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Superovulatory response and embryo quality of Holstein heifers treated with one or two injections of somatotropin¤

Respuesta superovulatoria y calidad de embriones de novillas Holstein tratadas con una o dos inyecciones de somatotropina

Resposta superovulatória e qualidade dos embriões de novilhas Holandesas tratadas com uma ou duas injeções de somatotropina

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Summary

Background: recombinant bovine somatotropin (rbST) is used in bovine embryo donors to improve superovulatory response and embryo quality. **Objective**: this study evaluated the effect of applying one versus two injections of 500 mg of rbST to donor Holstein heifers on estrus incidence (IE), diameter of the largest preovulatory follicle (dLPF), superovulation response (SR), embryo yield, and pregnancy rate in recipient Holstein cows (PRR). **Methods:** two superstimulation programs were conducted. Heifers were assigned to one of two treatments: 1) rbST-I, n = 5: heifers received one injection of rbST on day 0 (day of CIDR (controlled internal drug release) removal); 2) rbST-II, n = 5: heifers received the first rbST injection on day -8 and the second one on day 0. Thirty-eight cows were used as recipients and were assigned to receive one embryo from one of the two treatments. **Results**: there was no effect of treatment ($p>0.05$) on PRR, dLPF, IE, and SR. The number of oocytes increased ($p<0.05$) in the rbST-I treatment (1.3 ± 0.4 vs 0.2 ± 0.2), but there was no difference in the number ($p>0.05$) of degenerated (1.0 ± 0.5 vs 4.5 ± 3.0) or transferable (1.0 ± 0.5 vs 1.4 ± 0.7) embryos between heifers in the rbST-I and rbST-II treatments, respectively. Moreover, no oocytes or embryos were recovered from 36.8% of donor heifers in either treatment. **Conclusion**: the application of 500 mg of rbST on days -8 and 0 of the follicular wave synchronization program did not increase the superovulatory response but significantly reduced the number of oocytes recovered from superovulated Holstein heifers.

Keywords: *conception rate, donor heifers, incidence of estrus, preovulatory follicle.*

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Resumen

Antecedentes: la somatotropina bovina recombinante (rbST) ha sido aplicada en vacas donadoras de embriones con el objetivo de mejorar la respuesta superovulatoria y la calidad embrionaria. **Objetivo**: este trabajo de investigación evaluó el efecto de aplicar una versus dos inyecciones de 500 mg de rbST a novillas Holstein donadoras de embriones sobre la incidencia de celo (IE), diámetro del folículo preovulatorio de mayor tamaño (dLFP), respuesta superovulatoria (SR), producción de embriones y tasa de concepción en vacas Holstein receptoras de embriones (PRR). **Métodos**: se llevaron a cabo dos programas de superestimulación. En cada programa las novillas fueron asignadas a uno de dos tratamientos: 1) rbST-I, n = 5: las novillas recibieron una inyección de rbST el día cero (día de remoción del CIDR (dispositivo intravaginal liberador de progesterona); 2) rbST-II, n = 5: las novillas recibieron la primera inyección de rbST el día -8 y la segunda el día cero. Treinta y ocho vacas fueron utilizadas como receptoras. Cada receptora recibió un embrión proveniente de una donadora de uno de los dos tratamientos. **Resultados**: la aplicación de una o dos inyecciones de rbST no afectó ($p>0,05$) la PRR, dLFP, IE ni la SR. El número de ovocitos fue mayor ($p<0,05$) en el tratamiento rbST-I (1,3 ± 0,4 vs 0,2 ± 0,2), el número de embriones degenerados (1,0 ± 0,5 vs 4,5 ± 3,0) o transferibles (1.0 ± 0,5 vs 1,4 ± 0,7) no fue diferente ($p>0,05$) entre las vaquillas del tratamiento rbST-I y rbST-II. Además, del 36,8% de donadoras de ambos tratamientos no se colectó ningún ovocito o embrión. **Conclusión**: la aplicación de 500 mg de rbST los días -8 y cero del protocolo de sincronización de la onda folicular no incrementó la respuesta superovulatoria pero redujo significativamente el número de ovocitos recolectados de vaquillas Holstein superovuladas.

Palabras clave: *folículo preovulatorio, incidencia de celo, novillas donadoras, tasa de concepción.*

Resumo

Antecedentes: a somatotropina bovina recombinante (rbST) tem sido aplicada em vacas doadoras de embriões com o objetivo de melhorar a resposta superovulatória e a qualidade embrionária. **Objetivo**: o presente trabalho avaliou o efeito da aplicação de uma contra duas injeções de 500 mg de rbST em novilhas de raça Holandesa doadoras de embriões na incidência do cio (IE), diâmetro do maior folículo pré-ovulatorio (dLFP), resposta superovulatória (SR), produção de embriões e taxa de gestação em vacas receptoras de raça Holandesa (PRR). **Métodos**: foram feitos dois programas de superestimulação, em cada superstimulação as novilhas foram distribuídas em um de dois tratamentos: 1) rbST-I, n = 5: as novilhas receberam uma injeção de rbST no dia zero (dia de remoção do CIDR -dispositivo intravaginal de liberação controlada de progesterona). 2) rbST-II, n = 5: as novilhas receberam a primeira injeção de rbST no dia -8 e a segunda injeção no dia zero. Trinta e oito vacas foram utilizadas como receptoras e receberam um embrião proveniente de uma doadora de um dos dois tratamentos. **Resultados**: a aplicação de uma ou duas injeções de rbST não afetou ($p>0,05$) a PRR, dLFP, IE nem a SR. O número de oócitos foi maior ($p<0,05$) no tratamento rbST-I (1,3 ± 0,4 vs 0,2 ± 0,2), o número de embriões não-viáveis (1,0 ± 0,5 vs 4,5 ± 3,0) ou viáveis (1,0 ± 0,5 vs 1,4 ± 0,7) não foram diferentes ($p>0,05$) entre as novilhas do tratamento rbST-I e rbST-II. Além disso, do 36.8% das doadoras de ambos os tratamentos não foi possível coletar nenhum oócito ou embrião. **Conclusões**: A aplicação de 500 mg de rbST o dia -8 e zero do protocolo de sincronização da onda folicular não aumentou a resposta superovulatória, mas diminuiu significativamente o número de oócitos coletados de novilhas de raça Holandesa superovuladas.

Palavras chave: *folículo pré-ovulatorio, incidência do cio, novilhas doadoras, taxa de gestação.*

Introduction

Variability in the superovulatory response (number of ovulations) and number of good quality embryos among donors is an inconvenience in bovine embryo transfer programs. This variability has been associated with differences in size of the follicular populations at the beginning of the superstimulation treatment (Chupin and Saumande 1979). According to Ireland *et al*. (2007), the number of transferable embryos flushed per animal is larger in donors with high follicular populations than those with low populations. Thus, to increase yield of transferable embryos, selection of donors with high follicular populations must be considered. On the other hand, size of the follicular population can be manipulated by using recombinant bovine somatotropin (rbST), which produces an increase in the number of recruited small follicles (Gong *et al.,* 1993a) and follicular growth after recruitment (De la Sota *et al.,* 1993; Lucy *et al.,* 1994). Pretreatment of the donor with rbST appears to reduce donor superovulatory variability (Gong *et al.,* 1993b).

Therefore, the use of rbST may be a feasible way to reduce the superovulatory variability among donors by increasing follicular populations and production of embryos.

The effect of rbST on superovulatory response and embryo quality in bovines seems to be time-dependent. After administering a single injection seven (Kuehner *et al.,* 1993) or four (Neves *et al.,* 2005) days before and three days after (Márquez-Hernández, 2011) the beginning of the superstimulatory treatment, rbST improved embryo quality. However, donors given rbST five days before commencing (Gong *et al.,* 1993b) or at the beginning (Kuehner *et al.,* 1993) of the superstimulatory treatment showed an increase in the superovulatory response but not in embryo quality. We hypothesized that superovulatory response as well as the number of good quality embryos can be increased by the administration of two injections of rbST: the first to increase superovulatory response and, consequently, have a larger number of small follicles recruited before initiating the superstimulatory treatment, and the second to further support growth of the recruited follicles and improve embryo quality. Thus, the objective of the study was to determine whether superstimulated heifers receiving two injections of rbST before estrus would have higher superovulation response and more transferable embryos than those receiving one injection.

Materials and methods

Location

The experiment was conducted at "18 de julio" dairy farm of Universidad Autónoma Chapingo. The farm is near Tlahualilo, Durango, México, located at 25º 54´ N and 103° 35´ W, 1,137 m above sea level. The climate of the region is semiarid, with a mean annual temperature of 21.1 °C and 239 mm of rainfall per year (García 1988). The experiment complies with the current laws of Mexico and it was conducted following the guidelines set by the Canadian Council on Animal Care (2009).

Treatment and experimental design

The study was conducted, according to availability of donors and receptors, during the months of June and September, with average daily temperature and relative humidity of 28.3 °C and 40.5%, 25.2 °C and 41.3%, respectively. During each of these months, a superstimulation and embryo transfer program was conducted. In each month, twelve to fifteen month-old healthy cyclic Holstein heifers (June, n = 10, 382.4 ± 29.16 kg live weight and 3.2 ± 0.06 body condition score; September, n = 10, 342.7 ± 29.68 kg live weight and 3.1 ± 0.05 body condition score) and mature Holstein cows with ≥ 3 lactations (June, n = 19, 684.0 ± 5.34 kg live weight and 3.3 ± 0.05 body condition score; September, n = 19, 680.2 ± 5.34 kg live weight and 3.2 ± 0.05 body condition score) were used as donors and recipients, respectively. Donor heifers were randomly assigned to one of two treatments: T1, rbST-I (n = 5) and T2, rbST-II (n = 5). The donor heifers in T1 received a subcutaneous injection of 500 mg of rbST (Boosting-S®, MSD Animal Health, Mexico) in the tail depression on day 0 (day of CIDR removal). Donor heifers in T2 received a first injection of rbST on day -8 and a second injection on day 0 (Figure 1). Due to the limited number donors, there was no control group, and in an attempt to reduce variability in the results, only two experimental groups were formed. The recipient cows were randomly assigned to receive one embryo from one of the two treatments. Nineteen cows were used each month.

Estrus synchronization and breeding

The estrous cycle of donor heifers was synchronized with a progesterone release device containing 1.9 g of progesterone (CIDR 1900 CATTLE INSERT®, Zoetis, Mexico), inserted intravaginally for eight days and an intramuscular injection of 2 mg estradiol benzoate (Benzoato de estradiol®, International Prode, Jalisco, Mexico). Estrus behavior of donor heifers was induced by two intramuscular injections (am and pm) of 500 µg cloprostenol (Celosil®, MSD Animal Health, Mexico). The recipient cows were synchronized using the same protocol as the donor heifers, but they received only one injection of cloprostenol in the morning. After the CIDRs were withdrawn, the synchronized donor heifers and recipient cows were permanently monitored by direct observation for signs of standing estrus. The donor heifers were artificially inseminated three times at 12, 18, and 24 h after standing estrus with a single dose of sexed semen each time from a single bull of

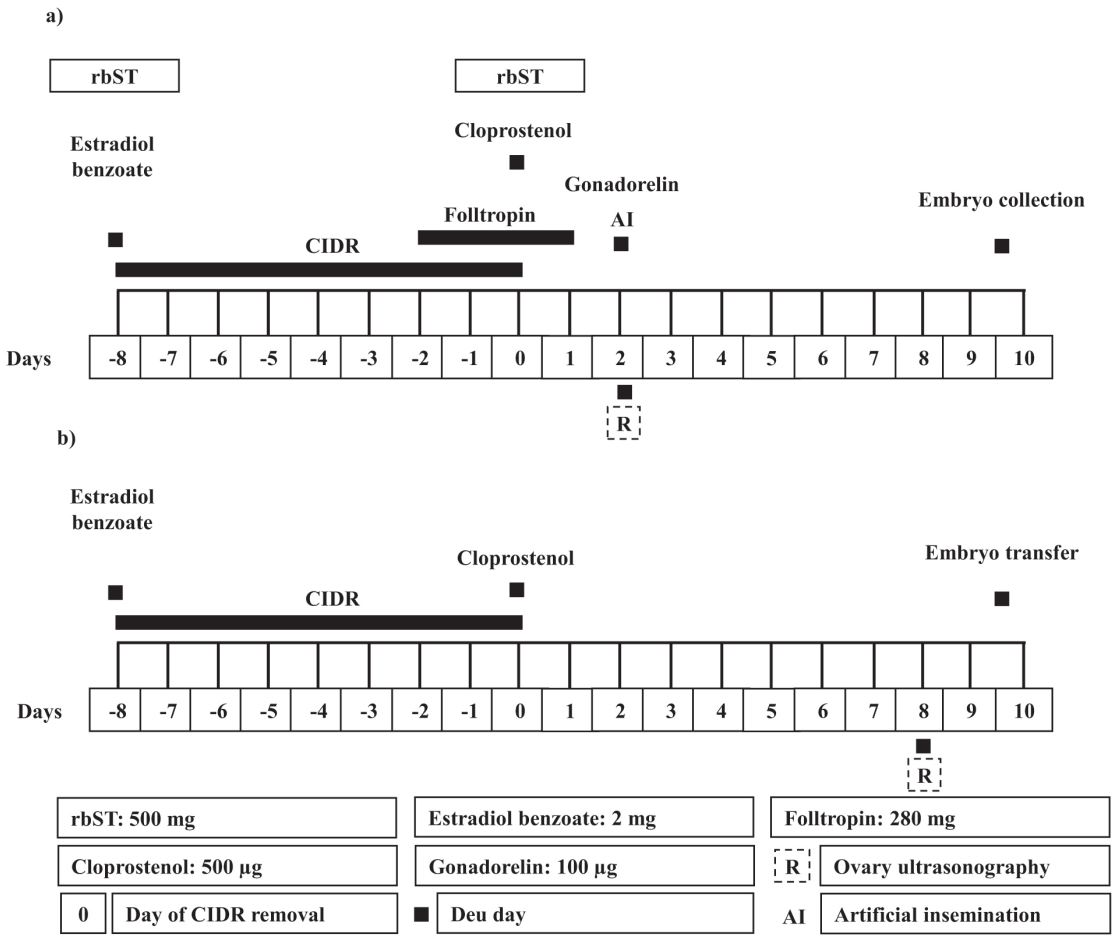

Figure 1. Superstimulation program in donors (a) and estrus synchronization in recipients (b).

proven fertility. To induce ovulation, an injection of 100 µg of a GnRH analogue (Cystorelin®, Merial, Queretaro, Mexico) was given to each donor heifer after the first AI (Figure 1).

Superstimulation program and embryo recovery

The superstimulation treatment of donor heifers was initiated on day -2 (Figure 1). A total of 280 mg of FSH (Folltropin®-V, Bioniche Animal Health, Ontario, Canada) was given twice daily as intramuscular injections in decreasing doses over four days (50, 50, 40, 40, 30, 30, 20, 20 mg). Embryo collection was performed 7.5 days after the onset standing estrus by standard non-surgical procedures. The retrieved embryos were morphologically evaluated and graded according to their stage of development, following guidelines by Wright (2009). Embryos graded quality 1 and 2 were considered transferable and the rest were discarded. One day before embryo collection, recipient cows were scanned (Medison SonoVet 2000, with 7.5 MHz, linear-array transducer; Universal Medical Systems Inc. Bedford Hills, NY, USA) and the ipsilateral uterine horn to the corpus luteum (CL) was established. The embryos were transferred to the ipsilateral uterine horn to the CL in the recipient cows.

Assessment of the effect of double rbST administration

To evaluate the effect of the two injections of rbST on Holstein heifer donors, the variables incidence of estrus (IE), diameter of the largest preovulatory follicle (dLPF), superovulation response (SR), number of oocytes, and number of degenerated and transferable embryos was

determined. Likewise, in recipient cows, the effect of the double rbST administration on pregnancy rate in recipient (PRR) was evaluated. The ovaries of the donor heifers were examined by transrectal ultrasonography (Medison SonoVet 2000, with 7.5 MHz, linear-array transducer; Universal Medical Systems Inc. Bedford Hills, NY, USA) at the beginning of standing estrus, and dLPF was calculated by the average of horizontal and vertical measurements. The SR of donor heifers was measured as the number of corpora lutea present in both ovaries. The number of corpora lutea was measured by transrectal palpation at the time of embryo collection and then categorized into three levels of superovulation response: 1) low (3-5 CL), 2) medium (6-8 CL) and 3) high (≥ 9 CL). Any donor heifer that did not show estrus or had a response of ≤ 2 CL was regarded as not responsive to the ST.

All recipient cows carrying an embryo were subjected to pregnancy diagnosis by rectal palpation 38 days after embryo transfer and PRR was determined.

Statistical analysis

All the response variables were analyzed using the SAS (2004) statistical software (SAS Inst. Inc, Cary, NC, USA) The effect of treatment on the dLPF was analyzed by the Student t-test using the TTEST procedure. The IE, SR, and PRR variables were evaluated by Chi-square test using the FREQ procedure. The number of oocytes as well as the number of degenerated and transferable embryos were compared among groups using the Wilcoxon rank-sum test with the NPAR1WAY procedure. Data are presented as $\bar{X}$ ± SE and were considered to be significant at $p<0.05$. The effect of month was not significant ($p>0.05$), therefore the data were analyzed disregarding this effect.

Results

The dLPF was not different ($p>0.05$) between both groups of donors (12.8 ± 0.8 vs 12.9 ± 0.9 mm for rbST-I and rbST-II, respectively). Administration of two rbST injections to donor heifers had no affect ($p>0.05$) on IE and SR; nineteen of the donor heifers exhibiting standing estrus produced ≥ 3 CL (47.37 ± 16.6 vs 52.63 ± 15.8% for rbST-I and rbST-II, respectively). The effect of treatment on percentage of superstimulated donor heifers relative to the level of superovulation response is shown in Table 1. There were no differences ($p>0.05$) in levels of superovulation between treatments.

Table 1. Average percentage ± standard error of superstimulated Holstein donor heifers with a low, medium or high superovulation response, assessed as the number of *corpora lutea* (CL), after receiving one (rbST-I) or two injections (rbST-II) of 500 mg of recombinant bovine somatotropin (rbST).

Superovulation response level	Treatment[1]	
	rbST-I	rbST-II
Low (3-5 CL)	33.3 ± 47.1 (1)	66.7 ± 33.3 (2)
Medium (6-8 CL)	50.0 ± 28.9 (3)	50.0 ± 28.9 (3)
High (≥ 9 CL)	50.0 ± 22.2 (5)	50.0 ± 22.2 (5)

[1]Number of donors within parenthesis. Significant differences ($p<0.05$) within rows are indicated by different letters.

The results regarding embryo production are shown in Table 2. The number of oocytes collected was higher ($p<0.05$) for the rbST-I treatment; 36.8% of the donors (six in the rbST-I and one in the rbST-II) produced at least one oocyte. The number of degenerated embryos was higher, but no significant ($p>0.05$), in rbST-II than in rbST-I treated heifers. On average, 36.8% of the donor heifers produced degenerated embryos (three from the rbST-I and four from the rbST-II). The number of transferable embryos was not different ($p>0.05$) between treatments; this result was obtained from 42.1% of the donor heifers (four from each group). Moreover, 63% of all transferable embryos recovered came from three donors.

On the other hand, no structures (oocytes or embryos) were recovered from 36.8% of the donor heifers (two from rbST-I and five from rbST-II) despite their positive SR. With regard to PRR, after embryos were transferred to recipients, a pregnancy rate of 20% ($p>0.05$) was obtained in both groups.

Table 2. Mean ± standard error of the number of oocytes, degenerated and transferable embryos in Holstein donor heifers treated with one (rbST-I) or two (rbST-II) injections of 500 mg of recombinant bovine somatotropin (rbST).

Variable	Treatment	
	rbST-I	rbST-II
Oocytes	1.3 ± 0.4[a]	0.2 ± 0.2[b]
Degenerated embryos	1.0 ± 0.5	4.5 ± 3.0
Transferable embryos	1.0 ± 0.5	1.4 ± 0.7

Significant differences ($p<0.05$) within rows are indicated by different letters.

Discussion

Diameter of the largest preovulatory follicle in donors and pregnancy rate in recipients

The size of the ovulatory follicle has a direct effect on oocyte quality and, therefore, on the likelihood of the female becoming pregnant. According to Machatkova *et al*. (2004), this could be due to the level of oocyte competence, which is higher in follicles measuring between 11 to 15 mm in diameter. In agreement with these authors, the probability of a cow becoming pregnant increases when the ovulated oocyte comes from 15.6 to 16.1 mm follicles, and is lower when the follicles are 14 to 15 mm (Lopes *et al.,* 2007), while in heifers the same occurs when the follicles are ≥ 12.8 mm and <10.7 or >15.7 mm, respectively (Perry *et al.,* 2007).

The diameter of the largest preovulatory follicle in both groups of donor heifers coincides with the size (≥ 12.8 mm) at which there is a high probability of conception. However, PRR was low in both groups of recipients. It is likely that the low PRR resulted from the negative effect of factors that indirectly harmed the quality of the oocyte in the donor heifers. These factors may include the simultaneous growth of multiple follicles and the use of GnRH analogues to induce ovulation in donor heifers. Superstimulation implies the growth of multiple follicles, but we measured the size of only the largest preovulatory follicle. The rest of the follicles, which were smaller, may have been able to ovulate in response to GnRH administration, but the oocytes did not end in pregnancy. According to Perry *et al*. (2005), GnRH-induced ovulation of follicles ≤ 11 mm in diameter resulted in decreased pregnancy rates and increased late embryonic mortality. Therefore, the low PRR may have been the outcome of transferring low quality embryos resulting from fertilization of oocytes ovulated from small follicles and having reduced competence levels.

Effect of rbST on superovulation response in donors

The proportion of donors that responded positively to the superstimulation treatment (95%) was higher than that reported by Borges *et al*. (2001), Moraes-Júnior *et al*. (2008) and Sales *et al*. (2008). However, the mean number of CL (9.3 ± 1.1 vs 9.5 ± 1.5 for rbST-I and rbST-II, respectively) was lower than that reported by Gong *et al*. (1993b) and Kuehner *et al*. (1993) in heifers, and by Herrier *et al*. (1994) in cows when rbST (320 to 640 mg/animal) was administered five days before or at the beginning of the superstimulation. These findings led us to assume that the superstimulation protocol used in our study is reliable for inducing positive SR in donor heifers.

Embryo yield of donors treated with rbST

The number of oocytes recovered from the donor heifers on the rbST-I treatment was 6.5 times larger than that from donors on the rbST-II treatment. However, since no structures were recovered from 50% of the donor heifers on the rbST-II treatment, it would not be appropriate to make any statement about this variable.

The production of degenerated embryos in the rbST-II treatment was 3.5 times larger than that in the rbST-I treatment. In contrast with the number of oocytes recovered, we speculated that if we had retrieved embryos from the donors that did not produce any structure, more degenerated embryos would have been collected. Consequently, a deleterious effect of the dose (62.5 mg/day) used in the rbST-II treatment on embryo viability may be suggested.

The high dose of rbST might have jeopardized oocyte quality and embryo development. Administration of rbST in cattle has shown to increase serum concentrations of IGF-I and insulin (Bilby *et al.,* 2004; Rivera *et al.,* 2010). Hence, the administration

of a higher dose of rbST led us to suggest that higher serum concentration of IGF-I and insulin may have occurred, but a final conclusion from our study cannot be drawn because analyses of these metabolites were not performed. However, if high concentrations of IGF-I and insulin in the rbST-II treatment did occur, they might have endangered oocyte quality (Adamiak *et al.,* 2005; Thomas *et al.,* 2007). Furthermore, high concentrations of IGF-I can cause early embryo death (Katagiri *et al.,* 1996) by reducing the energy supply to the cell (Chi *et al.,* 2000).

The administration of two injections of rbST in superstimulated Holstein donor heifers did not increase the production of transferable embryos. In fact, the number of transferable embryos in rbST-I and –II treatments was lower than those reported by Kuehner *et al.* (1993) and Herrier *et al.* (1994) in which only one injection of rbST was administered. The number of transferable embryos produced by donor heifers in our study was far from what we expected from the superstimulation treatment and much lower than that found in other studies without rbST administration and using sexed semen (Sartori *et al.,* 2004; Hayakawa *et al.,* 2009; Peippo *et al.,* 2009). The lack of recovery of structures from a large proportion of donor heifers receiving two injections of rbST might have contributed to the low values for production of transferable embryos. This response was observed in the present study and therefore a final conclusion on the practicality of applying two injections of rbST in donor heifers for the production of high quality embryos cannot be drawn. Further studies using larger number of donor heifers are necessary.

In embryo recovery studies, it is common that no oocytes or embryos are recovered from donors. Sartori *et al.* (2004) and Peippo *et al*. (2009) published that in 13 to 25% of the donors only one or none structures was collected. Nonetheless, these earlier results are lower than those obtained in our study.

The reason that oocytes or embryos are not recovered from donors still remains to be elucidated. It is plausible that the transport of viable embryos is different from the transport of oocytes or degenerated embryos (Sartori *et al.,* 2004). In cases of women with inexplicable infertility, Ahmad-Thabet (2000) and Roy *et al.* (2005) reported that abnormally short fimbriae that cannot reach the site of ovulation to catch the oocytes might be responsible for this condition. In addition, it is also possible that the increase in ovary size due to the superstimulation treatment makes it impossible for the fimbriae to cover all the ovulation sites and, as a consequence, the loose oocytes exited the reproductive tract. Some of these mechanisms could be involved in the high proportion of donor heifers from which no structures were recovered in our study.

It is concluded that the application of two injections of 500 mg of rbST, one on day -8 and the other on day 0 of the follicular wave synchronization protocol did not increase the superovulatory response, but significantly reduced the number of oocytes recovered from superovulated Holstein donor heifers.

Conflicts of interest

The authors declare they have no conflict of interest with regard to the work presented in this report.

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