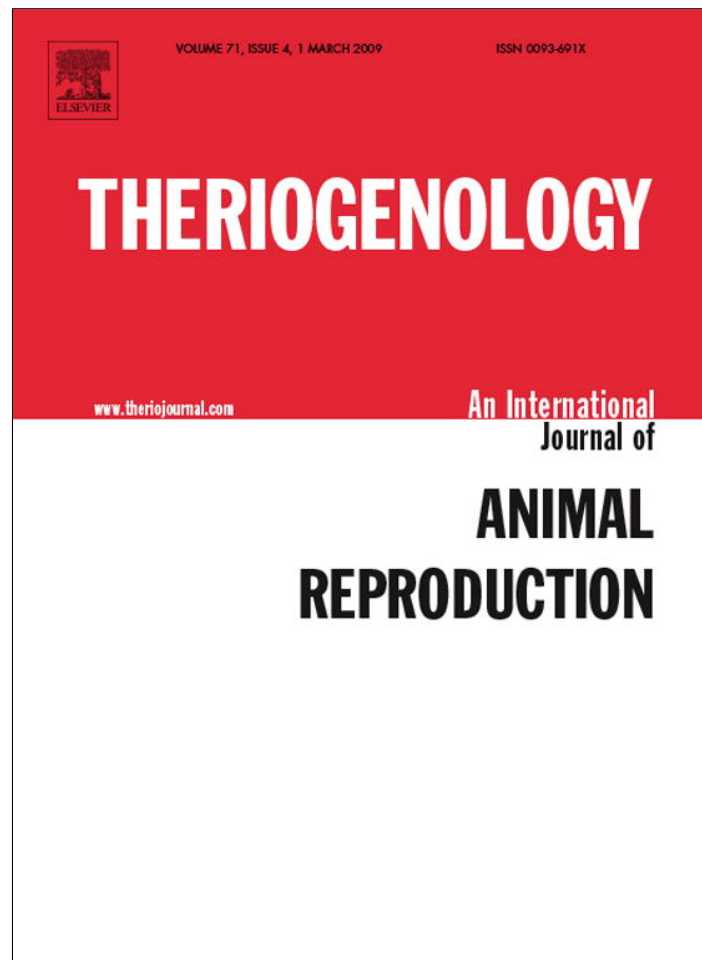


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Comparison of embryo yield and pregnancy rate between *in vivo* and *in vitro* methods in the same Nelore (*Bos indicus*) donor cows

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Abstract

To investigate why the preferred means to produce bovine embryos in Brazil has changed from *in vivo* to *in vitro*, we compared these two approaches in the same Nelore cows ($n = 30$) and assessed total embryo production and pregnancy rates. Without a specific schedule, all cows were subjected to ultrasound-guided ovum pick up (OPU)/*in vitro* production (IVP) and MOET, with intervals ranging from 15 to 45 d between procedures, respectively. To produce *in vivo* embryos, cows were superovulated and embryos were recovered nonsurgically from 1 to 3 times (1.4 ± 0.6), whereas OPU/IVP was repeated from 1 to 5 times (3.2 ± 1.2) in each donor cow during a 12-mo interval. Embryos obtained from both methods were transferred to crossbred heifers. On average, 25.6 ± 15.3 immature oocytes were collected per OPU attempt. The average number of embryos produced by OPU/IVP (9.4 ± 5.3) was higher ($P < 0.05$) than the MOET method (6.7 ± 3.7). However, pregnancy rates were lower ($P < 0.05$) following transfer of IVP (33.5%) versus *in vivo*-derived embryos (41.5%) embryos. Embryonic losses between Days 30 and 60 and fetal sex ratio were similar ($P > 0.05$) between *in vivo* and *in vitro*-derived embryos. We concluded that in Nelore cows, with an interval of 15 d between OPU procedures, it was possible to produce more embryos and pregnancies compared to conventional MOET.

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Keywords: *In vitro* fertilization; Ovum pick up; Embryo transfer; Cattle; Nelore

1. Introduction

In a recent report, Brazil was ranked as the second country in the world for the total number of embryos produced by MOET, and is at the top of the list for embryos produced by OPU/IVP [1]. There are several

reasons for the growing popularity of the OPU/IVP method for producing bovine embryos in this country. First, the commercial value of Nelore (*Bos indicus*), the most popular breed of cattle in Brazil, has been very high during the past 10 y. Second, there is increasing interest in other tropical countries to acquire Nelore genetics from Brazil [2]. Furthermore, Nelore cows normally have a larger numbers of ovarian follicles compared to *Bos taurus* breeds, with averages ranging from 18 to 25 recovered oocytes per OPU session [3–5]. This large population of follicles in this beef breed is

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present without the use of exogenous hormones or synchronization protocols. Consequently, more than 40 private IVP laboratories are currently operating in Brazil [6].

There are physiological similarities, as well as differences, between *B. indicus* and *B. taurus* breeds [7]. For instance, Nelore cows are similar to other *B. indicus* and *B. taurus* breeds when comparing the average embryo production by MOET [8,9]. However, *B. indicus* breeds tend to have more follicular waves [10,11] and a larger population of small follicles (< 5 mm) compared to *B. taurus* breeds [12]. It was also reported that Nelore cows have smaller dominant follicles and CL, and shorter estrus than *B. taurus* breeds [13,14]. Despite the physiological importance of these differences, the high number of oocytes obtained via OPU seems to be a unique characteristic of Nelore cows.

Little information is available in the literature regarding oocyte recovery, embryo production and particularly pregnancy rates in the Nelore breed. As indicated by Hansen [15], embryos produced *in vitro* could play a central role in dairy and beef production, but with the exception of Brazil, the use of *in vitro* embryo technologies is still very limited in the cattle industry. A better understanding of all aspects affecting *in vitro* embryo production in the Nelore breed should not only benefit the embryo industry, but also facilitate the application of other reproductive biotechnologies, such as transgenesis and cloning.

The aim of this study was to compare the efficiency of superovulation/embryo recovery versus OPU/IVP, using the same Nelore cows, on embryo production per donor and pregnancy rates over time.

2. Materials and methods

2.1. Animals

Non-pregnant, healthy and cycling Nelore cows ($n = 30$) were used as donors for oocytes and embryos. The donors cows included two heifers (24 mo old) and 28 multiparous cows (41 ± 7 mo old, range, 32–58) with 517 ± 13 kg (range, 447–631) live weight and average body score condition score of 3.3 ± 0.1 (range, 3.0–3.5; scale 0 to 5) [16]. The average postpartum interval was 284 ± 88 (range, 191–401 d). All animals were selected on the basis of genetic merit and had regular ovarian activity (based on transrectal palpation and ultrasonography). These cattle were kept on pasture, with mineral supplementation *ad libitum*. Experiments were conducted over a 12 mo interval,

in a commercial embryo production center. All cattle were submitted to both OPU (96 procedures) and embryo recovery (43 procedures). An average of 3.2 ± 1.2 (1 to 5) OPU and 1.4 ± 0.6 (1 to 3) superovulation and embryo recovery procedures were performed per donor cow. Because the experiments were conducted in a commercial embryo production center, animals were randomly used in OPU/IVP or MOET, without a specific schedule or predetermined sequence. For each donor cow, there was a minimum interval of 15 d between OPU/IVP and also when OPU/IVP was performed before or after MOET. The minimum interval between MOET was 45 d.

Crossbreed heifers (15 to 19 mo old) were used as recipients. Potential recipients were selected for body condition, normal cyclicity, and health status. Those with a detectable CL were given 500 μ g cloprostenol im (Ciosin, Schering-Coopers, Cotia, São Paulo, Brazil) to induce estrus. Those with a well-developed CL 6 to 8 d after standing estrus were used as recipients.

2.2. Donor preparation

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

2.3. Superovulation

All 30 donor cows received the same treatment, which consisted of an intravaginal progesterone implant (CIDR, Pfizer, Hamilton, New Zealand) and 2 mg estradiol benzoate im (Estrogin, Farmavet, São Paulo, São Paulo, Brazil) on Day 0. Between Days 4 and 7, FSH (Pluset, Serono, Rome, Italy) was administered twice daily in decreasing doses of 133, 100, 65, and 35 IU (total dose, 333 IU). In the afternoon of Day 6, donors were given 500 μ g cloprostenol im (Ciosin, Coopers, São Paulo, São Paulo Brazil) and the progesterone implants were removed 12 h later (morning of Day 7), with AI 36 and 48 h after progesterone implant removal.

2.4. Embryo recovery and transfer

Uterine flushing was performed 7 d after AI and embryos were collected using a two-way Foley catheter passed through the cervix. The catheter's tip was placed in the uterine body, caudally to the external bifurcation

of the uterus, and both horns were flushed simultaneously. The uterus was flushed three or four times using 1 L total volume of Dulbecco's Phosphate Buffered Saline (DPBS, Nutricell, Campinas, São Paulo, Brazil). Embryos were collected on a filter, counted and evaluated according to IETS criteria [17]. Embryos graded as 1, 2, and 3 were defined as viable. All viable embryos were individually transferred non-surgically to synchronous recipient heifers.

2.5. Follicle aspiration

Animals were used independent of their estrous cycle stage. Previously described procedures were used for follicular aspiration [18]. 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, Parana, Brazil) connected to a 50 mL conical tube (Corning, Acton, MA, USA) via a 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 10–12 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 µL/mL gentamycin sulfate (Schering-Plough, São Paulo, São Paulo, Brazil) and 10,000 IU/L sodium heparin (Sigma H-3149).

2.6. *In vitro* embryo production

Immediately after recovery, the aspirated material was filtered through an EmCon filter with phosphate buffered saline (PBS-Nutricell, Campinas, São Paulo, Brazil) supplemented with 5% FCS. Cumulus oocyte complexes were classified as follows: 1, more than three layers of compact cumulus cells; 2, at least one layer of cumulus cells; 3, denuded; and 4, atretic, with dark cumulus cells and signs of cytoplasmic degeneration [18]. After evaluation, only atretic oocytes were discarded. Prior to *in vitro* maturation (IVM), cumulus oocyte complexes (COC's) 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 (FSH-Folltropin, Vetrepharm, Belleville, ON, Canada), 1 µg estradiol (Estradiol 17β- Sigma E-8875), 2.2 µg pyruvate (Sigma P-4562), and 50 µg gentamycin/mL of medium. The COC's of each category were separately cultured for 24 h in 100 µL drops of maturation medium under mineral oil (D'Altomare, Santo Amaro, São Paulo, Brazil) at 39 °C and 5% CO₂ in air [19,20]. Only frozen semen from sires of known fertility was used, and the same sire was used for both *in vivo* and *in vitro* embryo production in each donor. For IVF, two straws were thawed for 20 s in a 35 °C water bath. Semen was then washed and centrifuged through a 90% - 45% Percoll gradient at 200 x g for 30 min. Sperm was capacitated using heparin (30 µg/mL) and motility was stimulated by the addition of 40 µL/mL of PHE [21]. Concentration was adjusted to 25 × 10⁶ live sperm/mL, and each fertilization drop received 4 µL of sperm (final concentration 100 × 10³ cells per drop) [18]. After maturation, COC's were washed three times in pre-fertilization medium TCM 199 supplemented with 25 mM hepes and 0.3% BSA (Sigma A-9647), and once in TALP fertilization medium supplemented with 10 µg/mL heparin and 160 µL PHE solution [22,23]. Presumptive zygotes had their cumulus cells stripped off (by pipetting) 20 h after insemination and were washed three times in pre-fertilization medium and once in Menezo's B2 medium (Pharmascience, Paris, France), supplemented with 10% FCS. Embryos were cultured in 50 µL drops of medium at 38.5 °C and 5% CO₂ in air for 48 to 72 h, when 50 µL of fresh development medium was added [24].

Embryos were classified according to IETS criteria [17], and only grades I and II embryos were used. Morula and blastocyst stage embryos from Days 6 to 7.5 post insemination were individually transferred to each recipient.

2.7. Pregnancy evaluation

Between Days 23 to 28 (hereafter designated Day 30) after embryo transfer, the pregnancy status of recipients was determined by ultrasound evaluation. Recipients diagnosed pregnant were re-evaluated by ultrasound 30 to 35 d later (designated Day 60) to confirm both pregnancy and fetal sex.

2.8. Statistical analysis

Statistical analysis was performed using the software Minitab 14 [25]. Means of procedures and embryos were compared by ANOVA, with differences analyzed

Table 1
Numbers of oocytes produced in Nelore donor cows.

| OPU | Donors (A–J) | | | | | | | | | |
|-----|--------------|----|----|----|----|----|----|----|----|----|
| | A | B | C | D | E | F | G | H | I | J |
| 1 | 46 | 37 | 11 | 22 | 20 | 13 | 19 | 61 | 44 | 14 |
| 2 | 48 | 23 | 35 | 60 | 5 | 15 | 18 | 75 | 40 | 13 |
| 3 | 33 | 24 | 13 | 68 | 40 | 21 | 15 | 65 | 23 | 15 |
| 4 | 39 | 25 | 13 | 46 | 17 | 21 | 34 | 46 | 28 | 13 |
| 5 | 30 | 19 | 8 | - | 32 | - | - | - | - | - |

Data represent the number of oocytes obtained in 4 or 5 OPU procedures from 10 donors (A–J), randomly selected from a group of 30 animals.

Intervals between follicular aspirations were at least 15 d.

by Tukey's test. Pregnancy rates were evaluated by Chi square. For all analyses, $P \leq 0.05$ was considered significant.

3. Results

A total of 2463 oocytes were collected in 96 OPU sessions performed in the 30 donor cows. On average, 25.6 ± 15.3 oocytes were obtained per procedure and 89.3% (2200/2463) were considered viable.

Oocyte production from 10 randomly selected donor cows is shown (Table 1). In some donors, the average number of oocytes produced per OPU was consistently high (>30 ; A and H) or low (<14 ; J), whereas others (E) had substantial variation (from 5 to 40 oocytes)

between OPUs. That the schedule between OPU/IVP and MOET was not predetermined and the intervals to obtain the oocytes were not controlled, these data must be cautiously interpreted.

Individual data of embryo production and pregnancy rates for both methods are shown (Table 2). Some cows (e.g., Donor III) with the usual average of embryos for the *in vivo* method (i.e, 6.5 per flushing) produced on average approximately four times more embryos (24.5) with the OPU/IVP method. Conversely, Donor VI produced an average of 5.3 embryos per uterine flushing and only 3.8 embryos per OPU/IVP session.

From 2200 oocytes submitted to IVF procedures 910 embryos (41.4%) were produced and transferred. Non-surgical collections of 43 superovulated donors yielded 376 embryo/ova and 289 viable embryos. This group of 30 Nelore cows produced an average of 9.4 ± 5.3 embryos per IVP session and 6.7 ± 3.7 embryos using the conventional embryo collection method ($P < 0.05$). Mean number of procedures per animal were 3.2 ± 1.2 and 1.4 ± 0.6 , for *in vitro* and *in vivo* systems, respectively.

Pregnancy rates were different between *in vivo* and IVP systems at Day 30 after embryo transfer (45.6 vs 37.4%, respectively) and at Day 60 (41.5 and 33.5%). For these systems, embryonic losses between Days 30 and 60 were 8.9 and 10.5% ($P = 0.12$). Embryos produced by IVP resulted in 52.8% male and 47.5% female calves, whereas *in vivo*-produced embryos

Table 2
Variation in embryo production among 6 Nelore cows (I–VI), comparing *in vitro* (OPU/IVF) versus *in vivo* (MOET) procedures.

| | Donors (I–VI) | | | | | |
|------------------------------------|---------------|------|------|------|------|------|
| | I | II | III | IV | V | VI |
| Total no. OPU IVF | 5 | 5 | 4 | 4 | 5 | 5 |
| Mean no. oocytes/collection | 36.6 | 25.6 | 49 | 29.7 | 22.8 | 16 |
| Mean no. viable oocytes/collection | 32.2 | 23.4 | 45.2 | 26 | 19.6 | 14.4 |
| Mean no. embryos/OPU IVF | 15.6 | 10.4 | 24.1 | 10.3 | 6.8 | 3.8 |
| Mean no. pregnancies/OPU IVF | 4.8 | 2.8 | 9.25 | 4.3 | 2.2 | 1 |
| Total no. MOET | 2 | 3 | 2 | 2 | 2 | 3 |
| Mean no. embryos/collection | 10 | 4.3 | 6.5 | 2 | 12.5 | 5.3 |
| Mean no. pregnant/collection | 5.5 | 2 | 1 | 1.5 | 6.5 | 1.3 |

Table 3
Pregnancy rates at Days 30 and 60, embryonic losses, and sex ratios from embryos obtained by *in vivo* or *in vitro* procedures from 30 Nelore cows.

| Method | Total no. transferred embryos | Pregnancies on Day 30 No. (%) | Pregnancies on Day 60 No. (%) | Embryonic loss No. (%) | Fetal gender diagnosed | |
|-----------------|-------------------------------|-------------------------------|-------------------------------|------------------------|------------------------|----------------|
| | | | | | Male No. (%) | Female No. (%) |
| <i>In vitro</i> | 910 | 341 (37.4) ^a | 305 (33.52) ^a | 36 (10.5) | 159 (52.5) | 144 (47.5) |
| <i>In vivo</i> | 289 | 132 (45.6) ^b | 120 (41.52) ^b | 12 (9.0) | 60 (50) | 60 (50) |

^{ab}Within a column, means without a common superscript differ ($P < 0.05$).

resulted in exactly 50% male and female calves (Table 3).

4. Discussion

To our knowledge, this is the first study comparing OPU/IVP and MOET in the same Nelore cows. These data should be of interest to the embryo production industry because they indicate that over the same interval, more embryos can be produced using *in vitro* compared to *in vivo* methods. Our findings were consistent with Brazil as the leading country in the world for number of embryos produced by OPU/IVP [1].

This study also confirmed the high oocyte production from Nelore cows, a unique aspect of this *B. indicus* breed. The number of oocytes produced per session (25.6) seemed much higher than results reported by Hasler et al. [26] and Bousquet et al. [24] in *B. taurus* breeds (average collection of 4.9 and 9.9 oocytes per OPU session, respectively). Furthermore, our previous studies with Holstein cows resulted in 4.1 oocytes collected per OPU session [18], which represented approximately 16% of the number obtained herein with Nelore cows. The average in the present work (25 oocytes/session) was similar to another report from our team [27].

Zebu cows have more follicles per wave in comparison to European breeds [28] but it is not clear if Nelore females have a larger follicular population, or just more recruited follicles per wave. Another possible aspect to be considered regarding the higher number of oocytes in Nelore is the predominance of three follicular waves per cycle, with reports of four waves [10,11], which generally exceeded the two or three waves described in Holstein cows [29,30]. Considering a better efficiency in oocyte collection when aspirating small follicles [18], more follicular waves results in a greater probability of finding small follicles in animals with three versus two follicular waves. In agreement with this are previous studies reporting the existence of a larger population of follicles <5 mm in diameter in *B. indicus* compared to *B. taurus* heifers [12]. We speculate that this contributes to the high number of oocytes recovered from Nelore cows.

The total number of follicles in Nelore ovaries remains to be better established. Apparently, the number of primordial and primary follicles in Nelore ovaries is similar to European breeds, but there are only a few reports describing this aspect [31]. Perhaps Nelore females have a larger number of germinal cells at the fetal stage or even a longer period of mitosis during the formation of oogonia. The controversial hypothesis of

follicular renewal [32,33] seems plausible, and was previously mentioned in the IVF context [26]. However, this new concept needs to be better established and receive greater acceptance before being considered [34,35]. That some Nelore cows produced more than 200 oocytes in one OPU procedure without receiving any kind of hormonal stimulation is difficult to explain. For instance, one OPU session performed in a Nelore cow by our team resulted in 251 oocytes (Seneda MM, unpublished data), with similar findings reported by several practitioners. Our current studies investigating pre-antral follicular population in Nelore females of different ages are expected to provide additional insights to explain these striking findings.

Consistent individual variation in oocyte production seemed to occur in Nelore cows. Indeed, such individual variation is frequently reported by practitioners and in field studies, there were specific donor cows producing a remarkably large number of oocytes, whereas other cattle, especially old cows, had very poor oocyte production [5]. That cows producing more oocytes are preferentially selected for OPU could also contribute to the superior rates of oocyte yield in Nelore cows.

In the present study, by performing only to five OPU per donor, it was not possible to evaluate if the large oocyte production per donor cow was consistently maintained over a prolonged interval. Attempts to obtain this information from other embryo production centers in Brazil were unsuccessful. However, based on only anecdotal information, it seems rare to find a donor cow that maintains excellent oocyte production for a prolonged interval under an OPU/IVF program. Due to contractual issues in the commercial embryo production centers, these high production cows are frequently used for few (i.e. one or two) OPU/IVP sessions, because the expected number of pregnancies is quickly achieved.

Regarding the MOET method, an average of 5 to 6 transferable embryos is well accepted for several European breeds [9,36]. In the present work, we obtained 6.7 viable embryos per recovery, which was very close to the average of 5 to 6 previously reported with Nelore cows in Brazil [8,37]. Under Brazilian conditions, a small variation was observed in other Zebu breeds, with 7.3 for Brahman, 4.1 for Gir, and 5.7 for Guzera [8]. We inferred that there was less variation with the MOET compared to the *in vitro* production method in Nelore cows, because similar means have been described when comparing Nelore with other European and Indian breeds on classical ET programs.

A comparison of OPU/IVP and MOET methods was previously conducted in Holstein cows, with averages of 4.3 and 4.7 embryos, respectively [27]. In the present

study, some cattle had good potential for OPU/IVP because they produced very high averages of oocytes and consequently the number of embryos and pregnancies were also high. Conversely, cows that produced lower averages of oocytes were less suitable for OPU/VIP; nevertheless they can still produce acceptable means of embryos by MOET. Perhaps it is possible to select Nelore donor cows that would better respond to *in vivo* or *in vitro* embryo production methods.

The pregnancy rate obtained in our study with IVP embryos (33.5%) seemed relatively lower compared than the rate reported in Holstein cows [26]. That only quality 1 and 2 embryos were transferred, we inferred that the recipients were a major cause of poor fertility. Due to the high value of pregnancies from top genetic quality Nelore cows, there has been increased demand and excessive value of cross-breed heifers to be used as embryo recipients [2]. Also, donors producing high number of embryos in one OPU/IVP procedure have imposed some difficulties for acquiring good recipient females at the appropriate time. This has probably influenced not only the pregnancy rate, but also the embryonic mortality rate.

As described previously [36], there are several differences between embryos generated *in vivo* and *in vitro*. In Holstein embryos, there was a higher degree of apoptosis at the blastocyst stage when they were obtained by the *in vitro* compared to the *in vivo* method [38]. Comparisons of embryos obtained by both methods from donor cows of European breeds showed higher rates of embryonic death in embryos produced *in vitro* [39]. Based on the current findings, there was an advantage of the *in vivo* over the *in vitro* method in terms of embryo quality, based on morphology and pregnancy rates (Table 3).

A big challenge for the embryo industry in Brazil is cryopreservation of Nelore embryos. Apparently there is a higher susceptibility of *B. indicus* embryos for freezing, as described for embryos obtained *in vivo* [40,41]. Pregnancy rates from *in vitro* Nelore embryos were usually very low and/or highly variable (personal communication with several practitioners). Consequently, only fresh embryos were used in the current study. Due to the difficulty in accessing suitable recipients, there is a large impetus to find an efficient method for freezing *B. indicus* embryos, but so far it is not well established. Although IVP was generally regarded as a mature technology [6], the difficult to successfully cryopreserve IVP Nelore embryos requires further investigation.

The synthetic oviduct fluid (SOF) culture medium has been largely used in many IVP systems worldwide

[6]. However, we used Menezo B2 medium, because it provided better results on embryo production in previous experiments performed in the embryo production center (Pontes J.H., unpublished data). A very important consideration regarding embryo culture medium is evaluation of fetal and postnatal development (after embryo transfer). Unfortunately, in the present study, it was not possible to obtain all the information to evaluate those parameters, because pregnant recipients were sent to various farms. The occurrence of Large Offspring Syndrome [42], or other problems such as abortions, dystocia or congenital malformations, were reported by very few farmers. Furthermore, despite the tendency of an altered sex ratio for animals generated by the *in vitro* method [36] our production system resulted in the proportion 1:1 for males and females.

The efficiency of embryo technologies depends on the costs to produce a live calf [36]. That this studies was conducted under commercial conditions, some economical issues are discussed. The cost of each embryo produced by OPU/IVP was approximately 1.5 fold higher compared to the MOET method, excluding the semen cost. Despite this difference, the OPU/IVP method offered other advantages that must be considered. For instance, embryos generated by the *in vitro* method have preferentially been produced using the most expensive semen. In such situations, a single dose was used to fertilize all the oocytes collected from more than 10 cows. It is also important to consider that the OPU/IVF method did not require any hormonal treatment or follicular stimulation. Finally, the OPU/IVP method can result in more pregnancies over a period of time. The use of the *in vivo* approach with embryo collections at 45-d intervals would produce approximately three pregnancies per month. Therefore, if compared over 1-y period, the OPU/IVP approach would result in many more pregnancies than the MOET method. Furthermore, with the high market value of Nelore breed in Brazil, it is easy to understand why the time has been considered as a key factor for choosing the method of embryo production.

In conclusion, our findings confirmed that Nelore cows produced high numbers of oocytes per OPU session, which seemed to be unique to this breed. The high oocyte production in this breed is an important component to explain how OPU/IVP became the preferred option for embryo production in Brazil. However, other factors, including cattle marketing and predominance of the Nelore breed, have also accounted for the expansion in the use of this technology for embryo production in Brazil.

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