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Research article
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Effect of embryo quality on pregnancy outcome in recipient cows and heifers

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ABSTRACT: This study was performed to compare the effects of embryo quality on pregnancy outcome in recipient cows and heifers. Embryos obtained from 83 *Holstein* donors were used in the study. In order to synchronize the recipients, 500 µg of cloprostenol were administered intramuscularly (i.m.), twice, at an interval of 11 days. In all recipients, one embryo was transferred to the upper 1/3 of the uterine horn ipsilateral to the ovary with the corpus luteum on the 7th day after oestrus onset (day 0). Each recipient received an i.m. injection of 5 µg of buserelin at the time ofembryo transfer. Pregnancy examinations were performed by ultrasonography on the day 30th post-transfer. A total of 345 (262 grade 1, 64 grade 2 and 19 grade 3) fresh embryos were transferred to 171 recipient cows and 174 recipient heifers. The pregnancy rates of the recipient cows and heifers were 41.52% and 52.29%, respectively ($p<0.05$).The pregnancy rates achieved with first, second and third quality grade embryos were 45.16%, 25.0% and 12.5% in the cows ($p<0.05$), and 55.11%, 41.66% and 54.54% in the heifers ($p>0.05$), respectively. In conclusion, the pregnancy rates was significantly higher in heifers than in cows ($p<0.05$) and, the embryo quality had a significant impact on recipient pregnancy outcome ($p<0.05$). The effect of embryo quality on pregnancy was significant in cows ($p<0.05$), but not significant in heifers ($p>0.05$).

Keywords: Embryo transfer, embryo quality, pregnancy rate, cows, heifers

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INTRODUCTION

Embryo transfer (ET) is a prominent technique used to shorten the generation interval of genetically valuable individuals, and thereby, to increase herd potential through the transfer of targeted traits to the offspring (Hasler, 2014). While artificial insemination enables genetic improvement with the use of valuable male breeders, ET renders it possible to make use of the genetic potential of high-producing female breeders (Tekin, 2007). Today, ET is a well-established method, which has found common use. Research on the use of multiple ovulation and embryo transfer (MOET) in the bovine has increased the pace of genetic selection and shortened the generation interval via the progeny of high-producing females and genetically superior bulls (Mapletoft and Hasler, 2005). The establishment of an industry specialized in commercial embryo production and transfer has brought about major advances in the technology and practical use of ET (Roper et al., 2018). Today, each year approximately 1.25 million transferable embryos are produced worldwide from high-producing females in vivo and in vitro. Of these embryos, nearly a million are transferred per year. According to data published by the International Embryo Transfer Society (IETS), as of 2016, the mean number of transferable embryos recovered in vivo per donor per flushing ranges from 6 to 7 (Perry, 2017).

Two important factors, affecting the success of ET in cattle, are the selection and the management of recipients. Although the improved management and nutrition of recipients increase the cost of ET, they also increase the profitability of ET by increasing the resulting pregnancy rate (Siqueira et al., 2009). The variability in the pregnancy outcome of embryo transfer is considered to be associated with embryonic, maternal and environmental factors and variances in the combination of these factors. The most significant among these factors are the skill and experience of the practitioner performing ET, the quality of the transferred embryo, and the fittingness of the recipient (Hasler, 2001). Multiple factors, including among others, poor embryo quality, temporal incompatibility between the recipient female, the embryo and uterine enviroment, and inadequate uterine-embryonic interaction, may cause embryonic death (Hasler, 2001). Furthermore, it is reported that the particular site, where the transferred embryo is deposited in the uterus, as well as the complication score of the transfer procedure and the length of time required for embryo transfer also influence the pregnancy outcome (Ferraz et al., 2016). Pregnancy rates achieved with ET in beef and dairy heifers are similar (Hasler, 2001). Nonetheless, pregnancy rates achieved with the transfer of fresh and frozen-thawed embryos are lower in dairy cows, compared to dairy heifers and beef cows and heifers (Putney et al., 1988).

This study was carried out at the premises of the Eastern Mediterranean Agricultural Research Institute under the “Project for the Improvement of the Anatolian Multi-coloured Cattle” with the aim to assess the superovulatory response of dairy donors and compare the pregnancy rates, achieved after the transfer of different-quality-embryos to recipient cows and heifers.

MATERIAL AND METHODS

Animals

Donor animals used in this study were regularly cycling 3 to 7-year-old, healthy, 3-3.5 body condition scored, 500-550 kg body weight, <8500 L first lactation milk yield. Holstein cowswhich were raised at the Research Farm of the Eastern Mediterranean Agricultural Research Institute; they were reproductively sound, regularly cycling, had given birth at least once once and were at <100 days of postpartum stage. The recipients were selected among healthy and reproductively sound Holstein cows (2 to 5 year-old, at <80 days of postpartum stage and in their 1st-3rd lactation) and heifers (over 370 kg body weight). All animals were fed a total mixed ration (TMR) that consisted of wheat straw, alfalfa hay and corn silage as roughage supplemented with concentrate. Donor and lactating recipient cows were fed with 63.21% roughage and 36.79% concentrate (containing 2700 kcal / kg ME and 19% crude protein). Recipient heifers were fed with 74.60% roughage and 25.40% concentrate (containing 2600 kcal/kg ME and 14% crude protein). Prior to the study, each animal underwent uterine and ovarian examination by ultrasonography (5 MHz, Honda HS-101V, Japan). The experiments on animals were conducted in accordance with local Ethical Committee laws and regulations as regards care and use of laboratory animals.

Superovulation

In total, 83 Holstein cows were used as donors. Some of the donors underwent superovulation treatment more than once (55 donors once, 18 donors twice, eight donors three times and two donors four times) a total of 120 uterine flushings were performed. For superovulatory treatment, between days 8-12 of

the sexual cycle, the donors received a total amount of 400 mg FSH (Follitropin V, Bioniche Animal Health Canada Inc, Canada), which was administered by i.m., twice daily for 4 days, at decreasing doses (Day 1 - 80/80 mg, Day 2 - 60/60 mg, Day 3 - 40/30 mg, and Day 4 - 30/20 mg). Two i.m. doses of 500 μg of cloprostenol (Esrumate, Schering Plough/Essex Animal Health, Sedelsberger Strasse 2, 26169 Friesoythe-Germany; Lutelen, Topkapı Pharmaceuticals and Premixes Industry and Trade Inc., Turkey) were co-administered with the 5th and 6th FSH injections. Oestrus monitoring and control of the donors were performed. Oestrus onset was observed 12 h after last FSH injection. Starting from the 12th hour after the last FSH injection, each donor was inseminated (bulls were selected from Holstein top 100 list and each dose contained at least 7 million motile spermatozoa) three times at an interval of 12 h. At the time of the second insemination, all cows were given 10 μg of buserelin (Receptal, Veterinary Pharmaceuticals Marketing and Trade Inc., Turkey) by i.m. route.

Collection and evaluation of embryos

Uterine flushings were performed seven days after the second insemination. Prior to this procedure, each donor cow underwent ultrasonographic examination (5 MHz, Honda HS-101V, Japan and 5 MHz, Honda HS- 2000VET, Japan) to determine the total numbers of corpora lutea (CL) and follicles in the ovaries.

Flushings were performed using 1000 ml of lactated Ringer's solution (Ringer-Fleks, Eczacıbaşı-Baxter Hospital Products Industry and Trade Inc., Ayazağa/Istanbul) containing 1% calf serum (Foetal Bovine Serum Sigma F 9665, Germany) and 0.1% kanamycin (Kanovet, Vetaş Veterinary Pharmaceuticals and Pesticides Joint Stock Company, Küçükçekmece/Istanbul) (13). Prior to the flushings, epidural anaesthesia was established with 4 ml of a local anaesthetic (L-Anestin, Alke Health Products Industry and Trade Inc., Turkey). After the flushings were completed, each donor received 500 μg of cloprostenol i.m. and 500 mg cephapirin benzathine (19 g, Metricure, MSD Animal Health, Turkey) intrauterinely.

Embryo quality was assessed based on morphological integrity, in line with the guidelines of the International Embryo Transfer Society (IETS) (Robertson and Nelson, 2010). The recovered flushing medium was examined under a stereomicroscope to grade the embryos for their quality and morphology. Embryos of first, second and third grade quality were considered to be transferable. The transferable embryos were washed three times in a solution containing TCM-199 (Sigma-M7528, Germany), L-glutamine (Sigma-G5763, Germany), Gentamicin (Sigma-G1264, Germany) and 20% Fetal Calf Serum (38.5 °C, 5% CO_2) and transferred to recipients within 6 hours.

Recipient synchronization

The recipient heifers and cows received two IM injections of 500 μg of cloprostenol, at an interval of 11 days, for oestrus synchronization. The animals were monitored twice daily for signs of oestrus. The oestrus starting time difference between the recipients and the donors was at maximum ±24 hours. The second cloprostenol injection to recipient cows was performed synchronous to the first cloprostenol injection to donors (5th FSH injection). In the case of recipient heifers, the second cloprostenol injection was performed synchronous to the second cloprostenol injection to the donors (6th FSH injection). Epidural anaesthesia was established prior to embryo transfer.

Embryo transfer

The embryos were deposited in the uterine horn ipsilateral to the ovary with the corpus luteum (CL) on the 7th day after oestrus (Day 0). Following the embryo transfer procedure, the recipients were administered with 5 μg of buserelin acetate by intramuscular route. Pregnancy examinations were performed by ultrasonography on the 30th day after embryo transfer.

Statistical analyses

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS) software, Version 20.0. The pregnancy rates of the recipient cows and heifers were compared with the t test. The Kruskall-Wallis test was used to determine the impact of embryo quality on recipient pregnancy outcome and to make a comparison of this impact between cows and heifers. The findings are presented as means and percentages, and mean values are expressed with their standard error or standard deviation.

RESULTS

In this study, 83 Holstein cows were used as donors. Some of the donors underwent superovulation treatment more than once (55 donors once, 18 donors twice, eight donors three times and two donors four times), and in total 120 uterine flushings were performed. Four of the donors did not respond to superovulation treatment, as was confirmed by the absence

of two or more corpora lutea in their ovaries on the day of flushing. On the other hand, from eight of the donors that responded to superovulation treatment, as was confirmed by the presence of two or more corpora lutea in their ovaries on the day of uterine flushing, yet no embryo was recovered from their flushing medium. Pre-flushing ovarian findings, post-flushing embryo recovery rates (Total Ova and Embryos / Total Number of CL x 100) and the numbers of embryos of the different quality grades are presented in Table 1.

Table 1. Ovarian findings, embryo recovery rate and numbers of the different quality grade embryos (Mean±SD), after superovulation of Holstein cows.

Parameter	Value
Number of Donors	83
Number of Uterine Flushings Performed	124
Total Number of CL	1264
Total Number of Follicles	334
Number of First Quality Embryos	471
Number of Second Quality Embryos	90
Number of Third Quality Embryos	21
Number of Unfertilized Oocytes	280
Number of Degenerate Embryos	94
Embryo Recovery Rate (%)	75.63
Mean Number of CL in Ovaries Per Uterine Flushing	10.24 ± 6.41
Mean Number of Follicles in Ovaries Per Uterine Flushing	2.70 ± 3.01
Mean Number of Total Ova/Embryos Achieved Per Uterine Flushing	7.72 ± 7.23
Mean Number of Transferable Embryos Achieved Per Uterine Flushing	4.71 ± 5.86
Mean Number of Dejenere Embryos Achieved Per Uterine Flushing	0.75 ± 1.49
Mean Number of Unfertilized Oocytes Achieved Per Uterine Flushing	2.25 ± 4.60

The pre-flushing total numbers of CL and follicles, and the mean numbers of transferable embryos, degenerate embryos and oocytes are presented in Table 2.

Table 2. Corpora Lutea and follicles recorded, and total ova/embryos, transferable embryos, degenerate embryos and oocytes collected (mean ± SE), after superovulation of Holstein cows.

Donor data	Total Number of Uterine Flushings (n=124) X± SE
Total Number of CL	10.24 ± 0.57
Total Number of Follicles	2.71 ± 0.27
Total Number of Ova/Embryos	7.72 ± 0.64
Transferable Embryos	4.71 ± 0.52
Degenerate Embryos	0.75 ± 0.13
Unfertilized Oocytes	2.25 ± 0.41

X: Mean, SE: Standard Error

In this study, a total number of 582 transferable embryos (of 1st, 2nd and 3rd grade quality) were recovered. Of these embryos, 174 were transferred to recipient heifers and 171 were transferred to recipient cows. The onset of oestrus in all recipients was detected ± 24 hours to that of the donors. The pregnancy number and rate achieved in the heifers were higher than those achieved in the cows (91 - 52.29% and 71 - 41.52%, respectively). The remaining embryos, which were not transferred, were frozen for future use. As can be seen in Table 3, the pregnancy numbers and rates of the recipient cows and heifers differed significantly ($P<0.05$).

Table 3. Single embryo transfers performed and pregnancy rates achieved in the recipient cows and heifers.

Recipient	Number of Transfers	Number of Pregnancies	Pregnancy Rate (%)	P Value
Heifer	174	91	52.29	0.045
Cow	171	71	41.52	0.045
Total	345	162	46.95	

The distribution of the pregnancy rates achieved with respect to the recipient group and embryo quality grade is presented in Table 4. Overall, it was determined that the impact of embryo quality on recipient pregnancy outcome was statistically significant ($p<0.05$). While embryo quality was observed to have a statistically significant effect on pregnancy in recipient cows ($p<0.05$), it was ascertained that the effect of embryo quality on pregnancy was statistically in significant in recipient heifers ($p>0.05$).

Table 4. Pregnancy rate distribution for the different recipient groups after single transfer of grade 1, 2 and 3 embryos.

Recipient	First Grade Embryos			Second Grade Embryos			Third Grade Embryos			P Value
	Number of Transfers	Number of Pregnancies	Pregnancy Rate (%)	Number of Transfers	Number of Pregnancies	Pregnancy Rate (%)	Number of Transfers	Number of Pregnancies	Pregnancy Rate (%)	
Heifer	127	70	55.11	36	15	41.66	11	6	54.54	0.359 P>0.05
Cow	135	63	45.16	28	7	25.0	8	1	12.5	0.025 p<0.05
Total	262	133	50.76	64	22	34.37	19	7	36.84	0.042 p<0.05

DISCUSSION

The success of embryo transfer (ET) procedures depends on the recovery of multiple high-quality embryos from donors and the achievement of the targeted pregnancy and calving rates with the transfer of these embryos to favourable recipients. Several studies have been carried out with the aim to increase both the number of embryos recovered from superovulated animals and the rate of pregnancy achieved after ET in recipients. In order to increase the response to superovulation, protocols have been developed after the synchronization of follicular wave emergence in the donors. In addition, fixed-time embryo transfer programs are implemented in which recipients can be synchronized (ovysynch or ovysynch + P4 etc.) without the need for oestrus detection (Bó et al., 2012).

Several researchers have reported embryo recovery rates of 60% to 70% (Barati et al., 2006; Bülbül et al., 2010). In our study, uterine flushing was performed by fixing the catheter 5 cm from the bifurcatio uteri, as indicated by Bülbül et al. (2010). These previously reported rates being lower than the mean embryo recovery rate in the present study (75.63%) could be attributed to possible differences in several factors, such as the day of embryo recovery, the type of catheter used for embryo recovery, the position of the catheter during uterine flushing, and the skill/experience level of the practitioner performing the flushing procedure, similar to the influential factors suggested in literature (Bülbül et al., 2010).

The success of embryo production in cattle depends on many factors. Donor age, breed, lactation status and milk yield are important. The hormones used for the induction of superovulation affect the success of the application. Another important factor is the difference between starting time of FSH administration (usually 8-12th days of the estrous cycle) and the time of follicular wave emergence (Kaymaz M, 2015). Inaddition, the number, method, dose and duration of FSH administration (Kaymaz M, 2015; Lovie et al., 1994), initiation of FSH administration during the emergence of different follicular waves (Lovie et al., 1994), the effectiveness of hormones and methods used to synchronize wave emergence are factors that affect embryo production (Bó et al., 1995; Wiley et al., 2019).

According to data published by the IETS (1998-2016), the mean number of transferable bovine embryos recovered per flushing ranged from 5.50 to 6.90 at a global level (Perry, 2013; Perry, 2014 ; Perry, 2015; Perry, 2016; Perry, 2017). The reasons for low mean numbers of transferable embryos recovered in the present study (4.71) compared to above studies of other researchers could be related to several widespread factors such as selected breed, yield, and superovulation method, as well as to animals non-responsive to superovulation and the donor animals from which embryos were not recovered. In addition, the IETS data report the average results of beef and dairy donors worldwide while only dairy cattle were used in our study. Kim et al. (2001) induced superovulation to Holstein donors after the aspiration of dominant follicle in the first group and on the 8th day of the estrous cycle in the second group. Their recorded 9.6±1.1;6.1±0.9 CL, 7.7±1.3;3.9±1.0 oocytes and embryos and 4.6±0.9;2.3±0.8 transferable embryos for each flushing, in the two study groups, respectively. The results obtained in the first group are consistent with our results. The inferior results obtained in the second group may be due to the differences in starting time of FSH administration in relation to the follicular wave emergence.

Another major factor, which affects the success of ET, is the recipient animal. In bovine ET practice, heifers and cows are used as recipients (Schmidt, 2010). It has been reported that pregnancy rates achieved in recipient heifers are 10% to 23% higher than those achieved in recipient cows (Hasler, 2014). On the other hand, the use of heifers as recipients presents with disadvantages such as dystocia and difficulties

in calving management and calf care. Although pregnancy rates achieved in cows are lower, 3 to 8-year-old cows are recommended to be used as recipients in view of the advantages they offer, including higher milk yield, known reproductive history, and colostrum production (Schmidt, 2010). It has been reported that the pregnancy rates achieved with ET in cattle range from 50% to 70% (Gordon, 2005). Assumed that the practitioners performing the procedure have the required technical skills, the main factors that affect the pregnancy rate achieved with ET are embryo quality and recipient suitability (Hasler, 2004). In addition, the synchronization between donor and recipient estrus is very important for pregnancy success in embryo transfer (Hasler, 2014). In cases where the difference in the time of estrus onset between the donor and recipient does not exceed ± 24 hours, the success of pregnancy is not affected highly (Kanagawa, 1995; Hasler, 2014).

In the present study, the mean pregnancy rate achieved in the recipient heifers (91/174, 52.29%) was significantly higher ($P<0.05$) than in the recipient cows (71/171, 41.52%). It has been reported that higher pregnancy rates are achieved with the transfer of either fresh or frozen-thawed embryos in heifers than in cows (Hasler, 2001). Lower pregnancy rates in cows have been attributed to lactation-related management requirements and metabolic factors (Hasler, 2005). High-producing dairy cows suffer embryonic death mostly during the first two weeks after fertilization due to physiological and metabolic changes associated with negative energy balance. This high early embryonic mortality has been attributed to the impact of the poor follicular microenvironment on oocyte and embryo quality as well as to the suboptimal uterine environment provided to the embryo and inadequate maternal-embryonic interaction (Lonergan et al., 2016). Köse et al. (2006) have reported pregnancy rates of 27.2% and 56.5% in Brown Swiss-cows and heifers, respectively, following the transfer of in vivo-produced fresh embryos. High pregnancy rates achived in the present study compared to previous studies, after the transfer of fresh embryos, could be related to embryo quality and supportive hormone treatment (GnRH) to increase progesterone levels of recipients at time of ET. Furthermore higher results previously obtained in recipient heifers compared to the present study could be related to different types of cattle breeds (meat or mixed-type) invovled. Julón et al. 2012 reported pregnancy rates of 54.1% and 34.6% in Brown Swiss recipient cows following the transfer of in vivo-produced fresh and frozen-thawed embryos, respectively. These better results could be attributed to differences in the type of cattle used (meat or mixed-type) and physiological and metabolical disturbances of high milk yielding cows included in the group. Greater success is achieved with ET in meat-type breeds than in dairy breeds (Putnry et. al., 1988). Wallacea et al. (2011) achieved a pregnancy rate of 66.3% in meat-type recipient cows with the transfer of in vivo-produced fresh embryos. The lower rate observed in the present study may be related, for the reasons explained above, to the high-producing dairy cows used in the present study.

Embryo quality has a significant role in pregnancy rates after ET. Low pregnancy rates are recorded when low quality embryos are transferred (Hasler, 2001). Nevertheless, in some studies, pregnancy rates achieved with the transfer of first and second quality embryos were not significantly different (Spell et al., 2001). In the present study, the mean pregnancy rates achieved in heifers and cows respectively after the transfer of embryos of different quality grades were 55.11% and 45.16%, for first quality embryos; 41.66% and 25.0%, for second quality embryos and 54.54% and 12.5%, for third quality embryos ($P<0.05$). While the effect of embryo quality on recipient pregnancy outcome was found to be statistically significant in cows ($P<0.05$), it was not significant in heifers ($p>0.05$). Freitas et al. (2004) reported pregnancy rates of 64.7% and 57.4% after the transfer of first and second grade quality in vivo-produced fresh embryos, respectively ($P>0.05$).

Today ET practice involves hormone administration to synchronized recipients, [human chorionic gonadotropin (hCG), gonadotropin-releasing hormone (GnRH) or luteotropic hormone (LH)] (Marques, 2003), concurrent with the transfer procedure, in order to generate an accessory CL, and thereby to increase the serum progesterone level and pregnancy rate. Wallacea et al. (2011) observed a higher pregnancy rate in recipients, which were treated with hCG at the time of ET (61.85%), compared to recipients that did not receive any supplementary hormone treatment (53.9%). Based on this result, they concluded that pregnancy rates achieved with ET could be increased by elevating serum progesterone levels through the generation of an accessory CL with hCG administration. On the other hand, Niles et al. (2019) reported that hCG administered to Holstein heifers during ET increased serum progesterone levels, but did not have

any effect on the resulting pregnancy rate. Torres et al. (2013) reported that treatment with hCG at ET significantly increased the survival rate of demi-embryos and the pregnancy rate of high-yielding lactating dairy cows (untreated 26% and hCG treated 53%, on day 42 pregnancy rate). In our study, pregnancy results in recipient cows were lower than the results in above studies, but higher than those obtained by Köse et al (2006) did not treat the recipients with GnRH, hCG or LH.

CONCLUSION

In conclusion, embryo production and transfer technology in cattle has shown great improvement over the last 20 years. Today, practical applications have become routine. With fresh embryo transfer applications in cattle breeding programs, successful breeding is possible in a much shorter time thus accelerating genetic progression on the female side. In this study, the results obtained with superovulation and ET meet MOET study requirements. In our study, the average number of transferable embryos obtained for each flush, which is an important criterion for the success of superovulation applications, was optimal for high yield dairy donors. This study demonstrated that the pregnancy rates achieved with ET inrecipient cows and heifers differed significantly. The overall impact of embryo quality on recipient pregnancy outcome was statistically significant. However, while the effect of embryo quality on pregnancy was significant in recipient cows, no such effect was observed in recipient heifers. In view of the results obtained in the present study, we suggest that if cows are used as recipients for embryo transfer, first quality embryos should be used to achieve the targeted pregnancy rate. Furthermore, in order to increase the success of embryo transfer, heifers should be preferred to be used as recipients. Moreover, if second and third quality embryos are to be transferred, heifers should be preferred as recipients in the first place.

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CONFLICT OF INTEREST

None declared.

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