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Article

Factors affecting the rate of pregnancy by embryo transfers (ET) by *in vitro* fertilization in multibreed heifers under Colombian tropical conditions

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Abstract:

Embryo transfer (ET) is currently considered a biotechnological tool with great importance to multiply and obtain individuals with productive potential and high genetic merit. The objective of this study was to determine the influence of factors such as THI, CL size, embryo development status and weight on the rate of pregnancy by embryo transfer by *in vitro* fertilization in multibreed heifers. Eight hundred forty heifers were selected as recipients, with an average age of 3 years, a weight of 346.5 ± 33.4 kg of live weight, to which a synchronization protocol for ET was applied, after recording the stage of the embryo, ultrasound monitoring and the environmental variables were monitored. The information was analyzed using a logistic regression model to determine the correlation between the independent variables and the dichotomous response variable pregnancy rate. The influence of corpus luteum (CL) size was determined given the significant differences ($P<0.05$) in the sizes of CL1 and CL2. Differences ($P<0.05$) were also found with the stages of BL and BX. In contrast, no statistical differences ($P>0.05$) were found in the other variables. The present study showed the impact of CL size and embryo development on the success of the ET technique.

Key words: Embryo transfer, Humidity-temperature index (THI), *In vitro* fertilization, Corpus luteum (CL).

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Introduction

Embryo transfer (ET) is currently considered a biotechnological tool with great importance to multiply and obtain individuals with productive potential and high genetic merit, capable of improving performance in bovine production systems[(1)]. Several studies show the impact of the factors that influence the effectiveness of the technique, the intrinsic factors which are specific to the animal and are directly related to its physiology, as well as factors related to the embryo [size of the corpus luteum (CL), stage of embryo development, bulls used for fertilization, among others]; and extrinsic factors that are related to the environment that surrounds the animals and that somehow affects the physiology of the animal (environment, nutrition, management, among others)[(2)].

Climatic conditions at levels outside the resting state of the animal frame the productivity of the individual, destabilizing the comfort conditions and subjecting it to alterations of physiological functioning that leads it to an environment of heat stress (HS)[(3)]. Over the years, many studies have established that the temperature-humidity index (THI) can specify, as a function of the combination of the variables temperature and relative humidity, the degree of HS suffered by animals[(4)]. The value of the THI varies between authors, but similarity is found when specifying that the values > 72 present a HS in animals. In this sense, different thresholds are considered to characterize the state of animal comfort, according to the value of the THI, the HS is characterized as: comfort (<68), mild discomfort (68 - 72), discomfort (72 - 75), alert (75 - 79), danger (79 - 84) and emergency (> 84)[(5)]. However, the improvement in the technique and the results of pregnancy rates remain the subject of research considering the number of factors that influence the success of biotechnology[(6)].

The objective of this study was to determine the influence of factors such as THI, CL size, embryo development status and weight on the rate of pregnancy by embryo transfer by *in vitro* fertilization in multibreed heifers.

Materials and methods

The study was conducted in Colombia, in the municipality of Puerto Boyacá, geographically located at 5°58´34´´ N and 74°35´15´´ W, belonging to the department of Boyacá. An observational experiment was carried out in order to measure the variables THI, CL size, embryo development status and weight, it was also estimated how they can affect the rate of pregnancy obtained by ET by *in vitro* fertilization. Recipient multibreed heifers (n= 840) with an average age of 3 yr, with an average weight of 346.5 kg, were selected; sanitary management was carried out prior to reproductive work, vitaminization, deworming, vaccination against reproductive diseases, antibiotic therapy and bath for external parasites. In the same sense, a control weighing was made every month, thus observing the weight gain of the animals. The feeding was implemented by extensive grazing, with the following species predominating: guinea grass (*Megathyrsus maximus*), sweet brachiaria (*Brachiaria humidicola*), para grass (*Brachiaria mutica*), water at will and mineralized salt at 8 % phosphorus, in this way a homogeneous management was provided for all animals. Variables such as breed were not evaluated given the conditions of high variability in the breed component of the recipient females.

The reproductive works began in March in continuous activities until December 2020; before starting the works, the recipients were examined rectally and by ultrasound, selecting animals that did not have anatomical problems of uterus, cervix and ovaries that limited the response to the transfer. The synchronization protocol for the recipients that was implemented was a combination of progesterone with estradiol benzoate as follows: on day 0 a bovine progesterone-releasing intravaginal device (0.6 g of progesterone P4) plus estradiol benzoate (2 mg). The device was removed on d 80 and 300 IU of eCG (equine chorionic gonadotropin), 150 µg of D-cloprostenol (synthetic analog of prostaglandin F2α) and 1 mg of estradiol cypionate were applied[7].

The embryo transfer was performed on d 17 of the start of the synchronization protocol, for this, palpation was first performed by transrectal ultrasonography in order to detect the presence of a corpus luteum in the ovary. Subsequently, for the execution of the transfer technique, 3.5 to 4 mL of lidocaine (epidural anesthesia) was applied to the recipient, the vulvar area was cleaned and then the embryo was placed in the most distal point of the uterine horn ipsilateral to the ovary that presents the corpus luteum[8]. The embryos used were by *in vitro* fertilization obtained and packed by professionals from the laboratory BIOEMBRIO FIV S.A.S. of about 7 d, who determined the stage of the embryo when packed. The embryos used in this study were of the Girolando breed (F1 and 3/8 Gyr X 5/8 Holstein). The technique was applied by a single veterinarian with experience, avoiding variability in the procedure. The detection of pregnancy was performed on d 28 after the transfer process, for which a Chison eco2-vet ultrasound machine with an L7V-A 6.5MHz linear transducer was used,

where it was determined if there was presence of the gestational vesicle in the uterus by ultrasound for the positive diagnosis.

Meteorological data

The environmental data were obtained from the Institute of Hydrology, Meteorology and Environmental Studies (IDEAM), through a meteorological station near the study area. The value of the index was calculated with the temperature and relative humidity data provided by that station. The temperature-relative humidity index (THI) was calculated using equation 1 reported by Habeeb[5]:

$THI = (1.8*At+32) - [(0.55-0.0055* RH) \times (1.8* At -26)]$
Where: RH= Relative air humidity (%) and At= Air temperature (ºC).

Data analysis

The data were analyzed using the statistical program Epi Info version 7[9], a logistic regression analysis was used to reveal the model of the relationship between independent variables (CL size, embryo stage, THI and precipitation) and the dichotomous response variable (pregnancy rate) which was obtained using embryo transfer; this statistical model uses Odds Ratios (OR). Standardized measures that allow comparing the level of influence or strength of the independent variables on the dependent variable, for the level of significance of the tests, $P<0.05$ was accepted. The GLM is described as $Y= \beta_0 + \alpha + e$. Where Y is the response variable 1= pregnant and 0= not pregnant, β_0 is the intercept, α is the effect of the categorical variables under study and e is the statistical error. Likewise, the continuous variable of weight was analyzed by descriptive statistics and an analysis of variance ANOVA, observing the degree of significance that this variable presented when associated with the response variable.

Results

A total of 2,259 synchronizations were performed and an overall response to the fixed time synchronization protocols of 75.6 % was obtained, which allowed carrying out a total of 1,710 embryo transfers by *in vitro* fertilization, where an overall pregnancy rate of 29 % was achieved.

Effect of CL size on pregnancy rate

The classification of the CL according to size allowed obtaining three categories CL1: <15 mm , CL2: 15-25 mm; CL3: >25 mm in diameter[10]. Table 1 shows that when associating the pregnancy rate obtained with the size of the corpus luteum, significant differences (P<0.05) are found for the sizes of CL1 and CL2. After the transfer, it was obtained that the probability of pregnancy (odds ratio, OR) is 0.46 for CL1; 0.62 for CL2 and 0.67 for CL3. This showed that there is 0.21 and 0.16 times more of obtaining a pregnancy with a CL3 and CL2 respectively, compared to CL1; in this way it is more likely to increase pregnancy rates when transferring embryos with larger sizes of CL 3 and 2 compared to CL1.

Table 1: Association of pregnancy rate with CL size

Variable	Pregnancy (%)	Odds ratio	95 % Confidence interval		*P*-value
Size CL1	25	0.4629	0.2636	0.8128	0.0073
Size CL2	31	0.6264	0.4051	0.9688	0.0355
Size CL3	32	0.6709	0.4342	1.0367	0.0722
Constant		*	*	*	0.1255

Underlined values present significant differences (P<0.05).

Effect of embryo stage on pregnancy rate

Embryo stage refers to the time and development of the embryo considering the classification guidelines established by the International Embryo Transfer Society (IETS)[11]; identified with the letters BI: initial blastocyst about 5 d of development, BL: blastocyst with 6 d of development, BX: expanded blastocyst with 7 d of development and BN: blastocyst in hatching, it is breaking the zona pellucida.

The analyses carried out to determine the effect of embryo stage on pregnancy show significant differences (Table 2) with the stages of BL and BX with *P* values of 0.0136 and 0.0000, respectively. It is also observed that the OR probabilities of obtaining a pregnancy by transferring embryos with development stages of BX and BL are 0.9 and 0.5 times more respectively, compared to BI. This may infer that, the greater development in embryos with the zona pellucida intact reflects their greater activity and viability, generating better pregnancy rates than when transferring late embryos, compromising fertility.

Table 2: Association of pregnancy rate with the effect of embryo stage

Variable	Odds ratio	95 % Confidence interval		*P*-value
Embryo stage BI	0.8290	0.5569	1.2342	0.3557
Embryo stage BL	1.4129	1.0737	1.8594	0.0136
Embryo stage BN	0.3060	0.0385	2.4337	0.2630
Embryo stage BX	1.7750	1.3727	2.2952	0.0000
Constant	*	*	*	0.0000

BI= initial blastocyst about 5 days of development; BL= blastocyst with 6 d of development; BX= expanded blastocyst with 7 d of development; BN= blastocyst in hatching.
Underlined values present significant differences $P<0.05$.

Effect of THI on pregnancy rate

Regarding the association of THI with pregnancy rates, no significant differences were found in the percentage of pregnancy with respect to the different values of THI at the time of transfer ($P>0.05$) (Table 3), although it was determined that, in the area where the study was carried out, the animals were in a range of 78 to 83 according to the index framed in danger zone according to the Armstrong classification, given the influence of a highly stressful environment where genetic and adaptive conditions can influence the response obtained.

Table 3: Association of pregnancy rate with the Temperature-Humidity Index (THI)

Variable	Odds ratio	95 % Confidence interval		*P*-value
THI 78 transfer day	1.0818	0.000	>1.0E12	1.0000
THI 79 transfer day	0.7575	0.000	>1.0E12	1.0000
THI 80 transfer day	1.0840	0.000	>1.0E12	1.0000
THI 81 transfer day	0.9087	0.000	>1.0E12	1.0000
THI 82 transfer day	0.9567	0.000	>1.0E12	1.0000
THI 83 transfer day	0.5751	0.000	>1.0E12	1.0000
Constant	*	*	*	1.0000

Effect of precipitation on pregnancy rate

According to the rainfall regime occurred during the period of study and reflected in the data of the meteorological station, it is classified as a period of transition of dry weather with the first rainy season of the year in March, obtaining a value of 184 mm per month; it can also be inferred that the months of greatest precipitation were April and September, with values of 380 and 578 mm, respectively. Likewise, the months with dry season were October, November and December, with values of 1, 71 and 0 mm respectively, of the year 2020. Table 4 shows the analysis of association of the pregnancy rate with precipitation, choosing to generate 4 categories that frame the months according to rainfall regime 1: rainfall 0 to 100 mm, 2: 101 to 200 mm, 3: 201 to 300 mm, and 4: > 301 mm. Taking into account the analyses carried out, no significant differences (P>0.05) were found between the precipitation variable with the response variable, but given the ORs, as the value is >1, the months categorized with the highest precipitation 3 and 4 are more likely to improve pregnancy rates compared to categories 1 and 2, being the least rainy months.

Table 4: Association of pregnancy rate with precipitation

Variable	Odds ratio	95 % Confidence interval		*P*-value
Precipitation 1	0.8368	0.0000	>1.0E12	1.0000
Precipitation 2	0.9175	0.0000	>1.0E12	1.0000
Precipitation 3	1.1640	0.0000	>1.0E12	1.0000
Precipitation 4	1.0937	0.0000	>1.0E12	1.0000
Constant	*	*	*	1.0000

Effect of recipient female weight on pregnancy rate

The average weight of the recipient females at the time of transfer showed an average of 344 ± 107 kg for animals that were not pregnant and 337 ± 42 kg for animals that became pregnant; the analysis of variance showed no statistical differences (P>0.05) that associate weight with pregnancy obtained (Table 5).

Table 5: Analysis of variance of weight associated with pregnancy rate

Variation	Sum of squares	df	Mean squared	F statistic
Between	14888.97033	1	14888.97033	1.79175
Within	12215293.50793	1470	8309.72347	
Total	12230182.47826	1471		

P-value= 0.18071.

Discussion

The results of the present study determined that the size of the CL has an effect on gestation rates after embryo transfer, it was observed that the pregnancy rate was higher as the CL size was larger. Gestation rates were higher in recipients with CL2 and 3 (31 % and 32 % respectively) compared to recipients with CL1 (25 %). Similar results were presented by Alkan *et al*[10] as they showed in their study that the diameter of CL had significant effects on the pregnancy rate during embryo transfer in meat heifers. Similarly, Baruselli *et al*[7] determined that the effect of CL size on progesterone concentration and conception rate in embryo recipients is established, since larger CLs secrete more P4 and this can have a positive effect on pregnancy recognition and, consequently, on effectiveness rates in ET programs. In contrast, other researchers[12] found no statistically significant effect for the physical traits of CL size and quality of the recipients on conception rate [CL volume (*P*= 0.20), CL side (*P*= 0.14)]. Similarly, in their study, Vieira *et al*[13] observed that there were no significant differences in the effect produced by the size of the CL on the percentage of pregnancy obtained.

In this study, significant differences were found for the variable of embryo development stage, obtaining better results when transferring embryos in blastocyst and expanded blastocyst. This can be explained since when transferring an embryo whose development has been faster, it could express more quickly the factors of recognition of pregnancy, compared to one of the same time and of smaller size. This may be supported by a study[13] in which it was observed that the degree of development of the embryo had an important impact on pregnancy outcomes in the recipients. In contrast, several authors[14] did not identify significant differences in pregnancy rates depending on the embryo development status BI, BL and BX. Bényei *et al*[15] reported that the effect that statistical analysis did not reveal significant differences in pregnancy obtained taking into account the stage in embryo development.

The statistical analysis of the present study found no significant differences in the effect of THI on pregnancy rate. In contrast, Silva *et al*[16] observed that from a THI greater than 72, Holstein cows begin to decrease milk production influenced by climatic variables. Other studies[17] conclude that the effects of the low pregnancy rate are influenced by the prolonged heat loads to which animals are exposed in their productive environment than to the THI on the day of service; in their study conducted in Queensland, Australia with lactating Holstein animals, THI 72 was established as the threshold for triggering negative reproductive effects, obtaining reduced conception rates due to the prolonged exposure to heat stress 5 and 1 week before and after service. Likewise, in their study with lactating cows, Schüller *et al*[18] found that the conception rate decreases as the animals are in a heat stress at the time of service as their prolonged exposure to these conditions. They also determined that 1 hour of exposure with a THI of 73 was enough for the conception rate to drop by 5 %.

In this experiment it was concluded that constant exposure to heat stress with THI 73 acts with negative effects on reproduction 42 and 31 d before and after the day of service respectively, decreasing pregnancy rates caused by heat stress. Cordeiro *et al*[19] in their study demonstrated that heat stress negatively affects the conception rates of crossbred (*Bos taurus* × *Bos indicus*) cows transferred to northern Brazil, where they had a decrease in conception rates when THI reached 75.7; in contrast, the month that obtained the highest conception rate presented the most favorable climate during the experiment (THI 73.1). It has been mentioned[20] that high THI levels have a negative effect on the resumption of ovarian activity and reproductive behavior in *Bos indicus* cows kept in grazing, especially if a high THI occurs during the last trimester of gestation.

Precipitation variability in animal production systems in tropical areas can have negative effects on forage growth and quality; representing an important economic condition by altering the productive and reproductive performance of the animal due to the low availability of nutrients[21]. In this study, no significant effect was found on the rate of pregnancy by embryo transfer associated with the rainfall regime occurred at the time of the experiment. In this sense, Mulliniks *et al*[22] obtained similar results as they concluded that the precipitation regime of the dry season and rainy season did not significantly influence (P>0.46) the reproductive activity and performance of the animals under study; also indicating that, given the variability in annual precipitation, animals that have a body condition score of 4 to 4.5 (rating from 1 to 9) may have reproductive performance similar to females with better body condition scores. Added to this, in the study conducted by Fernandes *et al*[23], no significant differences (P>0.05) were found, obtaining a similar gestation rate between the rainy and dry seasons with values of 42.3 *vs* 45.8 %, respectively (P>0.05). In contrast, Scasta *et al*[24] determined a positive impact that precipitation has on forage yield, which would indicate that cold and wet conditions offer a greater level of nutrition for grazing; thus, the different levels of precipitation experienced can be decisive during the key moments of gestation.

The statistical analysis of the study showed that there was no significant difference ($P>0.05$) in the variable of the weight of the recipient female with respect to the pregnancy rate. Similar data were obtained[23] when determining that there was no difference ($P>0.05$) in body weight (346.5 ± 33.4 kg) of the recipients with respect to the gestation rate. They also determined that individual variation in the potential to achieve daily weight gain (DWG) above 250 g/d was the main factor affecting pregnancy rates as DWG increases to 350 g/d, so they obtained this range in their experiment as an optimal threshold to improve results in the averages of pregnancies achieved. In addition, they concluded that pregnancy rates in embryo recipients reared under grazing in tropical climates could be improved by selecting females according to their potential for body weight gain. Contrary to this, in their study, Shorten *et al*[25] found significant difference ($P<001$) as they observed better pregnancy rates with higher body weight before mating (364 ± 77 kg) in females of the Angus breed.

Conclusions and implications

The study shows a significant effect of the size of the CL on the pregnancy rate, observing greater probabilities of a gestation when the embryo is transferred in the ipsilateral horn to a CL3 and 2, presenting a greater probability of not becoming pregnant when the embryo is transferred in a recipient that has a CL1 < 15 mm in diameter. In addition to this, there is a greater probability of obtaining a pregnancy by transferring embryos in a development stage of expanded blastocyst BX and blastocyst BL compared to developments of initial blastocyst BI and blastocyst in hatching BN. Variables such as THI, animal weight and precipitation did not present statistical differences that demonstrate the marked influence of the association of these variables with the pregnancy rate obtained. It is recommended to carry out further research to obtain more information about the effects of factors such as the breed component of recipients and embryos that influence the effectiveness of the embryo transfer technique.

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