	28.54 ± 2.96^{x}
A	252	12	44.76 ± 12.97^{b}	19.36 ± 2.04^{xwy}
B	169	9	45.33 ± 10.78^{b}	16.76 ± 1.72^{wy}
C	163	9	23.44 ± 6.66^{dc}	10.65 ± 1.05^{yz}
D	232	12	46.25 ± 19.19^{b}	18.37 ± 3.32^{wy}
E	184	10	33.97 ± 14.30^{cb}	12.40 ± 1.49^{yz}
F	203	9	16.85 ± 7.96^{d}	5.57 ± 0.44^{z}
G	476	25	64.79 ± 15.91^{a}	24.97 ± 2.17^{xw}
H	190	9	34.26 ± 12.77^{cb}	11.12 ± 1.00^{yz}
SEM			±2.60	±3.39

Means within same columns with different superscripts differ (P<0.05)

cleavage rates ranged from 16% to 68% in average. Some of these rates were similar to the authors of above with some bulls but some bulls were showed cleavage rates less than those of above authors. This can be interpreted as some bulls may not be sufficient capabilities in *in vitro* fertilization.

Schneider et al.[6] and Galli and Lazzari [13] have compared cleavage rates of bulls in their IVF studies, they have not seen a statistically significant difference in the rates of cleavage. However, Lu et al.[14] reported a significant difference cleavage rates at different fertility levels of the bulls. In this study, there were significant differences (P<0.05) among some of the bulls, but not others (P>0.05) in terms of rates of cleavage. Findings of Schneider et al.[6] and Galli and Lazzari [13] supported of our results for some bulls, the results of Lu et al.[14], was compatible with our conclusions. This situation can be interpreted with the variation of *in vitro* fertilization capability among bulls. Fertilization is not the standard capabilities among any bulls. Therefore, some bulls may have shown a significant difference among *in vitro* fertilization capabilities.

Al Naib et al.[15] have compared Holstein bulls with high and low fertility capabilities and reported that the cleavage rates of high fertility Holstein bulls were significantly better than those of low fertility Holstein bulls. The varying cleavage rates obtained in this study can be due to variable fertilizing capabilities among bulls. If a bull has a high *in vivo* fertilizing capacity, he can be successful for IVF.

Otoi et al.[16] have reported that the rate of *in vitro* embryo developments can even differ among straws of semen collected and frozen at once. Contrary to statement of Otoi et al.[16], each bull did not differ among the replications in this study. This difference can be explained by the fact that due to the different source of oocytes used.

Depending on the choice of medium or protocol, up to 25-30% blastocyst development can be obtained in IVEP [17]. Brackett and Zuelke [12] reported that developing up to morulae/blastocyst stage ranged from 27% to 38% in different cultural environments. Kato and Iritani [18] and Takahashi et al.[19] reached 13.0% and 22.4% blastocyst stage respectively. In this study, blastocyst development rates were similar to the findings of Brackett and Zuelke [12] but development to the blastocyst stage rates for some of the bulls were lower than those of those authors. Blastocyst development rates of two bulls were similar to the findings of Kato and Iritani [18]. While blastocyst development rates were lower than Kato and Iritani [18] for one bull but these rates higher than those authors for other six bulls. This can be explained with the variation at *in vitro* development capacity of bull depend on their fertilization capabilities.

Leibfried-Rutledge et al.[20] reported that IVF outcomes may be directly associated with the selected bulls for IVF. Wiemer and colleagues [21] stated that a large proportion of the success of the *in vitro* culture systems, depend on oocyte selection criteria, culture conditions and additives. The differentiations in blastocyst development observed in the study may be raised from male factor and such as *in vitro* embryo production protocols, culture media involved substances, difference in sperm processing methods as reported by researcher above.

Brackett and Zuelke [12] and Lu et al.[14] reported the highly good results of some bulls and there were significant differences among bulls in terms of blastocysts development rates. Galli and Lazzari [13] reported that there were great differences between bulls for developing to the blastocyst stage. Over 90% of bulls can be used for IVF but all the sires always not give good results for *in vitro* embryo development [13]. In our study, some of the bulls in terms of achievement rates of blastocyst were statistically significant (P<0.05) differences, but others were not significant (*Table 1*; P>0.05). There was a difference in terms of reaching the blastocyst rates of some of the bulls,

which was in agreement with the findings of Barckett and Zuelke [12] and Galli and Lazzari [13]. Al Naib et al.[15] have studied with high and low fertility of the semen of Holstein bulls in IVF studies and noted that their fertility level did not affect the rates of developing to the blastocyst stage. In the present study, the great variations in terms of achievement rates of blastocysts were observed among some bulls. This could be related to the IVF capabilities of the bull semen and sources of oocytes.

Tamassia et al.[22] reported that when they reached the high percentage of cleavage, they had the high proportion of blastocyst stage after *in vitro* embryo culture period in their study. Our findings on blastocyst development are compatible with the results of Tamassia et al.[22]. Because when bulls had high cleavage rate, their coming into blastocyst stage was high in this study.

Amount of heparin and caffeine added in the sperm processing medium plays very important role for the success of IVEP [23]. In the present study, a standard IVF protocol using optimal levels of heparin and caffeine were used. Bull semen were collected and frozen in one week and straws with same lot number were used in this study and statistically significant difference was not observed between replications of the same bull *(Table 1)*.

Gordon [1] has reported that there were differences at success rate depending on the female and male own condition even using standard protocols of IVEP studies. In the present study, the source of semen was a key determining factor for the success of IVEP as reported by Gordon [1].

In conclusion, bulls ought to be known whether it is suitable to use for IVEP, because when bulls are used for the purpose of IVEP these bulls may give different ratios of cleavage and blastocyst development depending on their *in vitro* fertilization capabilities. In addition, if cleavage rate is high for a bull, developing to the blastocyst stage is usually high for that bull, in other word, if a bull has a low cleavage rate for IVEP; he has got low blastocyst development capacity.

Bull semen is one of the important factors for IVEP success. Male may not suitable for IVEP as cleavage and blastocyst development rate; this should be taken into consideration. Thus, it would be better if the bull's semen, which has high level of cleavage and blastocyst development ratio, could be put into IVEP process.

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