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Pregnancy rate and birth rate of calves from a large-scale IVF program using reverse-sorted semen in *Bos indicus*, *Bos indicus-taurus*, and *Bos taurus* cattle

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ABSTRACT

Obtaining sexed sperm from previously frozen doses (reverse-sorted semen [RSS]) provides an important advantage because of the possibility of using the semen of bulls with desired genetic attributes that have died or have become infertile but from whom frozen semen is available. We report the efficiency of RSS on the pregnancy rate and birth rate of calves in a large-scale program using ovum pick-up and *in vitro* embryo production (IVEP) from *Bos indicus*, *Bos indicus-taurus*, and *Bos taurus* cattle. From 645 ovum pick-up procedures (Holstein, Gir, and Nelore), 9438 viable oocytes were recovered. A dose of frozen semen (Holstein, Nelore, Brahman, Gir, and Braford) was thawed, and the sperm were sex-sorted and cooled for use in IVF. Additionally, IVF with sperm from three Holstein bulls with freeze-thawed, sex-sorted (RSS) or sex-sorted, freeze-thawed (control) was tested. A total of 2729 embryos were produced, exhibiting a mean blastocyst rate of 29%. Heifers and cows selected for adequate body condition, estrus, and health received 2404 embryos, and 60 days later, a 41% average pregnancy rate was observed. A total of 966 calves were born, and 910 were of a predetermined sex, with an average of 94% accuracy in determining the sex. Despite the lower blastocyst rate with freeze-thawed, sex-sorted semen compared with sex-sorted semen, ($P < 0.05$), the pregnancy rate (bull I, 45% vs. 40%; II, 35% vs. 50%; and III, 47% vs. 48% for RSS and control, respectively; $P > 0.05$) and sex-sorted efficiency (bull I, 93% vs. 98%; II, 96% vs. 94%; and III, 96% vs. 97% for RSS and control, respectively; $P > 0.05$) were similar for each of the three bulls regardless of the sperm type used in the IVF. The sexing of previously frozen semen, associated with IVEP, produces viable embryos with a pregnancy rate of up to 40%, and calves of the desired sex are born even if the paternal bull has acquired some infertility, died, or is located a long distance from the sexing laboratory. Furthermore, these data show the feasibility of the process even when used in a large-scale IVEP program.

1. Introduction

Calf sex plays a key role in the productive performance of beef and dairy herds because many productive traits depend directly on gender. In this context, the use of sexed semen in association with reproductive biotechnologies represents a breakthrough in managing global livestock by enabling the predetermination of the animal's sex, optimizing production, and increasing profitability in the production of both beef and dairy herds.

The birth of offspring of predetermined sex clearly illustrates the importance of biotechnology of sperm sorting

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for ovum pick-up (OPU) and *in vitro* embryo production (IVEP) in bovines. Despite the use of artificial insemination (AI) and embryo transfer (ET), the most common application of sexed semen is IVF, which has satisfactory results and superior efficiency when compared with its use in other areas of biotechnology [1].

Conventionally, sexed semen are prepared starting with the collection of semen from bulls followed by sex sorting the sperm into those containing Y and X chromosomes and then freezing the semen. The possibility of obtaining sexed sperm from previously frozen doses (reverse-sorted semen [RSS]) represents a breakthrough in livestock management because of the possibility of its use in AI or IVF [2]. This technique represents an additional opportunity to produce embryos of predetermined sex, mainly because sorted doses can be shipped to fertilization facilities distant from a sorting facility, enabling the use of freeze-thawed, sex-sorted and cooled [3] or freeze-thawed, sex-sorted and re-freeze-thawed semen [4]. Another important advantage of this biotechnology is the sexing of semen from bulls with high genetic value that have died or become infertile but for whom frozen semen is still available.

Reverse-sorted semen has been associated with other biotechnologies, such as AI and IVEP, but with variable efficiency depending on the biotechnology and species used. In AI, the pregnancy rate for heifers was low, from 4% to 10% [5], with a birth rate of 14.2% (1/7) in cattle [2] and up to 36% (26/72) in sheep [6]. In IVEP, researchers observed no differences in blastocyst rates when oocytes were fertilized with sorted semen or RSS [4,7]. Additionally, studies have reported the birth of two calves by IVF [8] and one calf by AI [2], both using RSS re-frozen-thawed.

The aim of the present study is to report the efficiency of the large-scale use of RSS on the pregnancy rate and birth of calves using OPU and IVEP from *Bos taurus*, *Bos indicus-taurus*, and *Bos indicus* cattle. All results were obtained from In Vitro Brasil, a large commercial IVF production center.

2. Materials and methods

2.1. Animals and feed management

Data for this study were collected between January, 2010 and December, 2012. Nonpregnant, healthy, and cycling Holstein (*Bos taurus*), Gir (*Bos indicus*), and Nelore (*Bos indicus*) cows were selected on the basis of genetic merit to be used as oocyte donors. The mean body condition score was 3.5 ± 0.5 on a scale of 1 to 5 [9], and the animals had regular ovarian activity (based on transrectal palpation and ultrasonography).

The donors were from several dairy and beef farms located in different Brazilian states. The cows were randomly used in OPU (n = 645) without a specific schedule or predetermined sequence and without hormonal stimulation but with a minimum interval of 15 days between subsequent OPU sessions. Embryo production was conducted in a commercial embryo production center with laboratories located in Mogi Mirim, Sao Paulo, Brazil.

Heifers (18–24 months old) and cows (24–96 months old) located at the same farm as the donors and selected for adequate body condition score, normal estrous cycles, and health status were used as recipients.

Herds were maintained by continuous grazing (beef farm) or in stalls (dairy farm), and in both cases the animals had access to mineralized salt and water ad libitum.

2.2. Preparation of donor and OPU

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

Follicular aspiration was performed as previously described by Seneda, et al. [10]. Briefly, each visible follicle was aspirated using a real-time B-mode ultrasound scanner (Scanner 200 Vet; Pie Medical, Maastricht, The Netherlands) equipped with a 7.5-MHz convex array transducer and coupled to a follicular aspiration guide (Pie Medical) and a stainless steel guide. Follicular puncture was performed using a disposable 19-ga 1/2″ hypodermic needle (Becton Dickinson, Curitiba, Parana, Brazil) connected to a 50-mL conical tube (Corning, Acton, MA, USA) via silicon tubing (0.8 m; 2 mm inner diameter). Aspiration was performed using a vacuum pump (Cook Veterinary Products, Queensland, Australia) with a negative pressure of 10 to 12 mL of water per minute. 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 μL/mL gentamycin sulfate (Schering-Plough, São Paulo, Sao Paulo, Brazil), and 10,000 IU/L sodium heparin (Sigma H-3149).

2.3. Bulls and preparation of sperm

Frozen semen of *Bos taurus* (Holstein, n = 6), *Bos indicus* (Nelore, n = 2; Brahman, n = 1; and Gir, n = 1), and *Bos indicus-taurus* (Braford, n = 1) were shipped to a sorting facility, and the frozen semen was thawed, sex-sorted by flow cytometry, cooled (RSS), and returned to the IVF laboratory. Additionally, semen from *Bos taurus* bulls (Holstein, n = 3) was used for *in vitro* production with sperm that were first sex-sorted and then freeze-thawed (control) versus RSS. All bulls were selected on the basis of genetic merit and known fertility in IVF.

Reverse sorting was performed from conventional frozen doses ($1.5–3 \times 10^7$ per dose) thawed at 37 °C for 30 seconds, followed by centrifugation in a Percoll gradient of 45% to 90% (600× *g*, 20 minutes) and resuspension of the sperm pellet with TRIS medium (1:4, sperm:diluent) as previously described [2]. After thawed, an aliquot of semen was evaluated for motility and sperm morphology, to make sure that all samples presented appropriated levels of quality, i.e., 60% to 75% of motility morphologic abnormalities less than 25%. For sexing process, the semen was subjected to flow cytometry to sort X and Y sperm according to the technique previously described [11]. Subsequently, sorted doses (X or Y sperm) were cooled at 18 °C [3], shipped (maximum of 3 hours) to the IVF laboratory, and used for fertilization of oocytes. Immediately before

IVF, the motility was checked again, being the acceptable standard ranging from 40% to 60%.

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 PBS (Nutricell, Campinas, Sao Paulo, 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 [10]. Both good and regular oocytes were considered viable and used, whereas atretic follicles were discarded.

Before IVM, COCs were washed three times in TCM-199 HEPES (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% FCS and 50 μg gentamycin sulfate and once in bicarbonate TCM 199 (Gibco Life Technologies) supplemented with 10% FCS, 5 μg luteinizing hormone (LH-Ayerst, Rouses Point, NY, USA), 0.5 μg follicle stimulating hormone (Folltropin; Vetrepharm, Belleville, ON, Canada), 1 μg estradiol (estradiol-17α, Sigma E-8875), 2.2 μg pyruvate (Sigma P-4562), and 50 μg gentamycin/mL. The COCs of each category were separately cultured for 24 hours in 100 μL of maturation medium under mineral oil (D'Altomare, Santo Amaro, Sao Paulo, Brazil) at 39 °C and 5% CO_2 in air [12].

Sex-sorted, freeze-thawed semen (Holstein, n = 3), and freeze-thawed, sex-sorted, and cooled semen (Holstein, n = 6 and Gir, n = 1) were used to fertilize oocytes from Holstein and Gir cows. Oocytes from Nelore cows were fertilized with freeze-thawed, sex-sorted, and cooled sperm (Nelore, n = 2; Brahman, n = 1; and Braford, n = 1). Female sex-sorted sperm (X chromosome) was used in Holstein and Gir cows, whereas male sex-sorted sperm (Y chromosome) was used in Nelore females, all with a dose of 2×10^6 sperm. For IVF, straws were thawed for 20 seconds in a 35 °C water bath. The sperm were washed by centrifugation at 200× *g* for 30 minutes through a 45% to 90% Percoll gradient. The sperm were capacitated using heparin (30 g/mL), and motility was stimulated by the addition of 40 L/mL of penicillamine, hypotaurine, and epinephrine [13]. After a visual assessment of motility, the sperm concentration was adjusted to 2.5×10^7 motile sperm per mL, and each fertilization drop included 4 μL of sperm (final concentration 1×10^5 sperm per drop) [10]. After maturation, COCs were washed three times in prefertilization medium TCM 199 supplemented with 25 mM HEPES (Gibco Life Technologies) and 0.3% BSA (SigmaA-9647) and once in total alkaline phosphatase fertilization medium supplemented with 10 μg/mL heparin and 160 μL penicillamine, hypotaurine, and epinephrine solution [13,14].

Presumptive zygotes had their cumulus cells removed and were transferred to 100-μL drops of embryo culture medium (synthetic oviduct fluid - SOFaa containing 8 mg/mL BSA [free of fatty acid] and 1 mM glutamine) under the same temperature and atmosphere conditions used for IVF. Embryos were kept in these conditions until the moment of transfer to recipients.

The osmolarity of the culture medium was maintained at 270 to 280 Osm/L, and the pH was 7.4. Embryos at various developmental stages (Days 2–5; Day 0 of IVF) were chosen to be transported to farms, where the recipients were located. Because of the long distances between the laboratories and 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 on Day 7.

2.5. Embryo transportation

Embryos were produced in the state of São Paulo (southeastern Brazil) and were transferred to recipients located in several farms from different Brazilian states. Because of the different distances (up to 2000 km), embryos were shipped by car or airplane, transported inside microtubes containing 400 μL of the same embryo culture medium described above under 300 μL of mineral oil. The temperature and atmosphere were similar to those of the IVM. Each tube contained an average of 40 embryos. During transportation (24–48 hours) from the laboratory to the moment of transfer, all tubes were maintained inside an incubator designed for embryo transport (Ceafepe Tecnologia Veterinaria, Sorocaba, Sao Paulo, Brazil) [1]. Before transfer, each embryo was inserted into a 0.5-mL straw and nonsurgically transferred into a uterine horn ipsilateral to the CL. The developmental stage of the embryos was classified according to International Embryo Transfer Society criteria and only embryos of grades I or II were considered for transfer. From transferred embryos, the vast majority was at the morula or blastocyst stage.

2.6. Preparation of recipients and ET

In some situations, heifers and cows were evaluated for the presence of CL-detectable transrectal palpation and administered 500 mg of cloprostenol intramuscularly (Ciosin; Schering-Coopers, Cotia, São Paulo, Brazil) to induce estrus. Females with well-developed CL 6 to 8 days after the onset of estrus were used as recipients. However, in most cases, a fixed-time ET protocol with progesterone and estradiol benzoate–associated prostaglandin, eCG, and estradiol cypionate was used for recipient estrus synchrony, as previously described [1,15]. Without estrous detection, Day 10 was considered the day of estrus, and the embryos were transferred on Day 17. Before ET, the ovaries of each recipient were examined using ultrasonography (Aloka SSD 500 with 5-MHz linear transducer; Tokyo, Japan) to confirm the presence and size of the CL. Only recipients with a CL greater than or equal to 13 mm in diameter received an embryo.

2.7. Pregnancy diagnosis and calving

Recipients were assessed using transrectal ultrasonography between 23 and 28 days after ET (hereafter designated Day 30) to determine pregnancy status. Another ultrasound was performed 30 to 35 days later (designated Day 60) to confirm pregnancy, and calving data were recorded to calculate the sex ratio of the calves.

Table 1
Pregnancy rate and birth rate of calves in large-scale OPU and IVEP with freeze-thawed, sex-sorted (reverse-sorted) semen from different bulls (*Bos indicus*, *Bos indicus-taurus*, and *Bos taurus*).

Bull[a]	No. of OPU (n)	No. of viable oocytes (n)	No. of embryos (n)	Blastocyst rate (%)	Pregnancy rate (%)	% Sex-sorted efficiency[b] (calves born F or M/total)
I	8	224	79	35	43 (34/79)	97 (32 M/33)
II	9	259	100	39	48 (47/98)	92 (46 M/49)
III	10	238	114	48	52 (12/23)	100 (12 M/12)
IV	31	700	140	20	42 (50/117)	94 (46 F/49)
V	38	1184	259	22	23 (57/249)	96 (50 M/52)
VI	36	542	80	15	35 (28/80)	96 (25 F/26)
VII	52	668	165	25	32 (36/112)	88 (30 F/34)
VIII	56	797	255	32	39 (100/254)	95 (92 F/97)
IX	91	810	128	16	47 (61/128)	87 (41 F/47)
X	105	1362	522	38	43 (215/500)	93 (196 F/211)
XI	209	2654	887	33.5	47 (360/764)	95 (340 F/356)
Total or Mean	645	9438	2729	29	41 (986/2404)	94 (910/966)

Abbreviations: IVEP, *in vitro* embryo production; OPU, ovum pick-up.
[a] *Bos indicus* (Nelore, I and II; Brahman, III; and Gir, IV), *Bos indicus-taurus* (Braford, V), and *Bos taurus* (Holstein, VI–XI) bulls.
[b] The data in the table were confirmed after the birth of the calves.

2.8. Statistical analysis

The data are presented as proportions for descriptive statistical analyses. The embryo rate was obtained from the total aspirated oocytes. The blastocyst rate, pregnancy rate, and sex-sorted efficiency were compared between frozen-thawed, sex-sorted sperm and sex-sorted, frozen-thawed sperm of the same bull. Statistical analysis was performed using Minitab® statistical software 16.1.1, and the significance level for rejecting the null hypothesis was 5%. Therefore, a significance level of less than or equal to 0.05 was considered to be indicative of the effects of categorical variables and their interactions. A chi-square test was used to determine the differences in the qualitative data because of sperm type.

3. Results

A total of 9438 viable oocytes were recovered from 645 OPU procedures. After IVF with freeze-thawed, sex-sorted sperm, 2729 embryos were produced, a 29% average blastocyst rate (Table 1). For commercial reasons, 2404 embryos were transferred, and 60 days later, a 41% average pregnancy rate was observed. A total of 966 calves were born, and 910 were of the predetermined sex, an average 94% accuracy in determining the sex.

In vitro fertilization with freeze-thawed, sex-sorted sperm resulted in a smaller blastocyst rate compared with sex-sorted, freeze-thawed sperm for bull I (34% [74/219] vs. 50% [151/300], respectively; $P = 0.0001$), II (15% [80/542] vs. 35% [68/194], respectively; $P = 0.001$), and III (34% [542/1598] vs. 50% [136/330], respectively; $P = 0.0001$). However, the pregnancy rate was similar regardless of sperm type used in the IVF for bulls I (45% vs. 40%; $P = 0.517$), II (35% vs. 50%; $P = 0.065$), and III (47% vs. 48%; $P = 0.853$) for RSS and control, respectively. The sex-sorted accuracy was also comparable between RSS and control semen, varying between 93% and 98% ($P > 0.05$) (Table 2).

4. Discussion

We compiled data on embryo production, pregnancy rate and calves born from 645 OPU-IVEP procedures using freeze-thawed, sex-sorted sperm in a large-scale commercial program for IVEP from dairy (*Bos taurus*) and beef (*Bos indicus*) cattle. The results should help facilitate the expansion of OPU-IVEP programs in cattle because they demonstrate the viability of this process even when RSS is used at a large scale.

In the present study, the sexing of previously freeze-thawed sperm associated with IVEP biotechnologies produced viable rates of blastocyst and pregnancy, and calves of the desired sex were born. This technology enables the use of frozen sperm banks from breeding bulls that acquired infertility, died, or are located a long distance from

Table 2
Blastocyst rate, pregnancy rate (60 days), and birth rate of calves in large-scale *in vitro* production with sex-sorted, freeze-thawed (control) and freeze-thawed, sex-sorted (reverse-sorted) sperm of three different Holstein bulls in *Bos taurus* and *Bos indicus-taurus* cattle.

Bull/sperm type	Blastocyst rate (Embryos/viable oocytes)	Pregnancy rate	% Sex-sorted efficiency (females born/total)
A/reverse-sorted	34%[a] (74/219)	45%[a] (33/74)	93[a] (26/28)
A/control	50%[b] (151/300)	40%[a] (56/140)	98[a] (43/44)
B/reverse-sorted	15%[b] (80/542)	35%[a] (28/80)	96[a] (25/26)
B/control	35%[a] (68/194)	50%[a] (34/68)	94[a] (30/32)
C/reverse-sorted	34%[a] (542/1598)	47%[a] (228/484)	96[a] (74/77)
C/control	41%[a] (136/330)	48%[a] (64/132)	97[a] (61/63)

Rates followed by different superscript letters (a, b) for the same bull (A, B, or C) and within the same column were significantly different ($P \leq 0.05$).

the sexing laboratory. Furthermore, this process is feasible even in a large-scale program.

A recent study on the features of sexed sperm after thawing showed that the motility of RSS was similar to that of conventional, nonsexed sperm (control) when both were incubated at 15 °C and 37 °C for up to 2 hours. Furthermore, performing the sex-sorting process after thawing followed by incubation at 15 °C or 37 °C improved the motility, mitochondrial activity, acrosome integrity, and viability of the sperm membrane [16]. The viability of RSS was also assessed by Hollinshead, et al. [17], who observed a sperm motility pattern and distance traveled similar to that of nonsexed sperm in artificial vaginal mucus, suggesting the high functional capacity of RSS. This maintenance of the fertilization capacity was to be observed through the blastocyst rate, which averaged 29% in our study. Similar data with frozen-thawed, sex-sorted, re-frozen-thawed were observed in sheep [18] and cattle [4,7,8].

In the present study, the average rate of blastocyst formation using RSS was variable (16%–38%) depending on the bull selected for IVF. Sex-sorted, freeze-thawed sperm produced a higher blastocyst rate than RSS. Some studies found no difference in the blastocyst rate when oocytes were fertilized with freeze-thawed, sex-sorted or sex-sorted, freeze-thawed sperm of the same bull [4,7], but data differ among studies. Puglisi, et al. [8] obtained a blastocyst rate of 10.9%, most likely because oocytes from a slaughterhouse were used, and it has already been shown that oocyte quality is the main factor in blastocyst production [19–21]. Underwood, et al. [4] reported 26.9% blastocysts with selected donors, comparable with our 29%, with the best donors of the herd and bulls with known fertility selected. It is known that the bull has a decisive effect on the IVF results; therefore, to maximize the efficiency of the blastocyst rate, IVF laboratories usually perform previous evaluations of semen with IVF oocytes from the slaughterhouse.

We found an average pregnancy rate of 41% only with RSS. Similar data were reported by Pontes, et al. [1], who obtained a 39% pregnancy rate in a large-scale program with over 5000 OPU-IVEP procedures in *Bos taurus* and *Bos indicus-taurus* cattle using sexed sperm (sex-sorted, freeze-thawed). The present study also showed no difference in pregnancy rate when freeze-thawed, sex-sorted and sex-sorted, freeze-thawed sperm from the same bull were used for bull I (45% and 40%, respectively), bull II (35% and 51%, respectively), and bull III (47% and 48%, respectively). These results should be encouraging for companies because they are comparable with the results obtained using standard protocols for cryopreservation, sexing, maturation, and IVF. The similar pregnancy rates between freeze-thawed, sex-sorted sperm and sexed sperm (control) enable the large-scale use of RSS.

Geographical distance between sex-sorting facility and IVEP laboratories may be a limitation to the expansion of RSS in commercial programs. However, there is the potential to use re-frozen of RSS. Research shows that the RSS can be re-frozen without losing functional viability, including reports of births of lambs after IVEP and/or ET [18] and after AI [6]. In bovines, the birth of a calf after AI with frozen-thawed, sex-sorted, and re-frozen-thawed sperm was also reported [2]. However, Underwood, et al. [16] reported decreased motility, feasibility, and speed of sperm when sperm were exposed a new freezing procedure. The possibility of re-freezing RSS without losing their fertilization ability might overcome the limitation of distance between the sorting and IVEP embryo laboratories.

Currently, most of IVF is performed with sex-sorted, freeze-thawed sperm. The birth of offspring of predetermined sex from bulls with high genetic value that have died or become infertile but for whom frozen sperm is still available is a reality. This study showed the viability of RSS in a large-scale commercial program for the *in vitro* production of embryos with more than 900 calves born with predetermined sex using only this technique.

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References

[1] Pontes JHF, Silva KCF, Basso AC, Rigo AG, Ferreira CR, Santos GMG, et al. Large-scale in vitro embryo production and pregnancy rates from Bos taurus, Bos indicus, and indicus-taurus dairy cows using sexed sperm. Theriogenology 2010;74:1349–55.
[2] Underwood SL, Bathgate R, Maxweel WMC, Evans G. Birth of offspring after artificial insemination of heifers with frozen-thawed, sex-sorted, re-frozen-thawed bull sperm. Anim Reprod Sci 2010; 118:171–5.
[3] Avelino KB, Rossetto MR, Maraia AC, Lima MR, Mansano ATR, Garcia JM. Reports of *in vitro* production and pregnancy rates of bovine embryos produced by post-thawing sexed semen. Reprod Fertil Dev 2009;22:288 [abstract].
[4] Underwood SL, Bathgate R, Pereira DC, Castro A, Thomson PC, Maxwell WMC, et al. Embryo production after in vitro fertilization with frozen-thawed, sex-sorted, re-frozen-thawed bull sperm. Theriogenology 2010;73:97–102.
[5] Underwood SL, Bathgate R, Ebsworth M, Maxweel WMC, Evans G. Pregnancy loss in heifers after artificial insemination with frozen-thawed, sex-sorted, re-frozen-thawed dairy bull sperm. Anim Reprod Sci 2010;118:7–12.
[6] Graaf SP, Evans G, Maxwell WMC, Cran DG, O'Brien JK. Birth of offspring of pre-determined sex after artificial insemination of frozen-thawed, sex-sorted and re-frozen-thawed ram spermatozoa. Theriogenology 2007;67:391–8.
[7] Malcom V, Marfil M, Calvi M, Rigali F, Pugliese M, Gutierrez J, et al. Comparison of in vitro fertilizing capacity of frozen-thawed sex-sorted and sex-sorted frozen-thawed bull spermatozoa. Reprod Fertil Dev 2006;19:298 [abstract].
[8] Puglisi R, Vanni R, Galli A, Balduzzi D, Parati K, Bongiono G, et al. In vitro fertilization with frozen-thawed bovine sperm sexed by flow cytometry and validated for accuracy by real-time PCR. Reproduction 2006;132:519–26.
[9] Lowman BG, Scott NA, Somerville SH. Condition scoring of cattle, Revised edition. East Scotland College of Agriculture 1976; (Bulletin 6).
[10] Seneda MM, Esper CR, Garcia JM, Oliveira JA, Vantini R. Relationship between follicle size and ultrasound-guided transvaginal recovery. Anim Reprod Sci 2001;67:37–43.
[11] Schenk JL, Suh TK, Gran DG, Seidel Jr GE. Cryopreservation of flow-sorted bovine spermatozoa. Theriogenology 1999;52:1375–91.
[12] Pontes JHF, Melo Sterza FA, Bassoa AC, Ferreira CR, Sanches BV, Rubin KCP, et al. Ovum pick up, *in vitro* embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (*Bos indicus*) donors. Theriogenology 2011;75:1640–6.
[13] Parrish JJ, Susko-Parrish JL, Leibfriedge-Ruthedge ML, Critser ES, Eyestone WH, First NL. Bovine in vitro fertilization with frozen thawed semen. Theriogenology 1986;25:591–600.
[14] Bavister BD. A consistently successful procedure for in vitro fertilization of golden hamster eggs. Gamete Res 1989;23:139–58.

[15] Marinho LSR, Untura RM, Morotti F, Moino LL, Rigo AG, Sanches BV, et al. Large-scale programs for recipients of *in vitro*-produced embryos. Anim Reprod 2012;9:323–8.
[16] Underwood SL, Bathgate R, Maxweel WMC, Evans G. In vitro characteristics of frozen-thawed, sex-sorted bull sperm after refreezing or incubation at 15 or 37 °C. Theriogenology 2009;72:1001–8.
[17] Hollinshead FK, O'Brien JK, Maxwell WMC, Evans G. Assessment of in vitro sperm characteristics after flow cytometric sorting of frozen-thawed bull spermatozoa. Theriogenology 2004;62:958–68.
[18] Hollinshead FK, Evans G, Evans KM, Catt SL, Maxwell WMC, O'Brien JK. Birth of lambs of a pre-determined sex after in vitro production of embryos using frozen-thawed sex and re-frozen-thawed ram spermatozoa. Reproduction 2004;127:557–68.
[19] Rizos D, Ward F, Duffy P, Boland MP, Lonergan P. Consequences of bovine oocyte maturation, fertilization or early embryo development in vitro versus in vivo: implications for blatocyst yield and blastocyst quality. Mol Reprod Dev 2002;61:234–48.
[20] Rizos D, Gutiérrez-adán A, Pérez-garnelo S, de la Fuente J, Boland MP, Lonergan P. Bovine embryo culture in the presence or absence of serum: implications for blastocyst development, cryotolerance, and messenger RNA expression. Biol Reprod 2003;68:236–43.
[21] Lonergan P, Fair T. In vitro-produced bovine embryos: dealing with the warts. Theriogenology 2008;69:17–22.