

# Large-scale *in vitro* embryo production and pregnancy rates from *Bos taurus*, *Bos indicus*, and *indicus-taurus* dairy cows using sexed sperm

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Received 20 December 2008; received in revised form 22 May 2010; accepted 2 June 2010

## Abstract

Herein we describe a large-scale commercial program for *in vitro* production of embryos from dairy *Bos taurus*, *Bos indicus*, and *indicus-taurus* donors, using sexed sperm. From 5,407 OPU, we compared the number of recovered oocytes ( $n = 90,086$ ), viable oocytes ( $n = 64,826$ ), and embryos produced *in vitro* from Gir (*Bos indicus*,  $n = 617$ ), Holstein (*Bos taurus*,  $n = 180$ ), 1/4 Holstein  $\times$  3/4 Gir ( $n = 44$ ), and 1/2 Holstein-Gir ( $n = 37$ ) crossbred cows, and the pregnancy rate of recipient cows. Viable oocytes were *in vitro* matured (24 h at 38.8 °C, 5% CO<sub>2</sub> in air) and fertilized by incubating them for 18 to 20 h with frozen-thawed sexed sperm (X-chromosome bearing) from Gir ( $n = 8$ ) or Holstein ( $n = 7$ ) sires ( $2 \times 10^6$  sperm/dose). Embryos were cultured in similar conditions of temperature and atmosphere as for IVM, with variable intervals of culture (between Days 2 and 5) completed in a portable incubator. All embryos were transferred fresh, after 24 to 72 h of transportation (up to 2,000 km). On average,  $16.7 \pm 6.3$  oocytes (mean  $\pm$  SEM) were obtained per OPU procedure and 72.0% were considered viable. Total and viable oocytes per OPU procedure were  $17.1 \pm 4.5$  and  $12.1 \pm 3.9$  for Gir cows,  $11.4 \pm 3.9$  and  $8.0 \pm 2.7$  for Holstein cows,  $20.4 \pm 5.8$  and  $16.8 \pm 5.0$  for 1/4 Holstein  $\times$  3/4 Gir, and  $31.4 \pm 5.6$  and  $24.3 \pm 4.7$  for 1/2 Holstein-Gir crossbred females ( $P < 0.01$ ). The mean number of embryos produced by OPU/IVF and the pregnancy rates were 3.2 (12,243/ 3,778) and 40% for Gir cows, 2.1 (2,426/1,138) and 36% for Holstein cows, 3.9 (1,033/267) and 37% for 1/4 Holstein  $\times$  3/4 Gir, and 5.5 (1,222/224), and 37% for 1/2 Holstein-Gir. In conclusion, we compared oocyte yield from two levels of *indicus-taurus* breeds and demonstrated the efficiency of sexed sperm for *in vitro* embryo production. Culturing embryos during long distance transportation was successful, with potential for international movement of embryos.

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**Keywords:** Oocytes; Embryos; Ovum pick up; IVF; Cattle

## 1. Introduction

The *in vitro* embryo industry has been constantly improving during the last decade; currently, the numbers of embryos produced *in vivo* and *in vitro* are similar [1]. The use of IVP in Brazil is unique, with a

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strong impetus for replacing MOET by OPU/IVP [2]; an important reason is that *Bos indicus* (zebu) cattle have more ovarian follicles and more oocytes recovered by OPU than *Bos taurus* cattle [2]. There is no apparent biological explanation for this intriguing difference. However, most of the available information regarding OPU/IVP in Brazil was obtained from Nelore, a zebu beef breed that represents approximately 80% of the Brazilian herd (approximately  $200 \times 10^6$  animals).

Despite the importance of Nelore as a beef breed well adapted for tropical areas, there is also a growing interest for Zebu dairy breeds, such as Gir, considering their adaptability to produce large amounts of milk under stressful conditions, e.g. high temperature, parasites, and poor pasture. These characteristics are maintained in Gir-Holstein crossbred animals [3], usually named “Girolanda”, which are popular dairy cattle in Central and South America, and potentially other tropical areas.

Recently, the *in vitro* method for embryo production has been considered for Girolanda donors, due to the growing efficiency of using sexed sperm in IVF [4], which facilitates production of a large number of females for the dairy industry.

The objective of the present study was to investigate oocyte yield using OPU and *in vitro* embryo production with sexed sperm, and to determine pregnancy rates following extended transportation of embryos from *Bos taurus* (Holstein), *Bos indicus* (Gir), and *indicus-taurus* (Holstein  $\times$  Gir) breeds. All results were obtained from In Vitro Brasil, a large commercial IVF production center.

## 2. Materials and methods

### 2.1. Cattle

Non-pregnant, healthy, and cycling Gir (*Bos indicus*,  $n = 617$ ), Holstein (*Bos taurus*,  $n = 180$ ), 1/4 Holstein  $\times$  3/4 Gir ( $n = 44$ ), and 1/2 Holstein-Gir ( $n = 37$ ) cows, selected on the basis of genetic merit, were used as oocyte donors. The mean body condition score was  $3.5 \pm 0.5$  (scale, 1–5) [5], and the mean age was  $5 \pm 2.3$  y (range, 3–7 y). The median postpartum interval was 296 d. All donors had regular ovarian activity (based on transrectal palpation and ultrasonography). All cows were used for OPU (5,407 procedures), with a mean of  $6.2 \pm 2.4$  (range, 4–7) OPU performed per donor cow. Donors were randomly used in OPU/IVP without a specific schedule or predetermined sequence; the only constraint was a minimum interval of 15 d between subsequent OPU sessions.

None of the females were subjected to hormonal treatment before OPU/IVP. Embryo production was conducted over a 12-mo interval in a commercial embryo production center, with laboratories located in Mogi-Mirim, SP and Goiânia, GO, Brazil.

Crossbred heifers (15–19 mo old) and cows (24–48 mo old) were used as recipients. These females were located on one of eight farms in northern Brazil (states of Pará and Mato Grosso), approximately 2,000 km from the IVP laboratories. Recipients were selected for adequate body condition, normal estrous cycles, and health status.

### 2.2. Preparation of donor cows

Before each procedure, feces were removed from the rectum and the perineal area was cleaned with tap water and 70% ethanol. Prior to OPU, each cow received epidural anesthesia (7 mL of 2% lidocaine; Anestésico L, Pearson, São Paulo, SP, Brazil) to decrease peristalsis and discomfort.

### 2.3. Follicle aspiration

Previously described procedures were used for follicular aspiration [6]. Briefly, each visible follicle was aspirated using a real-time B-mode ultrasound scanner (Scanner 200 Vet, Pie Medical, Maastricht, The Netherlands), a 7.5-MHz convex array transducer fitted into the intravaginal device (Pie Medical), and a stainless steel guide. Follicular puncture was performed using a disposable 19-gauge 1/2” hypodermic needle (Becton Dickinson, Curitiba, PR, Brazil) connected to a 50-mL conical tube (Corning, Acton, MA, USA) via silicon tubing (0.8 m; 2 mm id). Aspiration was performed using a vacuum pump (Cook Veterinary Products, Queensland, Australia) with a negative pressure of 10 to 12 mL of water/min. The collection medium was TCM 199 (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 25 mM HEPES (Sigma H-0763), 5% fetal calf serum (FCS), 50  $\mu$ L/mL gentamycin sulfate (Schering-Plough, São Paulo, SP, Brazil), and 10,000 IU/L sodium heparin (Sigma H-3149).

### 2.4. In vitro embryo production

Immediately after recovery, aspirated material was washed and filtered through an Emcon embryo filter (Immuno Systems Inc., Spring Valley, WI, USA) with a phosphate buffer solution (PBS-Nutricell, Campinas, SP, Brazil). The cumulus oocyte complexes (COCs) were classified according to the presence of cumulus cells and the oocyte quality using the following criteria: good, more than three layers of cumulus cells; regular,

at least one layer; denuded, partly covered with cumulus cells or without cumulus cells; and atretic, dark cumulus oophorus and signs of cytoplasmic degeneration [6]. Both good and regular oocytes were considered viable and used, whereas atretic follicles were discarded.

Prior to *in vitro* maturation (IVM), COCs were washed three times in TCM-199 HEPES (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% FCS and 50  $\mu\text{g}$  gentamycin sulfate, and were washed once in bicarbonate TCM-199 (Gibco Life Technologies) supplemented with 10% FCS, 5  $\mu\text{g}$  luteinizing hormone (LH-Ayerst, Rouses Point, NY, USA), 0.5  $\mu\text{g}$  follicle stimulating hormone (FSH-Folltropin, Vetrepharm, Belleville, ON, Canada), 1  $\mu\text{g}$  estradiol (estradiol-17 $\beta$ , Sigma E-8875), 2.2  $\mu\text{g}$  pyruvate (Sigma P-4562), and 50  $\mu\text{g}$  gentamycin/mL of medium. The COCs of each category were separately cultured for 24 h in 100- $\mu\text{L}$  drops of maturation medium under mineral oil (D'Altomare, Santo Amaro, SP, Brazil) at 39 °C and 5% CO<sub>2</sub> in air [7,8]. Frozen-thawed sexed sperm (X-chromosome bearing,  $2 \times 10^6$ /dose) from Gir (n = 8) and Holstein (n = 7) sires of known fertility (based on previous utilization for IVF) were used. Sexed Holstein sperm were used for fertilization of oocytes from Gir, 1/4 Holstein  $\times$  3/4 Gir, and 1/2 Holstein-Gir cows, whereas sexed Gir sperm were used for Holstein females. For IVF, straws were thawed for 20 s in a 35 °C water bath. Sperm were washed by centrifugation at  $200 \times g$  for 30 min through a 90–45% Percoll gradient. Sperm were capacitated using heparin (30  $\mu\text{g}/\text{mL}$ ) and motility was stimulated by the addition of 40  $\mu\text{L}/\text{mL}$  of penicillamine, hypotaurine, and epinephrine (PHE) [9]. After a visual assessment of motility, sperm concentration was adjusted to  $25 \times 10^6$  motile sperm /mL and each fertilization drop received 4  $\mu\text{L}$  of sperm (final concentration  $1 \times 10^5$  sperm per drop) [6]. After maturation, COCs were washed three times in pre-fertilization medium TCM 199 supplemented with 25 mM HEPES (Gibco Life Technologies, Grand Island, NY, USA) and 0.3% BSA (Sigma A-9647), and were washed once in TALP fertilization medium supplemented with 10  $\mu\text{g}/\text{mL}$  heparin and 160  $\mu\text{L}$  PHE solution [9,10].

Presumptive zygotes had their cumulus cells removed and were transferred to 100  $\mu\text{L}$  drops of culture medium of embryos (SOFaa BSA, containing 8 mg/mL BSA [free of fatty acid] and 1 mM glutamine), under the same conditions of temperature and gaseous atmosphere used for IVF. Embryos remained under these conditions until the moment of transfer to recipients.

The osmolarity was maintained at 270 to 280 mOsmol and the pH was 7.4. The embryo rate was obtained from the total of aspirated oocytes. Embryos at various developmental stages (Days 2 to 5; Day 0 = day of IVF) were chosen to be transported to farms where the recipients were housed. Due to long distances from the laboratories to the recipients, the final stages of embryo culture were carried out during the transportation period, as described below. Despite the varying duration of transportation from the laboratory to the farm, all embryos were transferred at Day 7.

### 2.5. Protocol for embryo transfer

A fixed-time embryo transfer protocol was used for recipient estrus synchrony. Each recipient received an intravaginal progesterone implant (CIDR, Pfizer, Hamilton, New Zealand) and 2 mg of estradiol benzoate (Estrogin, Farmavet, São Paulo, SP, Brazil) on Day 0. Progesterone implants were removed on Day 8, when animals were also injected with 300 IU of eCG (Novormon, Syntex, Buenos Aires, Argentina), 150  $\mu\text{g}$  of d-cloprostenol (Preloban, Intervet, São Paulo, SP, Brazil), and 1 mg of estradiol cypionate (E.C.P., Pfizer, Guarulhos, SP, Brazil). No estrus detection was performed; Day 10 was considered the day of estrus. Embryos were transferred on Day 17. Before embryo transfer, the ovaries of each recipient were examined ultrasonographically (Aloka SSD 500<sup>®</sup> with 5 MHz linear transducer, Tokyo, Japan) to confirm the presence and size of a CL; only recipients with a CL  $\geq 13$  mm in diameter received an embryo.

### 2.6. Embryo transportation

Embryos were produced in the states of São Paulo (Southeast of Brazil) and Goiás (Center of Brazil) and were transferred to recipients located in eight farms in northern Brazil (Pará and Mato Grosso). Due to the long distances (up to 2,000 km), embryos were shipped on airplanes, transported inside microtubes containing 400  $\mu\text{L}$  of the same embryo culture medium described above, under 300  $\mu\text{L}$  of mineral oil. Temperature and atmosphere were similar to those of the *in vitro* maturation. Each tube contained an average of 40 embryos. During transportation (24 to 72 h), from the laboratory to the moment of transfer, all tubes were maintained inside an incubator designed for embryo transport (Ceafepe Tecnologia Veterinaria, Sorocaba, SP, Brazil). Prior to transfer, each embryo was inserted into a 0.5-mL straw and non-surgically transferred into a uterine horn, ipsilateral to the CL. The developmental stage

Table 1

Mean  $\pm$  SEM number of oocytes, embryos and pregnancy rate obtained from Gir (*B. indicus*), Holstein (*B. taurus*) and *indicus-taurus* donors submitted to OPU – IVP.

Type of donor	Total oocytes/ OPU (n)	Viable oocytes/ OPU (n)	Embryos/total oocytes % (n)	Embryos/ OPU-IVP (n)	Pregnancy/ OPU-IVP* (n)	Pregnancy rate (%)* (n)
Gir	17.1 $\pm$ 4.5 <sup>a</sup> (64617/3778)	12.1 $\pm$ 3.9 <sup>a</sup> (45838/3778)	18.9 (12243/64617)	3.2 <sup>a</sup> (12243/3778)	1.2 <sup>a</sup> (3113/2670)	40 (3113/7763)
Holstein	11.4 $\pm$ 3.9 <sup>b</sup> (12977/1138)	8.0 $\pm$ 2.7 <sup>b</sup> (9082/1138)	18.7 (2426/12977)	2.1 <sup>b</sup> (2426/1138)	0.7 <sup>b</sup> (604/822)	36 (604/1698)
¼ Holstein ¾ Gir	20.4 $\pm$ 5.8 <sup>c</sup> (5457/267)	16.8 $\pm$ 5.0 <sup>c</sup> (4472/267)	18.9 (1033/5457)	3.9 <sup>ac</sup> (1033/267)	1.3 <sup>ac</sup> (137/103)	37 (137/368)
½ Holstein ½ Gir	31.4 $\pm$ 5.6 <sup>d</sup> (7035/224)	24.3 $\pm$ 4.7 <sup>d</sup> (5434/224)	17.4 (1222/7035)	5.5 <sup>c</sup> (1222/224)	1.7 <sup>c</sup> (82/47)	37 (82/220)
Total	16.7 $\pm$ 6.3 (90086/5407)	12.0 $\pm$ 4.4 (64826/5407)	18.8 (16924/90086)	3.1 (16924/5407)	1.1 (3936/3642)	39 (3936/10049)

<sup>a–d</sup> Within a column, means without a common superscript differ ( $P < 0.05$ ).

\* Pregnancy data of 10 049 embryos from the total of 16 924 transferred (results of 6 876 transferred embryos were not available).

of embryos was not recorded at the time of transfer, but the vast majority were at the morula or blastocyst stage.

### 2.7. Pregnancy evaluation

Between 23 and 28 d after embryo transfer (hereafter designated Day 30), pregnancy status of recipients was determined by ultrasonography. Recipients diagnosed pregnant were re-evaluated with ultrasonography 30 to 35 d later (designated Day 60) to confirm pregnancy.

### 2.8. Statistical analysis

Statistical analysis was performed using the software Bioestat 5.0 [11]. The oocyte yield per donor cow and the number of embryos produced per donor cow were normally distributed and were analyzed by ANOVA. Comparisons between breeds were done with a Tukey test. The number of viable oocytes per donor cow was not normally distributed and therefore was analyzed using the Kruskal-Wallis test, with a Dunn test for comparisons between breeds.

## 3. Results

A total of 90,086 oocytes were collected in 5,407 OPU sessions. We performed 3,778 ultrasound-guided follicular aspiration in Gir cows, 1,138 in Holstein cows, 267 in 1/4 Holstein  $\times$  3/4 Gir cows, and 224 in 1/2 Holstein-Gir crossbreed cows. On average, 16.7  $\pm$  6.3 oocytes were obtained per procedure and 64,826 (72.0%) of the 90,086 oocytes were considered viable, generating an average of 12.0  $\pm$  4.4 viable oocytes per procedure.

The mean number of recovered oocytes (17.1  $\pm$  4.5 vs. 11.4  $\pm$  3.9) and viable oocytes (12.1  $\pm$  3.9 vs. 8.0  $\pm$  2.7) per procedure was greater ( $P < 0.01$ ) in Gir than Holstein cows (Table 1). The oocyte yield from *indicus-taurus* donor cows was greater ( $P < 0.01$ ) than from *Bos indicus* donors. For 1/4 Holstein  $\times$  3/4 Gir cows, the mean number of recovered (20.4  $\pm$  5.8) and viable (16.8  $\pm$  5.0) oocytes per procedure was lower ( $P < 0.01$ ) than for 1/2 Holstein-Gir crossbreed females, which had the highest number of recovered (31.4  $\pm$  5.6) and viable (24.3  $\pm$  4.7) oocytes per procedure (Table 1).

Individual results of embryo production are shown (Table 1). A total of 64,826 oocytes were used for IVF, producing 16,924 embryos (26.1%) that were transferred into recipients. The mean number of embryos produced per IVP session from 1/2 *indicus-taurus* (5.5,  $n = 1,222/224$ ) donor cows was greater ( $P < 0.01$ ) than from *Bos indicus* (3.2,  $n = 12,243/3,778$ ) cows. For 1/4 Holstein  $\times$  3/4 Gir cows, the mean number of embryos (3.9,  $n = 1,033/267$ ) produced per donor cow was similar ( $P > 0.05$ ) to 1/2 Holstein-Gir (5.5,  $n = 1,222/224$ ) crossbreed females per procedure (Table 1). The mean number of procedures per donor was 2.9  $\pm$  1.4.

Because of the magnitude of this embryo transfer program, we could not recover data for all transferred embryos. Based on 16,925 transferred embryos, 10,049 (59.3%) resulted in pregnancies. The overall pregnancy rate for all embryos transferred was 39% (Table 1). Percentage of cleaved oocytes, embryos per oocyte, and embryos per cleaved oocyte on a per-bull basis are shown (Table 2). Considering all aspects of such a large program, it was not possible to retrieve detailed information for each bull regarding the efficacy of the sexed

Table 2

Percentage of cleaved bovine oocytes, hatched blastocysts per oocyte and hatched blastocysts per cleaved oocyte obtained with sexed sperm from various Holstein (H) or Gir (G) sires.

	Holstein sires								Gir sires								
	H1	H2	H3	H4	H5	H6	H7	Mean	G1	G2	G3	G4	G5	G6	G7	G8	Mean
Blastocysts/total oocytes (%)	49	59	35	45	51	51	48	46	42	21	53	44	16	13	33	59	45
Blastocysts/cleaved oocytes (%)	86	98	67	72	100	91	89	67	74	40	80	75	51	28	45	82	74
Cleaved (%)	69	69	61	71	54	63	61	78	82	57	72	67	34	54	84	81	68
Replicates	0	466	695	2647	274	330	31	—	2	37	283	342	21	26	2	107	—

sperm. The overall rate of female calves (based on information obtained from the farmers) was 91% for Holstein and 87% for Gir.

#### 4. Discussion

To our knowledge, this is the largest program of *in vitro* embryo production in dairy *indicus* cattle using sexed sperm. We compiled data embryo production and pregnancy rates in Holstein (*Bos taurus*), Gir (*Bos indicus*), and Holstein-Gir donor cows, based on >5,000 OPU/IVP procedures. We also described a protocol for transporting embryos long distances prior to transfer. It is expected that this information can be used to facilitate the expansion of OPU/IVF programs in cattle.

In the present study, the number of recoverable oocytes from Holstein cows ( $11.4 \pm 3.9$ ) seemed much greater than previous reports [12,13] of approximately 4 oocytes collected per OPU session. However, the number of recovered oocytes from Holstein donors in the present study seemed similar to the results reported by Bousquet et al [13], who reported a mean of 9.5 (4,145/437) oocytes collected per OPU session. We concluded that the higher number of oocytes collected herein ( $11.4 \pm 3.9$ ) was more representative of this breed, given the large number (1,138) of procedures performed in this study. We credit two factors for our successful results; the use of donors that were reproductively sound, and the experience of our OPU technicians. In that regard, veterinarians from the embryo center usually perform 15 to 20 follicle aspirations every workday, an unusual situation when compared with most operators.

The number of recovered oocytes per OPU session from Gir cows ( $17.1 \pm 4.5$ ) seemed higher than previously reported by Viana et al [14]. These authors collected an average of 11.6 oocytes per OPU session from Gir cows. However, the present results seemed similar to those reported by the same authors [15] in another

study, collecting an average 18.8 oocytes per procedure. Based on the number of OPU procedures performed in the present study (3,778), we concluded that a collection of 17 to 18 oocytes was more representative for this breed. We could not find references in the literature regarding oocyte yield from Holstein-Gir donors. For these donors, we obtained means of oocytes ranging from 20 (3/4 Holstein-Gir,  $n = 267$ ) to 31 (1/2 Holstein-Gir,  $n = 224$ ). Perhaps heterosis promoted an increase in the number of follicles/oocytes, considering that crossbred cattle had higher averages than purebred cattle.

Considering only Zebu cows, the variation in oocyte yield seemed to be related to the gene sequence, at least for the Nelore breed [16]. These authors performed studies with genetic sequencing according to oocyte yield. The genes *GDF9*, *FGF8*, *BMP15*, and *BMP15* receptors were analyzed. Considering only the *FGF8* effect, these authors reported an increase of  $2.3 \pm 1.1$  oocytes on average and a possible variation of  $7.4 \pm 1.1$  oocytes when all genes were considered together.

Despite this promising study, there are several factors yet to be addressed pertaining to oocyte yield in zebu cattle. High oocyte yield seems to be a unique aspect of *Bos indicus* breeds; many hypotheses have been presented to account for this. For instance, perhaps *Bos indicus* females have a greater number of germinal cells at the fetal stage, or maybe a longer period of mitosis during the formation of oogonia. Distinctive mechanisms of follicular atresia could also explain the high oocyte yield from *Bos indicus* females. The controversial hypothesis of follicular renewal [17,18] could also be considered for Zebu cows. However, this new concept needs to be further investigated before being accepted [19,20]. We are currently studying the pre-antral follicular population in Zebu females to determine the validity of this concept.

In the present study, donor cows used for oocyte collection were not given exogenous hormones. As described earlier by our team, the number of oocytes

obtained from Nelore females by ultrasound-guided follicular aspiration did not increase with ovarian superstimulation [7]. This is an intriguing aspect that needs to be further investigated. Another unexplained factor is a tendency to produce fewer embryos when performing MOET [8].

It is obvious that the variation in oocyte yield was reflected by *in vitro* embryo production (Table 1). For Holstein cows, our average of 2.1 embryos/OPU-IVP was lower when compared with the average of 4.3 described by Bousquet et al [13]. Yet the embryo production system was more efficient in those studies, considering that their oocyte yield per OPU (9.5) seemed lower than ours (11.4). This variation can be explained by the logistics of our large embryo program, i.e., the distance from the laboratory to the recipients, and the use of embryos in various developmental stages.

For Gir and Gir-Holstein donors, we could not find other work describing *in vitro* embryo production and corresponding pregnancy rates. Given the large number of procedures performed, we concluded the averages of embryos presented in this work to be representative of these types of donors under similar conditions of OPU/IVP. We demonstrated similar rates of embryos/oocytes (Table 1) for all types of donors; *indicus*, *taurus*, or *indicus-taurus*; therefore, we inferred that the *in vitro* system of embryo production yielded similar results, regardless of the donor source of oocytes.

The successful use of sexed sperm was efficient for producing large numbers of females. Our team has used sexed sperm to produce zebu embryos, but the present work was our first attempt to generate a large number of *in vitro* embryos from Holstein and Gir sires. Other teams [4] have already described the efficiency of sex-sorted sperm to produce *in vitro* embryos in Holstein donors, with apparently higher averages of embryo development, but those authors used X-sorted sperm from a single, selected Holstein bull, whereas our results came from 15 sires. It was very clear that there was large individual variation among bulls, as previously reported [4].

Our general pregnancy rate was nearly 40%, which seemed higher than the 33.5% recently described in another study by our team [2] and very similar to previous reports with sexed sperm (40 and 41%) [4]. Considering the large number of embryos transferred in the present study, as well the unusual logistics for transporting embryos over long distances, we concluded our results were commercially acceptable. Our success may be due to the protocol for fixed-time em-

bryo transfer. This fixed-time protocol has improved considerably in Brazil [21] in recent years.

Regarding the transportation of embryos at early stages of embryonic development, we could not find similar reports in the literature. This strategy was proposed at the beginning of the project, due to the long distance from the laboratory to the recipients. At least under the presented conditions, we clearly showed it was possible to transport embryos at an early stage of development over long distances and extended intervals, resulting in acceptable pregnancy rates.

In conclusion, we clearly demonstrated the influence of *indicus* cattle on oocyte yield by comparing two levels of *indicus-taurus* crossbreds. In addition, we demonstrated the efficiency of sexed sperm for large production of dairy female calves. The protocol for culturing embryos during transportation over long distances and prolonged intervals yielded promising results with wide potential applications for the international movement of bovine embryos.

## Acknowledgements

The first two authors contributed equally to this work. The authors thank In Vitro Brasil and the National Council for Scientific and Technological Development (CNPq).

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