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Factors affecting embryo production in superovulated Nelore cattle[1]

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Abstract

Understanding the factors involved in embryo production in superovulated cows is fundamentally important in order to plan a program of embryo transfer. The present study analyzed the relevance of both intrinsic and extrinsic factors related to embryo production in Nelore cows (*Bos taurus indicus*). We studied 884 superovulation inductions in 318 donor cows ranging from 2 to 21 years of age, on six farms located in the state of Mato Grosso do Sul, Brazil. The data were analyzed using a General Linear Model in the SAS software package. The number and quality of the recovered embryos were not affected ($P > 0.05$) by continuous superovulation treatment (i.e., inducing superovulation up to six times), by the source of the hormones used (Pluset ™, Super-Ov ™, Ovagen ™, FSH-P ™), by the hormone dosage (50, 75 or 100%), or by synchronization of follicular wave emergence with progestogens. Donor age negatively affected the number and quality of the embryos. Senile (>14 years) cows produced, on average, 5.0 ± 0.2 fewer total embryos and 3.0 ± 0.1 fewer transferable embryos than young cows ($P < 0.001$). The farm management increased the number of transferable embryos by 2.0 ± 0.4. We conclude that farm management and donor age are the main factors that should be considered when implementing a program of embryo transfer in Nelore cows submitted to superovulation treatment.

Keywords: *Bos indicus,* donor age, farm effect, FSH, ova/embryos, superovulation.

Introduction

Approximately 100,000 transfers of *in vivo*-produced bovine embryos are conducted annually in Brazil (Thibier, 2006). These embryos result from the uterine flushing of approximately 20,000 superovulated donor cows per year. However, although there has been some progress in the methods of superovulation and embryo collection (including improvement of the flushing methods, the elimination of follicular dominance, and the use of progestogens associated with gonadotrophin), the available superovulation protocols continue to give extremely variable responses, making it impossible to accurately predict embryo production from a given donor. Indeed, the average of 5 to 6 transferable embryos per collection from *Bos taurus* (Hasler, 1992) and *Bos indicus* (Baruselli *et al*., 2006) breeds consider a very large range of variation, from zero to more than 50 embryos.

Although still poorly known, the factors responsible for this variability can be considered either intrinsic or extrinsic to the donor (Alvarez, 1994; Kafi and McGowan, 1997). The intrinsic factors include genetics (breeds and individual animals that are more or less sensitive to gonadotrophin), physiological characteristics (including age, ovary conditions or follicular dominance, and the population of follicles at the time of superovulation), nutritional status (body condition and deficits or excesses of nutrients) and sanitary conditions (ovary, uterus and oviduct pathologies). The extrinsic factors include the use of different commercial preparations of FSH (recombinant or pituitary-derived FSH with varying amounts of LH, eCG, HMG, and inhibin), dosage, route of application, season, and farm management (Breuel *et al*., 1991; Alvarez, 1994; Kafi and McGowan, 1997).

Strict evaluation of the influence of these factors on embryo production can contribute to better planning of embryo transfer programs, with greater attention given to the most relevant factors. We chose to perform this study in Nelore cows because of the high number of embryos collected from this breed in Brazil (Baruselli *et al*., 2006). The objective of the present study was to evaluate the effect of physiological factors (age), environmental factors (farm and season), and factors related to the superovulation protocol (hormone type and dosage, timing of the procedure, repeated superovulations) on the embryo production of Nelore cows.

Materials and Methods

Data description

The data used in the present study were collected on six farms located in the state of Mato Grosso do Sul. A total of 1,040 superovulations were

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analyzed between 1995 and 1998. The elimination of incomplete data reduced the total to 884 superovulations performed in 318 Nelore donor cows (*Bos taurus indicus*), ranging in age from 2 to 21 years old. The animals were handled similarly on each farm, grazing in sown grasslands dominated by the grass species *Brachiaria brizantha* and *Brachiaria decumbens*. Mineral salt and water were provided *ad libitum*. Occasionally, some of the animals were fed both forage and concentrates when taken to cattle fairs.

Besides the genetic, phenotypic and economic characteristics, the main variables used to select donors were the manifestation of at least two regular heats before the beginning of superovulation. To induce superovulation, commercially available porcine or ovine pituitary extracts were injected in the muscle in decreasing doses every 12 hours for 4 days. The commercial products used were Pluset™ (Serono, Rome, Italy), Super-ov™ (Ausa International Inc., Tyler, TX), Ovagen™ (ICPbio Ltd, NZ) and FSH-P™ (Burns-Biotec, Omaha, NE). The doses of each hormone varied from 200 to 400 IU for Pluset™, from 28 to 75 IU for Super-ov™, from 10 to 16 mg for Ovagen™ and from 16 to 36 mg for FSH-P™. The superovulation protocol began between the day 8 and day 12 of the estrous cycle, after one heat as a reference.

On two farms, some of the animals were treated with a progestogen implant (Syncro-Mate-B™, Sanofi Inc., Overland Park, KS, USA) tagged in the ear at any stage of the estrous cycle. Five milligrams of estradiol valerate was injected intramuscularly and the cow was tagged. The superovulation protocol began 5 days later. On the 3rd day after the beginning of superovulation, animals received two intramuscular injections of 150 mcg prostaglandin F2 alpha analog, cloprostenol (Veteglan, Lab. Serono, Rome, Italy), within a 12-hour interval. The tag was removed at the time of the second cloprostenol injection.

Artificial insemination (AI) of the donors was performed 12 and 24 hours after the first estrus signal. The ovarian response (number of corpora lutea) was evaluated by rectal palpation at the time of embryo collection, 6 to 7 days after AI. Cows that had fewer than 3 corpora lutea (CL) were considered to be non-responsive to the superovulatory treatment, and were excluded from the embryo production data analysis. The embryos were recovered by flushing the uterine horns with PBS solution (Dulbeco's phosphate-buffered saline, Nutricell - Brazil) through a Foley catheter.

The evaluation and morphologic classification of the embryos were done according to criteria of the International Embryo Transfer Society (Robertson and Nelson, 1998). In the present work, embryos graded I, II and III were considered transferable (or viable), and embryos of grade IV were considered non-transferable (or non-viable). Besides the morphologic quality, the capacity of the embryos to continue their development after transfer into recipient heifers was also evaluated.

Estrus of cyclic heifers was induced by intramuscular injection of 125 mcg Veteglan 1 day before the first Veteglan injection of the donors. Thus, 1,958 fresh embryos were transferred non-surgically to the recipient heifers, whose estrus was more or less synchronized with that of the donors, using the French inovulator (IMV, L'Aigle, France). Pregnancy diagnosis was performed by ultrasound scanning on days 30 to 45 after embryo transfer, and confirmed by rectal palpation 30 days later.

Statistical analyses

The data were analyzed using the General Linear Model (GLM) implemented in the SAS/STAT software. It was estimated that the variability of the data in response to the superovulation protocol was similar within and among donors, so each superovulation treatment was used as an individual observation and we did not make adjustments for correlation among observations in the same donor.

The mathematical model was the following:

$Y_{13} = \mu + A_1 + \varepsilon_{13}$

Y_{13} = Characteristics studied (total number of ova/embryos by collection; number of transferable embryos; pregnancy rate after transfer).

μ = General means.

A_1 = Effects considered fixed (donor age; season; type and dosage of the hormones; farm; progestogen used and number of previous superovulations).

ε_{13} = residual.

Pregnancy rate of recipients transferred with embryos from old and young cows was analyzed by Chi-square test using the frequency procedure of SAS. A probability value of $P \leq 0.05$ was considered statistically significant.

Results

Embryos were collected only from animals with at least three detectable corpora lutea after the superovulation protocol. From 826 collections (93.4% of animals superovulated), 663 (75.0%) resulted in the recovery of at least one transferable embryo, 130 (14.7%) produced no transferable embryos (i.e., produced only oocytes and degenerate embryos) and 33 (3.7%) did not produce any ova/embryos. On average, 10.1 ± 0.3 ova/embryos were recovered and 5.1 ± 0.2 were transferable embryos. Considering only the donors from which embryos were collected, the average number of recovered ova/embryos was 10.8 ± 0.3, with 5.5 ± 0.2 transferable embryos.

The analysis of the results from the 204 donors that had been superovulated more than once, with an interval between treatments of 60 days, did not show any influence of this variable on embryo production (Table 1).

Table 1. Total number of ova/embryos and transferable embryos collected from Nelore cows submitted to consecutive superovulations.

Number of superovulations	n	Ova/Embryos (mean ± SEM)	Transferable embryos (mean ± SEM)
1	204	11.8 ± 0.6	5.8 ± 0.4
2	193	11.0 ± 0.5	5.5 ± 0.4
3	111	10.7 ± 0.6	6.2 ± 0.5
4	66	9.5 ± 0.9	4.5 ± 0.5
5	43	10.0 ± 1.3	5.2 ± 0.7
6	24	10.2 ± 1.3	5.0 ± 0.9

The regression analysis showed a quadratic effect of donor age on the total production ($y = 14.24 + -0.38x^2$ r = -0.39) and on the quality ($y = 7.66 + -0.25x^2$ r = -0.25) of recovered embryos. A decrease was observed in the total number of collected ova/embryos and the number of viable embryos as donor age increased. However, when those embryos were transferred, there was no significant (P > 0.05) effect of donor age on the subsequent pregnancy rate (Table 2).

Table 2. Embryo production of Nelore donors from different classes of age and pregnancy rate of heifers transferred with transferable embryos.

Donor class	n	Ova/Embryos (mean ± SEM)	Transferable embryos (mean ± SEM)	Pregnancy rate (%; n)
2 to 8 years	426	12.0 ± 0.4[a]	6.2 ± 0.3[a]	49.8 (495/1010)[a]
9 to 14 years	294	10.2 ± 0.4[b]	5.1 ± 0.3[a]	51.2 (345/687)[a]
>14 years	106	7.6 ± 0.6[c]	3.4 ± 0.4[b]	49.7 (133/251)[a]

Lower case letter in the same column do not differ (P > 0.05).

There were significant (P < 0.05) differences among farms for the total number of ova/embryos and transferable embryos collected. Farm 5 presented the highest number of recovered ova/embryos (12.3 ± 1.2), while farm 3 had the lowest mean (6.7 ± 1.0). Conversely, farm 2 recovered the highest number of viable embryos (6.1 ± 0.2) and farm 5 the lowest number of viable embryos (4.4 ± 0.7).

Embryo production was not influenced (P > 0.05) by either the type or the dose of the various commercial brands of FSH that were used (Table 3).

Table 3. Total ova/embryos and transferable embryos collected according to the source of commercial hormone and dosage used to induce superovulation.

Hormone	Dose*	n**	Ova/Embryos (mean ± SEM)	Transferable embryos (mean ± SEM)
Pluset™	200 IU	12	8.8 ± 2.1	4.4 ± 1.1
	250IU	123	10.3 ± 0. 6	5.8 ± 0.4
	300 IU	119	10.2 ± 0.7	5.2 ± 0.5
	400 IU	28	9.9 ± 1.5	6.3 ± 1.1
Super-Ov™	28 to 50 IU	18	12.1 ± 2.2	4.2 ± 0.9
	60 IU	35	11.4 ± 1.4	5.0 ± 0.9
	75 IU	18	9.6 ± 1.8	4.4 ± 1.1
Ovagen™	10 and 12 mg	26	11.3 ± 1.7	4.8 ± 1.1
	14 and 16 mg	15	8.3 ± 2.2	4.4 ± 1.3
FSH-P™	16 to 28 mg	25	10.8 ± 1.7	4.6 ± 1.1
	32 and 36 mg	11	8.0 ± 1.6	4.1 ± 1.2

*1 mg is equivalent to approximately 16 IU.
**The dosages and source of hormones with fewer than five replicates were excluded from the analyses.

There was no effect of season on embryo production and quality ($P > 0.05$). Total recovered ova/embryos and transferable embryos were, respectively, 10.6 ± 0.5 and 5.2 ± 0.4 in spring, 10.8 ± 0.5 and 5.7 ± 0.4 in summer, 10.5 ± 0.5 and 5.4 ± 0.3 in fall and 11.4 ± 0.6 and 5.6 ± 0.4 in winter.

There was no significant ($P > 0.05$) difference between the females that began the superovulation protocol in the middle of the estrous cycle (11.5 ± 0.7 total ova/embryos and 5.3 ± 0.5 transferable embryos) and those that were treated with a progestogen implant at any stage of the cycle (10.3 ± 0.9 total ova/embryos and 4.8 ± 0.7 transferable embryos).

Discussion

The results of the superovulatory responses (ovarian stimulation and embryo production) in the present study are similar to those previously reported in *Bos taurus* (Lerner *et al*., 1986) and *Bos indicus* (Baruselli *et al*., 2006) cattle, and confirm that approximately 20% of the donors under the superovulation process do not produce viable embryos. According to Stroud and Hasler (2006), the great variability in individual embryo production may be a result of the unnatural process of superovulation itself. Indeed, under normal conditions cows usually ovulate one ovum per estrous cycle, and the injection of exogenous gonadotropins induces the maturation and ovulation of several follicles that would normally undergo atresia or degeneration (Alvarez *et al*., 1994). This results in fertilization rates ranging from 50 to 70% in superovulated cattle, compared to more than 90% in single-ovulating, non-superstimulated cattle (Hasler, 1992). In addition, conventional superstimulation in the cow requires a 4 to 5 day period of gonadotropin treatment, followed by estrus detection and AI (Alvarez *et al*., 1994). All of these steps provide opportunities for error. As a result, fewer than 20% of the donors produced more than 15 embryos, while the great majority had fewer than four viable embryos.

The data demonstrated that successive superovulations using FSH (pituitary extracts from swine and sheep can be conducted in the same donor without influencing the yield of viable embryos. This confirms previous results for *Bos indicus* (Lamberson and Lambeth, 1986; Zanenga and Silva, 1988) and *Bos taurus* (Dorn *et al*., 1991) breeds. Some previous studies have demonstrated that, when superovulation is induced with eCG (cows, hamsters) or rhFSH (rabbit does) more than once in the same animal, the response to treatment may be reduced (Saumande *et al*., 1978; Loseke and Spanel-Borowski, 1996; Cortell and Viudes de Castro, 2008). This reduced response may be related to an increase of anti-gonadotropin antibodies (Combarnous, 1997). Nevertheless, since there were females in which ovulation rate diminished without an increase in sera antibodies, it is clear that reproduction failure after consecutive superovulation treatments can be caused by different factors, which should be studied in the future. Additionally, we observed that although some cows did not respond to the first superovulation treatment, they often did respond to the second treatment, and vice-versa. This "unpredictability" of the response to treatment was found even after the sixth superovulation. Consequently, within the inherent restrictions of management problems or possible genetic characteristics, a female should not be excluded from the program of embryo transfer based on not having responded to the first superovulation attempt.

The negative effect of donor age on embryo production shown in this experiment has been previously highlighted in *Bos taurus* (Lerner *et al*., 1986; Breuel *et al*., 1991; Malhi *et al*., 2007) and *Bos indicus* (Oliveira *et al*., 2002) breeds. The lesser embryo production from aged cows may be related to follicular and endocrine changes that occur as age increases. Although the follicular wave pattern in older animals is similar to that in young cows, old cows have fewer small ovarian follicles recruited into a follicular wave (Malhi *et al*., 2005), and have fewer large follicles after ovarian superstimulation (Malhi *et al*., 2006). On the other hand, old cows have elevated circulating concentrations of FSH (Malhi *et al*., 2005), similar to aged women with a low storage of ovarian oocytes (Klein *et al*., 1996).

In contrast to equines, in which pregnancy rates of 52 and 23% were reported following transfer of embryos originating from young (3 to 10 years old) and senile (>19 years old) mares, respectively (Ball *et al*., 1989), the age of the cow does not seem to influence the subsequent survival of embryos of apparent good quality when transferred to young recipients. This result seems to confirm recent data of Malhi *et al*. (2007) for crossbred Hereford cows, which suggests that fertilization/cleavage rates are lower in oocytes from old cows than those from young ones, but that embryos that reached the morula/blastocyst stage of development had similar developmental potential regardless of donor age.

In the present study, the farm was the major extrinsic factor responsible for differences in embryo production. In a previous study, Hahn (1992) found that the farm contributed approximately 65% of the variation in embryo production. This emphasizes the importance of farm management in the donor ovarian response. More recently, Stroud and Hasler (2006) evaluated the performance of some farms in the USA for embryo production, and concluded that among the analyzed factors (management of the donor, genetic composition, nutrition, age, lactational phase, quality of the semen and timing of the insemination), the donors' management was the most important isolated variable that affected the results of the superovulation. This is most likely because the animals' well being is related to management practices involving sanitation, food supply and environmental characteristics.

According to Mapletoft (2002), variability in ovarian response is related to differences in superovulatory treatments, such as gonadotropin preparation, batch and total dose, duration and timing of treatment, and the use of additional hormones in the superovulatory scheme. One potentially important difference among the commercial hormones evaluated in this study is their LH content. It has been suggested that high LH levels during superstimulation may adversely influence embryo quality due to premature activation of the oocyte (Moor *et al*., 1984). In a study done with Holstein cows, Donaldson (1990) found greater embryo production with the use of Super-ov™ in comparison with FSH-P™, and attributed this finding to differences in FSH/LH ratio. However, the present study did not find differences in embryo production attributable to the source of FSH. It is possible that the divergence in these results is due to the difference in the sample size or to the use of different hormone batches.

It is usually accepted that the dose of FSH needed to induce superovulation in *Bos taurus* is 30 to 50% higher than the dose recommended for *Bos indicus* (Visintin *et al*., 1999; Barros *et al*., 2003; Barati *et al*., 2006) cattle. Our results confirm this, and indicate that Nelore cows can be treated with up to 60% of the dose recommended for *Bos taurus* without compromising embryo production. This lower amount of FSH needed to induce superovulation in Zebu cattle might be related to the smaller size of the ovarian structures (follicles and corpora lutea), as shown by Figueiredo *et al*. (1997). However, in contrast to Visintin *et al*. (1999), who observed greater embryo production in Nelore heifers superovulated with 60% compared to those superovulated with 100% of the dose recommended for *Bos taurus* beef cattle, the higher dose used in the present study did not negatively affect the yield or quality of the recovered embryos.

A study performed in Mexico with Holstein cows found that the number of transferable embryos was affected negatively by the rainy season, although the ovulation rate was not affected (Monty Jr and Racowsky, 1987). In the present study, however, embryo quality was not affected by the season of the year. There is evidence that *Bos indicus* embryos are less sensitive to heat stress than *Bos taurus* embryos (Krininger *et al*., 2003). The superior thermoregulatory ability of *Bos indicus* cattle makes these animals better adapted for hot climates than many *Bos taurus* breeds (Beatty *et al*., 2006). Thus, it seems that the thermoregulatory mechanisms of *Bos indicus*, as well as some adapted *Bos taurus* breeds (Barati *et al*., 2006), are not severely affected by heat stress. As a result, these breeds are able to respond to the superovulation treatment even in a relatively warm environment.

The superovulatory response in the progestogen-estradiol-treated cows was equivalent to that of control groups superstimulated on days 8 to 12 of the cycle. This result confirms previous findings (Meyer *et al*., 2000; Bo *et al*., 2004) and demonstrates that elective induction of follicle wave emergence offers the advantage of initiating superstimulatory treatment at a time that is optimal for follicle recruitment. Thus, the full extent of the estrous cycle is available for superstimulation, without the necessity of waiting 8 to 12 days to initiate gonadotropin treatments.

Based on the information analyzed above, we conclude that in the planning of an embryo transfer program with Nelore cows submitted to a superovulation protocol, special attention should be given to the farm management as well as to the age of donor cows. These two factors had the greatest effect on the number and quality of the recovered embryos.

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