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Donor, Recipient and Embryo Contributions to Pregnancy in a Commercial Cattle Embryo Transfer System

Rabie L. Abdel Aziz* · **Abdel-Tawab. A.Y. Khalil** · **Ahmed F.M. Badr**

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Department of Theriogenology, Faculty of Veterinary Medicine, Beni-Suef University, Beni Suef 62511, Egypt.

Correspondence
Rabie L. Abdel Aziz, Department of Theriogenology, Faculty of Veterinary Medicine, Beni-Suef University, Beni Suef 62511, Egypt.
E-mail: rabea.ramadan@vet.bsu.edu.eg

Abstract
Two experiments were designed to investigate factors affecting the risk of pregnancy in recipient Holstein heifers after transfer of *in vivo* produced embryos. The first experiment determined interactions of donor (n=16, multiparous Holstein cows), recipient (n=95 heifers) and embryo associated factors contributing to pregnancy establishment in recipients. Donors were superstimulated using pFSH and embryos were collected on day 7 after insemination, graded and transferred fresh to synchronous recipients. Effects of donor superovulation response (SR, high vs. low), embryo quality grade (first, second or third) and circulating progesterone in recipients at ET (High vs. low) on pregnancy rate per embryo transfer (PR/ET) were evaluated. High SR of donors was associated with higher numbers of all embryo quality grades ($P<0.05$). Recipient heifers receiving 2nd and 3rd grade embryos from low SR donors achieved greater PR/ET (70%), compared to recipients receiving 2nd and 3rd grade embryos from high SR donors (17.65%). In experiment 2, recipient Holstein heifers were allocated into a control (n=48) and a flunixin meglumine (FM) treated group (n=21) in which individual heifers were administered with 10 ml FM at ET to test effects of FM on PR/ET. Recipient temperament was evaluated (calm vs excitable). Excitable recipient temperament at ET was associated with lower PR/ET. Pregnancy tended to be higher (66.67% vs 60.42%) in treated recipients. Conceivably, SR influenced establishment of pregnancy in recipients depending on the quality of transferred embryos. In addition, treatment of embryo recipients with FM at ET may improve PR/ET in excitable recipient dairy heifers.

Keywords
Embryo quality, Flunixin meglumine, Pregnancy, Progesterone, Recipient

1. Introduction
In vivo production and transfer of bovine embryos is a very suitable tool for genetic improvement in cattle populations **(Jaton et al., 2019)**. This reproductive program adds genetic superiority characteristics and ensures dissemination of genetics of top preforming cows **(Abdel Aziz et al., 2017)**. In spite of these facts, prediction of donor response to ovarian super-stimulation and its effects on pregnancy establishment in biological as well as recipient dams is still representing a major challenge facing this technology **(Kasimanickam et al., 2019)**. In addition, successful interaction between embryo and recipient's uterus is very critical for pregnancy establishment in recipients after ET **(Madureira et al., 2022)**. The recipient's uterine environment varies according to variations of progesterone levels and recently based on variations of its temperament at ET with consequences on the risk of pregnancy establishment **(Kasimanickam et al., 2019)**. Other factors related to recipients including nature of estrus (induced vs spontaneous; expressed or not), body measurements, age as well as daily gain at ET may also influence pregnancy outcome in embryo recipient heifers **(Funston and Summers, 2013; Madureira et al., 2022)**. Donor factors affecting establishment of pregnancy in recipients have been extensively studied in cows, however, contribution of recipient heifers to success of pregnancy after ET requires further research, particularly research using therapeutic interventions as flunixin meglumine (FM) **(Kasimanickam et al., 2018, 2019)**. Two experiments were

carried out in the present study. In experiment 1, contributions of donor cows (high vs low SR), recipient heifers and embryos to establishment of pregnancy after ET were studied. In experiment 2, we investigated effects of FM treatment of recipient heifers at ET on PR/ET taking into account several recipient factors including recipient's temperament at ET.

2. Materials and Methods

2.1. Animals, Feeding and Management

All animal handling procedures including drug administrations, embryo flushing, embryo transfer, blood sampling and pregnancy diagnosis were approved according to the guidelines provided by the institutional animal care and use committee, Beni-Suef University, Egypt **(BSU-IACUC: 022-304).**

Experiment 1 was conducted to investigate donor (Mixed parity Holstein cows more than 60 days postpartum; n=16), recipient (Nulliparous Holstein heifers, average age at ET was 14.5±0.5 Months, average daily gain was 0.57±0.05 Kg; n=95) as well as embryo-related factors contributing to pregnancy establishment in an in vivo embryo production-transfer system in a well-managed dairy herd. In experiment 2, nulliparous Holstein heifers were enrolled with similar criteria to embryo recipients included in experiment 1. Donor cows were selected based on excellent production and reproduction traits during the previous lactations. Recipient heifers were selected after meeting the criteria of heifer selection for breeding. Animals were fed totally mixed ration based on their nutrient requirements according to **Nutrition Research Council (2001).** Donor cows were milked thrice daily using an automatic milking parlor.

2.2. Superovulation, Embryo Evaluation and Transfer

Embryos were produced by superovulation of donor cows following the standard protocol employed by **Abdel Aziz et al. (2017)** using a twice daily decreasing dose of porcine follicle stimulating hormone, pFSH (Folltropin-V, Agtech inc, USA). On day zero, each cow received a CIDR insert (CIDR insert, Zoetis animal health, USA). Two days later, each cow received an intramuscular injection of GnRH (12 Ug Busrelin, 3ml Receptal, MSD, New Cairo, Egypt). The protocol of superovulation started on day 4 and included injection of each donor cow with pFSH daily at 6:00 am and 6:00pm starting with 80 mg on the first day of FSH injection and ending with 20mg on fourth day of superovulation. The regimen of injection was 80-80, 60-60, 40-40 and 20-20 accounting for a total dose of 400mg FSH. To induce estrus, PGF2 alpha was administered on day 7 and 8 after the beginning of superovulation. CIDR was removed on day 7. On day 8, another dose of GnRH was administered to each cow. Cows were inseminated using frozen-thawed semen from approved sires twice at 48 and 60 hours after CIDR removal.

On day 7 after insemination, before flushing, the ovaries were scanned by trans-rectal ultrasound to determine the number of corpora lutea. Embryos were recovered non-surgically by flushing the uterine horn using Vigro complete flushing medium (Agtech Inc, USA). Approximately, 250ml solution were used to flush each uterine horn separately using a two-way Foley catheter connected to a Y-shaped connector. Embryos were received onto an embryo filter and were searched under the stereomicroscope. The grading process of embryos was done according to the international embryo transfer criteria **(Stringfellow and Givens, 2010).** After the process of flushing had been completed, SR was determined for individual donor cows. When the total structures collected from a donor cow was greater than 10 structures, it was categorized as high SR donor. On the other hand, when the total structures collected from a donor cow was less than 10 structures, it was classified as low SR donor.

Embryos were non-surgically transferred to previously synchronized recipient heifers on day 7 of recipient's estrous cycle. Recipient heifers were selected based on satisfactory CL quality on day 6 after estrus according to ultrasono-graphic findings. Recipient heifers were examined using 6MHz B-mode linear array transducer (Easi-scan, BCF technology). At embryo transfer, the perineal region of the recipient was washed and dried using a paper towel and was administered with a light dose of epidural anesthesia (4 ml lidocaine HCl). Embryos were loaded into mini-straws and were transferred into the anterior third of the uterine horn ipsilateral to the ovary bearing CL using an embryo transfer gun.

2.3. Pregnancy Diagnosis in Recipient Heifers

Establishment of pregnancy in recipient heifers in the two experiments was diagnosed using ultrasonography on day 30 after embryo transfer. Pregnancy was confirmed by visualization of the embryo surrounded by the anechoic amniotic fluid within the rounded smooth echogenic amniotic membrane **(Kasimanickam et al., 2018).**

2.4. Data Collection

2.4.1. Experiment 1

For donor cows, SR (total structures collected) was recorded as high SR (more than 10) or low SR (less than 10). The number as well as the percentage of each embryo quality grade (grade 1: Excellent embryo; grade 2: good embryo; grade 2: fair embryo; Grade 4: degenerated embryo) was recorded for individual donor cows as well. For recipient heifers, the stage (Morula or blastocyst) and quality grade of the transferred embryo as well as recipient progesterone level (High vs low) at ET were recorded.

2.4.2. Experiment 2

This experiment included 69 Holstein heifers divided into two groups. The control group comprised 48 control recipients which did not receive any treatment at ET. Individual recipient heifers in the treated group (n=21) were administered with 10ml I/M FM (Flunixin injection, Norbrook, 106 King Faisal st., Giza, Egypt) at ET. The following data were recorded for each recipient heifer at ET in experiment 2: age, body weight, wither height, heart girth,

average daily gain, type of estrus, and type of CL, size of CL, heifer temperament one week prior to and at ET (calm vs excitable), quality grade and stage of transferred embryo. The classification of recipient heifers was confirmed at ET. Recipient heifers were classified according to their chute exit score into heifers with calm temperament (calm, slow chute exit, walk) and those with excitable temperament (excited, fast chute exit, jump, trot or run) according to **Kasimanickam et al., (2018).**

2.5. Sampling and Progesterone Analysis

In experiment 1, individual blood samples were obtained from recipient heifers at ET and serum was separated by centrifugation at 1600rpm for 20min. The obtained serum samples were kept frozen till being assayed for progesterone according to the method described by **Mohamed et al., (2015)** using radioimmunoassay.

2.6. Statistical Analysis

2.6.1. Experiment 1

Shapiro-Wilk test was used to test data for normal distribution via the (Descriptive statistics-explore) command of SPSS program version 22. Variations of embryo quality grades between high and low SR donors were tested using independent samples T test. Differences between PR/ET under high and low SR were compared using chi square analysis. Similarly, PR/ET under high and low recipient progesterone concentrations as well as under different embryo quality grades were tested using Chi square analyses. A *p* value <0.05 was considered significant.

2.6.2. Experiment 2

Data were tested using SPSS version 22. Normal distribution of data was confirmed by Shapiro Wilk test. A binary logistic regression analysis was carried out to determine possible predictors of pregnancy in recipient heifers. The outcome variable was pregnancy (Pregnant: 1, Not pregnant: 2). The independent variables included categorical variables heifer temperament at ET (Calm: 1, Excitable: 2), type of recipient heat (Spontaneous:1, Induced: 2), FM treatment (Treated: 1, Control: 2), CL type (Compact: 1, Cavitory: 2), embryo quality grade (Excellent: 1, Good: 2, Fair:3) and embryo stage (Morula: 1, Blastocyst: 2). The continuous variables included in the model were heifer's age, heifer's body weight, heifer's average daily gain, heifer's heart girth, heifer's wither height and size of the CL. A *p* values less than 0.05 was considered significant.

3. Results

3.1. Experiment 1

3.1.1. Effects of Donor's Superovulatory Response on Embryo Quality Grades

As demonstrated in **Table (1),** donor cows with high SR had significantly greater mean numbers of all embryo quality grades including degenerated embryos (P< 0.05). But the percentages of each embryo quality grade were not significantly different between high or low SR donors (P> 0.05) as demonstrated in **Table (2).**

Table 1. Effect of superovulation response of donors on quality grade embryos (mean ± SEM) in super-stimulated Holstein cows (n=16)

Item	High SR (n=6)	Low SR (n=10)	P value
First grade embryo	4.33±1.09	2.20±0.53	0.07
Second grade embryo	2.83±0.70	0.90±0.28	0.01
Third grade embryo	2.33±0.72	1.20±0.44	0.17
Degenerated embryo	5.83±1.45	2.20±0.74	0.03
Transferable embryo	9.50±1.45	4.20±1.45	0.004

SR: Superovulation response; n: Number.
High SR: Total structures collected per donor cow >10; Low SR: Total structures collected per donor cow <10.

Table 2. Effect of superovulation response of donors on percentages of embryo quality grades in super-stimulated Holstein cows (n=16)

Item	High SR (n=6)	Low SR (n=10)	P value
First grade embryo	44.52	46.23	0.90
Second grade embryo	29.14	22.30	0.50
Third grade embryo	26.34	23.97	0.87
Degenerated embryo	37.53	34.81	0.87
Transferable embryo	62.47	65.19	0.86

SR: Superovulation response; n: Number.
High SR: Total structures collected per donor cow >10; Low SR: Total structures collected per donor cow <10.

3.1.2. Effects of Donor's Superovulatory Response on PR/ET in Embryo Recipient Holstein Heifers

Table (3) illustrates the PR/ET in embryo recipients after transfer of embryos recovered from donors with high or low SR. First grade embryos recovered from donors with high SR achieved similar PR/ET to those recovered from donors with low SR (78.95% vs 70%, respectively). In spite of that, PR/ET achieved by 2nd and 3rd grade embryos obtained from low SR donors (70%) was greater (P< 0.01) than that achieved by 2nd and 3rd grade embryos recovered from high SR donors (17.65%). Overall, PR/ET was numerically greater after transfer of embryos obtained from low SR donors (70%), compared to 60.00% after transfer of embryos obtained from high SR donors.

3.1.3. Effects of Embryo Quality Grade on Establishment of Pregnancy in Recipient Heifers

Table (4) clarifies PR/ET in recipient Holstein heifers based on quality grade of transferred embryos regardless of donor's SR category. Transfer of first grade embryos was associated with the greatest PR/ET (79.17%), followed by second grade embryos (65.38%) and the two grades achieved significantly greater PR/ET (P< 0.05) that third grade embryos (33.33%).

3.1.4. Effect of Recipient's Circulating Progesterone Concentration at ET on PR/ET

As demonstrated in Table (5), the overall PR/ET tended (P=0.06) to be greater in high progesterone recipients (77.72%), when compared to recipients with low progesterone levels (59.38%). Numerically greater PR/ET were achieved after transfer of first grade embryos to high progesterone recipients and the same result was observed after transfer of second and third grade embryos.

3.2. Experiment 2

3.2.1. Characteristics of Pregnant and Non-pregnant Recipient Holstein Heifers after Embryo Transfer

Table (6) displays variations in recipient factors between pregnant and non-pregnant Holstein heifers after ET. All the studied recipient factors were similar (P> 0.05) between pregnant and non-pregnant recipients except for CL size which was greater in non-pregnant recipients (2.34±0.06cm), compared to 2.18±0.04 cm in pregnant recipients.

Table 3. Effects of donor superovulatory response (SR)* on pregnancy rate per embryo transfer in recipient Holstein heifers

	High SR	Low SR	P value
PR/ET for first grade embryos %(n/n)	78.95 (30/38)	70 (7/10)	0.41
PR/ET for second and third grade embryos %(n/n)	17.65 (3/17)	70 (21/30)	0.001
Overall PR/ET %(n/n)	60 (33/55)	70 (28/40)	0.22

n: number; PR/ET: Pregnancy rate per embryo transfer, calculated as number of recipient heifers established pregnancies, divided by the total number of recipients under each category.
* High SR: total structures collected greater than 10; Low SR: total structures collected less than 10.

Table 4. Effects of quality grades of embryos transferred to recipient Holstein heifers on pregnancy outcome and sex of the newborn.

Embryo quality grade	First grade	Second grade	Third grade	P value
Transfers (n)	48	26	21	NA
Established pregnancies (n)	38	17	7	NA
PR/ET (%)	79.17[a]	65.38[a]	33.33[b]	0.01

n: Number; NA: not applicable; PR/ET: Pregnancy rate per embryo transfer, calculated as number of recipient heifers established pregnancies, divided by the total number of recipients under each category.

Table 5. Effect of recipient progesterone on day of embryo transfer on recipient pregnancy rate.

	High progesterone	Low progesterone	P value
Serum progesterone at ET (ng/ml)	10.02±1.73	5.89±0.84	0.01
PR/ET for first grade embryos % (n/n)	88.24 (15/17)	74.19 (23/31)	0.22
PR/ET for second and third grade embryos % (n/n)	64.29 (9/14)	45.46 (15/33)	0.22
Overall PR/ET % (n/n)	77.72 (24/31)	59.38 (38/64)	0.06

Values were expressed as (mean ± SEM) or as percentages; n: Number; PR/ET: Pregnancy rate per embryo transfer, calculated as number of recipient heifers established pregnancies, divided by the total number of recipients under each category

3.2.2. Predictors of PR/ET in Recipient Holstein Heifers

None of the variables included in the logistic regression model were associated with significant variations (P>0.05) in PR/ET (Table, 7). It was observed that FM treatment at ET tended to affect PR/ET (P=0.08) in the binary logistic model. It is important to notice that excitable recipients that did not receive FM at ET had lower PR/ET (42.12%), as compared to calm recipients not that did not receive FM at ET (72.41%) or to excitable recipients that received FM at ET (66.67%).

Table 6. Characteristics (Mean ± SEM) of pregnant and non-pregnant recipient Holstein heifers after embryo transfer in experiment 2.

Item	Pregnant recipients	Non-pregnant recipients	P value
Age (year)	1.24±0.012	1.24±0.015	0.97
Body weight (Kg)	312.82±1.99	316.46±2.96	0.30
Daily gain (Kg/day)	0.95±0.053	0.92±0.056	0.72
Wither height (cm)	121.77±0.33	122.62±0.52	0.16
Heart girth (cm)	173.51±0.88	175.31±0.98	0.18
CL size (cm)	2.18±0.04	2.34±0.06	0.03

Table 7. Predictors* of PR/ET in recipient Holstein heifers submitted to experiment 2.

Variable	B	SE	Wald	P value
Age	-4.97	4.79	1.08	0.29
Wither height	-0.02	0.13	0.02	0.87
Body weight	0.02	0.03	0.38	0.54
Daily gain	0.31	0.95	0.10	0.75
Heart girth	0.07	0.07	0.96	0.33
Heat type	1.32	0.82	2.6	0.11
Temperament	-1.16	0.78	2.1	0.14
Treatment	1.48	0.82	3.17	0.08
CL type	-0.71	0.66	1.14	0.29
CL size	0.57	1.23	0.21	0.65
Embryo stage	-0.57	0.70	0.65	0.42
Embryo grade	-0.40	0.44	0.85	0.36

* A binary logistic regression model was built where pregnancy was included as the independent variable, while independent variables were included as: categorical variables including heat type, temperament, treatment, CL type, embryo stage and embryo grade. Meanwhile, age, body weight, wither height, heart girth, daily gain and CL size were included as continuous numerical variables.

4. Discussion

The present study aimed to determine the contribution of donor, recipient and embryo to PR/ET in a commercial MOET program in a well-managed dairy herd. Two experiments were designed to fulfill the purpose of this study. In the first experiment, interactions among donor's SR, embryo grade and recipient progesterone level at ET and their effect on the risk of pregnancy establishment in embryo recipient heifers were studied. A second experiment was carried out on two groups of recipient heifers to determine effects of FM treatment at ET on PR/ET. In the second experiment, several recipient factors have been included in a statistical model to test for predictors of PR/ET.

In the first experiment, donor cows with high SR (Total structures collected greater than 10) had greater means of all embryo quality grades including degenerated embryos when compared to donors with low SR. Antral follicle count is correlated with SR of donor cows **(Singh et al., 2004).** Thus, it might be possible that donors with high SR have had greater antral follicle populations at the beginning of the protocol. Modulation of serum metabolic profile as well as uterine progesterone levels by superovulation on day of embryo recovery with possible effects on number of transferable embryos has been reported in super-stimulated Holstein cows **(Rasolomboahanginjatovo et al., 2014).** Higher numbers of degenerated embryos in donor cows with high SR were previously recorded by **Hussein et al., (2014).** The higher number of degenerated embryos recovered from high SR cows might be due to lack of optimal developmental plasticity.

When embryos recovered from High SR donors were transferred to recipient heifers, the overall PR/ET was numerically lower than that achieved in heifers receiving embryos recovered from low SR donors as presented in **Table (3).** In addition, the pattern of pregnancy differed after transfer of different embryo quality grades. Notably, second and third grade embryos recovered from donors with low SR achieved greater PR/ET (P<0.01). It is likely that the higher numbers of embryos in the uteri of high SR donors had been an obstacle against normal developmental plasticity of inferior quality embryos either in the uteri of donors during the first seven days before being collected or after being transferred to recipient heifers. Regardless of the quality grade of transferred, compared to day 7-embryos produced by AI, there is a greater risk of pregnancy loss after transfer of day 7- embryos produced by superovulation **(Demetrio et al., 2007).** An embryo produced by AI of single ovulating cow will have the opportunity to benefit from the entire uterine environment during the most critical period (first week) after fertilization. So, it is plausible that higher numbers of embryos in the fallopian tubes and the uteri during the first 7 days after fertilization in high SR donor cows might have been associated with absence of optimal conditions that support their further development after transfer to recipients. In a similar context, gene expression analysis studies comparing embryos produced after MOET and those produced after AI of single ovulating cows proved that genetic characteristics of blastocyst have been altered by alterations in uterine environment under the effects of superovulation **(Gad et al., 2011)** and that genes related to stress responses have been upregulated in embryos produced by MOET.

Regardless of the donor SR category, the quality grade of the transferred embryo significantly influenced the risk of pregnancy establishment in recipient heifers in the present study **(Table, 4).** PR/ET were greater after transfer of First grade and second grade than after transfer of third grade embryos (P<0.05). Several studies reported significantly greater PR/ET after transfer of first grade embryos, when compared to second grade embryos **(Ferraz et al., 2016; Erdem et al., 2020).** These findings contradict the results reported in the present study likely due to the relatively small numbers of each embryo quality grade category in this study.

The overall PR/ET tended (P=0.06) to be greater in recipients with high circulating progesterone at ET (70.72%), compared to recipients with low circulating progesterone at ET (59.38%) regardless of the quality of transferred embryo. Based on the quality of transferred embryos, PR/ET was numerically greater when first grade embryos or second and third grade embryos were transferred to recipients with high progesterone. **Oshba et al., (2019)** reported that recipients with cavitory CL expressed greater serum progesterone

levels and achieved greater PR/ET. Contradictory results were reported by **Niemann et al., (1985)** who demonstrated that variations of plasma progesterone concentration on the day of ET did not affect the resultant PR/ET in embryo recipients.

Table (6) demonstrates recipient factors in pregnant and non-pregnant heifers included in experiment 2. Based on the information displayed, none of the studied recipient factors varied between pregnant and non-pregnant heifers except for CL size on the day of ET. The CL size at ET was greater ($P< 0.05$) in non-pregnant recipients than in pregnant herdmates. In accordance, **Oshba et al., (2019)** reported that recipients with cavitory CL had smaller CL diameters and achieved greater PR/ET. However, **Nogueira et al., (2012)** in beef recipients showed that PR/ET was increased by increased CL size. Other studies declare no significant correlations between the size of recipient CL at ET and the risk of pregnancy establishment **(Spell et al., 2001; Rodriguez et al., 2007).** Differences in reproductive physiology in different recipient breeds, lactation status and other factors might be the underlying etiologies of such contradiction.

In experiment 2, recipient heifers were classified into a treated group administered with 10 ml FM at ET and a control group. It is important to notice that treated heifers (n=21) had excitable temperaments, meanwhile control heifers had calm (n=25) and excitable temperaments (n=22). Results of the logistic regression analysis revealed that treatment of recipient heifers using FM at ET tended ($P=0.08$) to affect risk of pregnancy. PR/ET in treated recipients was 66.67%, compared to 60.42% in control recipients. In accordance, **Kasimanickam et al., (2018)** reported that PR/ET was numerically greater in beef recipients treated with FM at ET. In that study, there was a treatment by recipient temperament interaction where excitable recipients without treatment expressed significantly lower PR/ET, compared to other groups, as reported herein. Furthermore, regardless of FM treatment, we demonstrated that PR/ET was numerically greater in recipients with calm temperament than in excitable recipients (72.41 vs 55%, respectively). FM is anti-PGF2 and a compelling body of evidence supports its use in beef and dairy embryo recipients to improve PR/ET without altering the return to cyclicity in non-pregnant recipients **(Kasimanickam et al., 2019).**

5. Conclusion

In this study, we declare that the degree of response of embryo donor cows to ovarian superstimulation plays a significant role in pregnancy establishment after transfer of in vivo produced embryos to recipients. In addition, we infer that FM may help to improve PR/ET in recipient dairy heifers with an excitable temperament.

6. Author Contributions

All authors contributed equally to the study including design of the experiments, methodologies, analysis and interpretation of results and drafting the manuscript.

7. Conflict of Interest

The authors declare no conflict of interest.

8. Acknowledgements

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