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Corpus luteum diameter and embryo developmental stage are associated with pregnancy rate: data analysis from 17,521 embryo transfers from a commercial *in vitro* bovine embryo production program

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Abstract

The objective of this study was to assess the association between CL diameter, embryo developmental status and recipient pregnancy rate after *in vitro*-produced embryo transfer. Data from a total of 17,521 embryo transfers from a commercial program were analyzed. The information was organized in pivot tables and the frequency of variables was determined with contingency tables. Additionally, a logistic regression analysis was performed to determine the odds ratio and the degree of association between the variables. Higher pregnancy rates were achieved when the CL was larger than 24 mm (39.7%) and 22 mm (33.7%) in diameter, whereas lower pregnancy rates were associated with a CL of 14 mm (24.2%). Moreover, pregnancy rate was 30.5% when the recipients had a CL greater than 20 mm in diameter, whereas pregnancy rate was 32.0% with a CL smaller than 20 mm ($P < 0.01$). Furthermore, an association between high conception rates and CL diameter was found. With regard to embryo developmental status, higher pregnancy rates were associated with expanded blastocyst (37.0%) and blastocyst (28.8%) transfer. Low pregnancy rates were achieved when morulae were transferred (15.4%). The present results suggest that variables such as CL diameter and embryo developmental status are significantly important and must be considered for the success of an *in vitro*-produced bovine embryo transfer program.

Keywords: corpus luteum diameter, embryo developmental stage, *in vitro* embryo production.

Introduction

In vitro embryo production (IVP) potentially increases the number of offspring from a donor cow during its life (Palma, 2001). However, one of the major disadvantages of this technique is the high cost, which might increase with conception failure after the embryo transfer (ET; Gonçalves *et al*., 2002; Rodriguez-Martinez, 2012). Although this biotechnology has grown exponentially in Colombia, many producers are still reluctant to use IVP due to its low efficiency. Consequently, research has been undertaken to find strategies to reduce costs and increase pregnancy rates of IVP programs in tropical environments (López *et al*., 1995; Dias *et al*., 2006; Ramos *et al*., 2006) and, specifically, under Colombian tropical conditions (Duica *et al*., 2007; López *et al*., 2007; Urrego *et al*., 2008).

Several factors must be considered for an ET program since they might affect the likelihood of pregnancy in the recipient female (Hasler, 2001; Benyei *et al*., 2006; Camargo *et al*., 2006). In cows, the procedure usually involves confirming the presence of a "functional" CL (according to its diameter) and transferring the embryo to the ipsilateral horn. CL diameter has traditionally been used to classify the recipients (Demetrio *et al*., 2007; Duica *et al*., 2007); however, the results of several studies are contradictory. Some studies showed that when luteal diameter increased, the pregnancy rate increased as well (Baruselli *et al*., 2001; Duica *et al*., 2007), but others studies did not find statistical evidence to indicate this finding. When comparing two female recipients under similar conditions, the recipient with the larger luteal structure had a higher progesterone concentration (Kerbler *et al*., 1997; Mann, 2009) and therefore a more suitable uterine environment (Ashworth *et al*., 1989; Lonergan *et al*., 2007; Okumu *et al*., 2010). Progesterone plays a major role during early pregnancy and allows the endometrium to achieve a productive phase in which the endometrial glands synthesize histotroph (Gray *et al*., 2001; Wang *et al*., 2007; Lonergan, 2011). This is required for embryo development, migration, and implantation since it provides growth factors, amino acids, and carbohydrates, among other necessary substances (Barnes, 2000; Spencer *et al*., 2004; Morris and Diskin, 2008). Despite this, several studies in which exogenous progesterone was supplemented or hCG was used to induce diestral ovulations during early pregnancy have been inconclusive (Mann and Laming, 1995; Santos *et al*., 2001; Looney *et al*., 2006; Mann *et al*., 2006).

Another factor that has been considered for ET is the embryo developmental stage (Peixoto *et al*., 2007). With IVP, it is commonly found that not all the embryos are in the same developmental stage at the time of ET; this may constitute a source of variation in results. Generally, the embryo may be at the morula,

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initial blastocyst, blastocyst, expanded blastocyst, or hatched blastocyst stage at the time of ET. Once again, several studies in which embryo developmental stage was analyzed are contradictory (López *et al*., 1995; Peixoto *et al*., 2007).

The aim of this study was to determine the association between CL diameter, the embryo's developmental stage and pregnancy rate in a commercial IVP program in the Colombian tropics.

Materials and Methods

Localization

This study analyzes data collected from January 2003 to August 2009 at the CTELCA: Central de Embriones, Tecnología de Semen y Núcleo de Mejoramiento Genético Las Camelias, located in Puerto Araujo, Santander, Colombia. All animals were under the commercial management of an IVP-ET program.

Population

Data from 17,521 ETs were analyzed. A total of 1,399 (Gyr = 847; Guzerat = 552) healthy, nonpregnant and cyclic cows under grazing conditions (water and mineral salt *ad libitum*) were used as donors. Exogenous hormones for synchronization or stimulation of follicular waves were not used prior to ovum pickup (OPU). A reproductive examination was performed by rectal palpation and ultrasound prior to all OPUs. Additionally, the timing of follicular aspiration depended upon the arrival of the donor at the center and on the client's demands; therefore, the animals were at random stages of the estrous cycle. Less than 15 days passed between OPU sessions for the same cow. A total of 473 OPU sessions were recorded, and the number of OPU sessions for each donor ranged from 1 to 15.

Ovum pickup

For each OPU session, an ultrasound (SSD 500, Aloka, Japan) with a 7.5 MHz microconvex transducer coupled to an intravaginal probe with a stainless steel guide were used. For follicular puncture and oocyte recovery, a disposable needle (16-gauge) coupled to a disposable 50 ml conical tube (Falcon, BD Bioscience) through a silicon hose (100 cm) were employed. Aspiration was conducted using an electric vacuum system (WTA) with 10-12 ml of water/minute pressure. The collection medium was TCM-199 (Sigma Aldrich) supplemented with 25 mM HEPES (Sigma Aldrich) and sodium bicarbonate.

In vitro embryo production and embryo transfer

Embryos were produced for *in vitro* fertilization by a commercial company (Vitrogen, Colombia) using standard protocols previously reported (Dayan *et al*., 2002; Nasser *et al*., 2004). On the day of the transfer (day 7 of embryo development), embryos were evaluated and classified by stage (morula, initial blastocyst, blastocyst, expanded blastocyst, hatching blastocyst, hatched blastocyst) and quality. The latter was based on the symmetry and compactness of the blastomeres. Only structures classified as transferable were used (1: excellent; 2: good). A total of 17,521 virgin and healthy heifers of different breeds (Simmental/Brahman, Gyr/Brahman, Holstein/Brahman and Brahman) were used as recipients. These heifers were synchronized with Crestar and heat was detected using penis-deviated bulls. Additionally, a reproductive check was performed by palpation and ultrasound prior ET, and the presence, position (left or right ovary) and diameter (mm) of the corpus luteum were assessed. To facilitate data analysis and interpretation, the corpus luteum was measured and then categorized by diameter within a 2 mm range (<14 mm, 14 mm, 16 mm, 18 mm, 20 mm, 22 mm, 24 mm, and >24 mm).

Pregnancy diagnosis

Transrectal ultrasound examination was performed between days 30 and 45 on all recipients to determine the pregnancy status. A confirmation examination was performed 60-70 days post ET.

Statistical analyses

Pregnancy rate was analyzed using a logistic regression methodology to detect the degree of importance of each variable and to quantify the variations in pregnancy probability associated with the changes in those variables. The recipients' pregnancy status was included in the model as a binomial distributed variable with the probability (Pi) of the ith recipient becoming pregnant after ET and the probability (Qi=1_Pi) of the ith recipient not becoming pregnant. The logistic regression model was used to produce the maximum likelihood estimates of regression coefficients and to calculate the odds ratio to determine the effect of the independent variables on pregnancy status. The model was created using the PROC LOG procedure of SAS 9.0 (Statistical Analysis System Institute, Cary, NC, USA).

Results

Sixty days after ET, a total of 5,495 recipients (31.4%) were pregnant. CL diameter ranged from <14 to 26 mm, with 18 mm being the most common diameter. The most common embryo developmental stage at transfer was expanded blastocyst. Results from a contingency table showed that 39.7% of recipients with a CL larger than 24 mm in diameter were pregnant, suggesting a positive relation between CL diameter and pregnancy. Also, it was observed that the highest conception rate was achieved when an expanded blastocyst was transferred (Table 1).

Table 1. Pregnancy rate of the recipients after *in vitro* embryo produced transfer, corpus luteum size, and embryo developmental stage.

Variable	Level	Pregnancy rate (%; n)
Corpus luteum diameter	<14 mm	24.18 (91)
	14 mm	29.53 (508)
	16 mm	30.14 (3,205)
	18 mm	31.08 (4,208)
	20 mm	31.21 (4,095)
	22 mm	33.72 (2,432)
	24 mm	31.49 (2,836)
	>24 mm	39.73 (146)
Embryo developmental stage	morula	15.41 (836)
	initial blastocyst	21.33 (2,489)
	blastocyst	28.83 (4,561)
	expanded blastocyst	37.04 (8,900)
	hatching blastocyst	31.56 (488)
	hatched blastocyst	30.43 (247)

A logistic regression model was constructed to confirm the existence of associations between the variables. The odds ratio results for the embryo developmental stage are given in Table 2. Regarding developmental states, ET at the morula stage was associated with the lowest pregnancy rates ($P < 0.05$). Likewise, the transfer of initial blastocyst embryos showed the second-lowest pregnancy rates and was different from blastocyst, expanded blastocyst and hatching blastocyst ($P < 0.05$). The highest pregnancy rates were found when expanded blastocysts were transferred ($P < 0.05$). Finally, no significant differences were found when comparing the pregnancy rates of transferred blastocyst and hatching blastocyst embryos.

Table 2. Confidence interval of the odds ratio for embryo developmental stage and pregnancy rate.

Embryo developmental stages	Estimated	Confidence limits (95%)	
Initial blastocyst *vs*. blastocyst	0.671	0.597	0.753*
Initial blastocyst *vs*. expanded blastocyst	0.461	0.415	0.512*
Initial blastocyst *vs*. hatching blastocyst	0.586	0.473	0.726*
Initial blastocyst *vs*. hatched blastocyst	0.624	0.457	0.852*
Initial blastocyst *vs*. morula	1.494	1.214	1.837*
Blastocyst *vs*. expanded blastocyst	0.687	0.636	0.742*
Blastocyst *vs*. hatching blastocyst	0.874	0.714	1.069
Blastocyst *vs*. hatched blastocyst	0.931	0.687	1.26
Blastocyst *vs*. morula	2.227	1.834	2.705*
Expanded blastocyst *vs*. hatching blastocyst	1.272	1.046	1.547*
Expanded blastocyst *vs*. hatched blastocyst	1.355	1.004	1.827*
Expanded blastocyst *vs*. morula	3.242	2.685	3.915*
Hatching blastocyst *vs*. hatched blastocyst	1.065	0.749	1.515
Hatching blastocyst *vs*. morula	2.549	1.956	3.323*
Hatched blastocyst *vs*. morula	2.394	1.689	3.391*

*Indicates the presence of statistical differences.

The statistical association between luteal diameter and pregnancy status of the recipient was calculated using an odds ratio analysis (Table 3). A CL larger than 24 mm was associated with higher pregnancy rates. This was statistically significant when compared to <14 mm, 14 mm, 16 mm, 18 mm, 20 mm, and 24 mm CLs, but not significant when compared to 22 mm CLs. When the pregnancy rates achieved for the 22 mm luteal diameter recipients were analyzed, a significant difference was found when comparing with <14 mm, 16 mm, 18 mm, and 20 mm CLs ($P < 0.05$), but there were no significant differences when compared with 14 mm and 24 mm CLs.

Finally, when grouping all data from recipients with CLs larger than 20 mm and smaller than 20 mm, it was found that the first group had higher pregnancy rates (32.0%) when compared with the second group (30.5%; $P < 0.01$; Table 4).

Table 3. Confidence interval of the odds ratio for corpus luteum diameter and pregnancy rate.

Corpus luteum diameter	Estimated	Confidence limits (95%)	
<14 mm *vs*. 14 mm	0.702	0.417	1.182
<14 mm *vs*. 16 mm	0.693	0.425	1.131
<14 mm *vs*. 18 mm	0.656	0.403	1.07
<14 mm *vs*. 20 mm	0.655	0.402	1.067
<14 mm *vs*. 22 mm	0.585	0.358	0.957*
<14 mm *vs*. 24 mm	0.646	0.395	1.054
<14 mm *vs*. >24 mm	0.439	0.243	0.791*
14 mm *vs*. 16 mm	0.987	0.802	1.214
14 mm *vs*. 18 mm	0.935	0.762	1.146
14 mm *vs*. 20 mm	0.932	0.76	1.143
14 mm *vs*. 22 mm	0.834	0.675	1.029
14 mm *vs*. 24 mm	0.919	0.746	1.133
14 mm *vs*. >24 mm	0.625	0.424	0.92*
16 mm *vs*. 18 mm	0.947	0.856	1.047
16 mm *vs*. 20 mm	0.944	0.853	1.045
16 mm *vs*. 22 mm	0.845	0.753	0.947*
16 mm *vs*. 24 mm	0.931	0.834	1.04
16 mm *vs*. >24 mm	0.633	0.449	0.893*
18 mm *vs*. 20 mm	0.997	0.908	1.095
18 mm *vs*. 22 mm	0.892	0.801	0.993*
18 mm *vs*. 24 mm	0.984	0.887	1.091
18 mm *vs*. >24 mm	0.669	0.475	0.942*
20 mm *vs*. 22 mm	0.894	0.803	0.997*
20 mm *vs*. 24 mm	0.986	0.889	1.095
20 mm *vs*. >24 mm	0.67	0.476	0.944*
22 mm *vs*. 24 mm	1.103	0.981	1.239
22 mm *vs*. >24 mm	0.75	0.53	1.06
24 mm *vs*. >24 mm	0.68	0.481	0.96*

*Indicates the presence of statistical differences.

Table 4. Pregnancy rate associated with CL diameter in recipients with a diameter of more or less than 20 mm.

CL diameter	Frequency			Rate	
	Pregnant	Not pregnant	Total	Pregnant	Not pregnant
<20 mm	2,446	5,566	8,012	30.53%	69.47%
>20 mm	3,049	6,460	9,509	32.06%	67.94%
Total	5,495	12,026	17,521	31.36%	68.64%

Discussion

The pregnancy rates observed in this study (31.4%) were similar to those obtained by Benyei *et al*. (2006; 34.2%) and Aller *et al*. (2000; 31.5%), but lower when compared to those obtained in studies using embryos produced *in vivo* by superovulation; Peixoto *et al*. (2007; 63.7%) and Farin *et al*. (2004; 79%). This is evidence that the IVP process must be improved to achieve higher pregnancy rates in the future. Some problems related to the IVP embryos include a higher male to female ratio, abnormal intracellular lipid accumulation, mitochondrial density alteration and anomalies in the inner cell mass compaction (Farin *et al*., 2004; Looney *et al*., 2006).

The present results indicated a predisposition for increased pregnancy rates with increased luteal diameters in the recipients. Thus, a higher pregnancy rate (39.7%) resulted when recipients had a CL >24 mm in diameter; recipients with a 22 mm luteal diameter showed similar pregnancy rates (33.7%). These findings are comparable to those previously reported by Baruselli *et al*. (2001). They found that recipients with a CL >20 mm in diameter had 2.44 ng/ml plasma progesterone concentrations and a 58% pregnancy rate; the group with a CL of 15 mm in diameter had 1.75 ng/ml and a 41% pregnancy rate; and the animals with a CL less than 15 mm in diameter had 1.19 ng/ml and a 31% pregnancy rate. Other studies, unlike ours, did not find a positive correlation between pregnancy rates and luteal diameter. Spell *et al*. (2001), using data from 763 ETs, and Rodriguez and Giraldo (2007), using data from 174 ETs, did not find evidence of the luteal effect on gestational rate. Also, results reported by Benyei *et al*

(2006), in which only recipient females with 5 (excellent: large, prominent and >2.0 cm) and 4 (good: good quality, defined and >2.0 cm) luteal scores were included, did not find a positive relationship. All this suggests that there are many factors involved in the recipient's pregnancy. This work concludes that luteal diameter could be an important factor. However, there must be other variables involved in the recipient's selection process. It is very important to perform a thorough reproductive test of the recipient, ideally by ultrasound, since rectal palpation alone may not yield accurate luteal size determination (Looney *et al*., 2006). It is accepted that animals with larger luteal diameters synthesize higher progesterone concentrations (Kayacik *et al*., 2006; Looney *et al*., 2006; Siqueira *et al*., 2009), which in turn promotes endometrial adaptation to become receptive. Kayacik *et al*. (2006) found a significant and positive correlation (r: 0.92) between luteal diameter and progesterone serum levels in normal-cyclic cows. Also, Siqueira *et al*. (2009) concluded that the luteal tissue area was highly correlated with plasma progesterone concentrations ($r = 0.86$; $P < 0.001$). Progesterone prepares the uterine environment to receive and stimulate the embryo's development, involving growth factors and nutrients for the success of the preimplantation period (Gray *et al*., 2001; Spencer *et al*., 2004; Gonella *et al*., 2010).

The present study also evaluated the importance of the embryonic developmental stage as related to pregnancy rates. Expanded blastocyst and blastocyst stage embryos were related to higher pregnancy rates (37.0 and 28.8%, respectively), whereas the lowest results were found after morulae were transferred (15.4%). Similar results were found by Peixoto *et al*. (2007), who conducted a logistic regression model to analyze data from 5,627 ETs and found that the lowest pregnancy rates resulted after morulae and hatched blastocysts were transferred, and higher pregnancy rates occurred when early blastocysts and blastocysts were transferred. There is no clear evidence suggesting why pregnancy rates are higher when a blastocyst is transferred. However, several authors recommend transferring embryos after the compact morula stage and before the hatching blastocyst stage (Schneider *et al*., 1980; Hasler, 2001).

Considering the amount of data analyzed and the results of this study, it can be concluded that CL diameter and embryonic developmental stage are important factors affecting pregnancy rates in commercial IVP programs. Additionally, it was established that transferring embryos at the morula stage is less effective and must be avoided when the number of recipients is limited. Since a positive relationship between luteal diameter and pregnancy rate was found, it is concluded that a reproductive exam of the recipient is important for identifying those with a luteal diameter larger than 20 mm to achieve higher pregnancy rates.

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