Fertility analysis of bovine semen by in vitro fertilization

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Abstract
The aim of the present study was to evaluate the efficiency of using in vitro fertilization to validate semen fertility for artificial insemination. Cryopreserved semen from ten bulls (five Nelore and five Brangus bulls) was evaluated using in vitro production of embryos (IVPE) and via fixed-time artificial insemination (FTAI). There was variation (p < 0.05) in the IVPE (20.9 to 53.7% of blastocyst production) and in the FTAI (42.0 to 56.0% of pregnant cows) results among the semen evaluated. According to the results, there was a positive correlation (rs = 0.8378; p = 0.0001) between the rate of blastocyst production (using IVPE) and the rate of pregnancy (using FTAI) using Nelore bull semen. Variation (p < 0.05) was also found using semen from Brangus bulls, in the rates of blastocyst production (36.5 to 47.0%) and pregnancy (45.6 to 52.2%) via FTAI. There was also a positive correlation (rs = 0.8786; p = 0.0001) between the rates of blastocyst production (IVPE) and pregnancy (FTAI) when using Brangus bull semen. According to the results, IVPE may be used in addition to conventional semen analysis to evaluate and validate the semen fertility of bulls for artificial insemination programs.

Keywords Brangus · Embryo production · Artificial insemination · Nelore · Pregnancy

Introduction
Biotechniques such as artificial insemination or in vitro fertilization are commonly used in cattle to promote genetic improvement of the herd (Pellegrino et al. 2016). The semen from the bulls used for this purpose is mostly cryopreserved and purchased from artificial insemination centers. However, the fertilization potential of this semen is highly variable due to the limitations of predicting the actual fertility status. This variation directly affects the herd’s productivity.

The ability of a sperm to fertilize an egg in vivo is acquired as the gamete passes through the female’s reproductive tract via a process known as “capacitation” (Binelli et al. 2018; Jin and Yang 2017). In vitro, sperm capacitation is acquired through the use of drugs (Jin and Yang 2017; Parrish 2014; Samardzija et al. 2006). Regardless of whether it is in vivo or in vitro, the ability and fertility potential of the semen is fundamentally important for the advancement of breeding biotechniques (Garcia-Alvarez et al. 2009). However, the evaluation procedures used in artificial insemination centers essentially consist of a subjective diagnosis of the motility, concentration, and morphology of the semen, and of the integrity of the plasma and acrosomal membranes (Dias et al. 2009; Khalil et al. 2019). However, several other evaluations are also used, including the computerized sperm movement assessment procedure, morphofunctional analysis by fluorescent probes, flow cytometry, incubation testing, and sperm separation (de Arruda et al. 2015; Dias et al. 2009; Khalil et al. 2019; Rodriguez-Martinez 2007). Although artificial insemination centers employ various evaluations that are indispensable and even irreplaceable (de Arruda et al. 2015), there is a deficiency in our ability to predict the fertilization result of a sample from a given bull that is used for artificial insemination or in vitro fertilization. This uncertainty leads to concerns about the reliability of semen fertility and its correlation with field fertility.

A more reliable method for testing the fertility of a semen sample may be artificial insemination itself. However, artificial insemination can be time consuming and costly. In vitro embryo production could provide a viable and promising alternative. Using this tool it is possible to perform several
simultaneous evaluations of different bulls, which would reduce the time required to validate semen fertility and possibly reduce costs. The aim of the present study was to evaluate the efficiency of in vitro fertilization as a method for validating semen fertility for use in artificial insemination.

Materials and methods

Oocyte selection

Chemicals were purchased from Sigma Chemical Company (St. Louis, MO, USA) unless otherwise specified. For in vitro embryo production, the ovaries of cows were collected from the slaughterhouse and transported in NaCl solution (0.9%) plus 50 μg/mL gentamicin at a temperature of approximately 30 °C. Ovaries containing follicles with diameters of 3–8 mm were aspirated using a needle and syringe (Fidelis et al. 2020). Oocyte categorization was performed in a petri dish (Corning Incorporated, USA, 430,487) under a stereomicroscope (Nikon, SMZ1000). Oocytes with more than two cumulus cell layers and a uniform cytoplasm were selected (de Almeida Barros et al. 2019).

In vitro maturation

The selected oocytes were transferred to TCM-199 (M4530) maturation medium supplemented with 10% fetal bovine serum, 50 μg/mL gentamicin, 0.2 μM pyruvate, 100 μM cysteamine, and hormones (5.0 μg/mL Lutropin and 0.5 μg/mL Folitropin; Bioniche Animal Health USA, Inc.). In vitro maturation was performed in a Petri dish (Corning, 430,166) in 100 μL drops of maturation medium (with 25 oocytes per drop), which were covered with mineral oil, for a period of 22 h in a cell culture incubator at 38.5 °C, with an atmosphere containing 5% CO₂ (Sovernigo et al. 2017).

In vitro fertilization and culture

The cryopreserved semen from each bull used for both in vitro fertilization and fixed-time artificial insemination were from the same batch/set. For the in vitro fertilization study, semen of five Nelore bulls (N1–5) and five Brangus bulls (3/4 Red Angus and 1/4 Brahman; B1–5) was thawed at 36 °C in a water bath for 1 min and prepared according to the BoviPure density gradient technique (Nidacon International AB, Sweden). Oocytes and sperm (2 × 10⁶ cells/mL) (Adona et al. 2020) were co-incubated in 100 μL droplets of fertilization medium (Parrish 2014) covered in mineral oil (Corning, 430,166) for a period of 18 h. The study was conducted in six experimental repeats.

After in vitro fertilization, the oocytes were partially stripped with the aid of an automatic peptide and transferred to the in vitro culture medium (Holm et al. 1999; Sovernigo et al. 2017). In vitro cultivation was performed at 38.5 °C in an atmosphere containing 5% CO₂. The embryo (blastocyst) production rate was evaluated under a stereomicroscope (Nikon, SMZ1000) on the seventh day of cultivation.

Fixed-time artificial insemination

The fixed-time artificial insemination (FTAI) procedures were performed during the reproductive season in a property located in the city Aragarças, Goiás State (GO), Brazil (15°53′51″ S, 52°15′03″ W). The climate is Aw according to the Köppen classification, with an average annual temperature of 25.7 °C and precipitation of 1,579 mm.

The cows were separated according to breed (Nelore [Zebuino] and Brangus [3/4 Red Angus and 1/4 Brahman]). The cows had body condition scores higher than 2.5 (1–5 scale) and were kept on a Brachiaria brizantha pasture, with access to water and mineral salt ad libitum. Sanitary control was performed according to the technical manual of the Ministry of Agriculture, Livestock and Food Supply (BRASIL 2009).

For the FTAI, the cows (Nelore and Brangus) were synchronized at a random estrous cycle stage using an intravaginal(progesterone device (Sincrogest, Ourofino Animal Health, Brazil) and the intramuscular application of 2 mg estradiol benzoate (Estrogin, Pfizer Animal Health, Brazil) on day 0. On day 8, the intravaginal device was removed, and 150 g cloprostenol (Preloban, Intervet, Brazil), 300 IU equine chorionic gonadotropin (eCG; Novormon, Syntex, Argentina), and 1 mg estradiol cypionate (ECP, Pfizer, Brazil) was administered intramuscularly. The artificial insemination (single dose) was performed 48 h later. The semen (lot/set the same as used in in vitro fertilization) of five Nelore (N) and five Brangus (B) bulls was purchased from registered artificial insemination centers. Thirty days after artificial insemination, a pregnancy diagnosis was performed by transrectal ultrasound using a DP-2200Vet ultrasound coupled to a Linear Transducer (Mindray of Brazil, Trade and Distribution of Medical Equipment Ltd.). The study was conducted in six experimental repeats.

Statistical analysis

Statistical analyses were performed using the Shapiro-Wilks test followed by the Kruskal–Wallis test to compare the rates of blastocyst production (via in vitro production of embryos [IVPE]) and pregnancy (via fixed-time artificial insemination [FTAI]). The Spearman’s rank correlation test (rs) was used to verify the degree of association between the variables IVPE (blastocysts) and FTAI (pregnancy). P values lower than 0.05 were considered significant.
Results

There were no significant differences \((P > 0.05)\) in the rates of blastocyst production \((IVPE)\) using the semen of Nelore (N) bulls N1 and N2. However, the rate of blastocyst production of bull N1 semen was superior \((P < 0.05)\) to that of bulls N3, N4, and N5. The semen of the bull N2 not significantly different \((P > 0.05)\) to bull N3, and the semen of the bull N3 not significantly different \((P > 0.05)\) to bull N4 in the rate of blastocyst production. However, the IVPE of bull N4 was significantly different \((P < 0.05)\) from that of bulls N1, N2, and N5. The semen of the Bull N5 had the lowest \((P < 0.05)\) blastocyst production rate compared to the other bulls (Table 1).

The pregnancy rate \((FTAI)\) using the Nelore bull semen did not differ significantly \((P > 0.05)\) between the bulls N1, N2, and N3. The pregnancy rate of bulls N1 and N3 were also similar to that of bull N4 \((P > 0.05)\), which was similar to that of bull N5 \((P > 0.05)\). The pregnancy rate of bull N5 was significantly different \((P < 0.05)\) to that of the bulls N1, N2, and N3 (Table 1). There was a positive correlation \((rs = 0.8378; P = 0.0001)\) between the blastocyst production rates \((IVPE)\) and the pregnancy rates \((FTAI)\) using Nelore bull semen (Fig. 1A).

With regard to the Brangus (B) bulls, the rate of blastocyst production did not differ significantly \((P > 0.05)\) between bulls B1, B2, B3, and B4 (Table 2). However, bull B5 had the lowest \((P < 0.05)\) production of blastocysts compared to the other bulls evaluated (B1, B2, B3, and B4).

The pregnancy rate \((FTAI)\) significantly differed between bulls B1 and B5 \((P < 0.05)\). However the pregnancy rate of both bulls (B1 and B5) did not significantly differ \((P > 0.05)\) from that of the other bulls evaluated (B2, B3, and B4). The pregnancy rates of the bulls B2, B3, and B4 did not significantly differ \((P > 0.05)\) from each other (Table 2). There was a positive correlation \((rs = 0.8786; P = 0.0001)\) between the IVPE and FTAI results using Brangus bull semen (Fig. 1B).

Discussion

Knowledge of the ability or fertility potential of semen is of fundamental importance for the advancement of breeding biotechniques (Garcia-Alvarez et al. 2009). The evaluations performed in this study found significant variation in semen fertility, regardless of the breed (Nelore or Brangus) or the method used (IVPE or FTAI). This variability in semen fecundity is inherent to the bovine species and may be related to ejaculates from the same bull, age of the

![Table 1 Evaluation of in vitro production of embryos (IVPE) and fixed-time artificial insemination (FTAI) using the semen of five Nelore bulls (N1–5)](image)

| IVPE   | Oocytes N | Blastocysts* N (%) ± SD | FTAI   | Cows N | Pregnancy** N (%) ± SD |
|--------|-----------|-------------------------|--------|--------|------------------------|
| Semen N1 | 149       | 80 (53.7 ± 2.0)a         | Semen N1 | 349     | 180 (51.6 ± 3.9)ab      |
| Semen N2 | 150       | 77 (51.3 ± 3.0)ab        | Semen N2 | 341     | 191 (56.0 ± 5.1)a       |
| Semen N3 | 150       | 70 (46.7 ± 2.1)bc        | Semen N3 | 214     | 108 (50.5 ± 6.3)bc      |
| Semen N4 | 150       | 68 (45.3 ± 2.1)c         | Semen N4 | 321     | 155 (48.3 ± 4.2)bc      |
| Semen N5 | 148       | 31 (20.9 ± 3.2)d         | Semen N5 | 131     | 55 (42.0 ± 5.5)c        |

Different letters in the same column a-d indicate a significant difference \((P < 0.05)\) between bulls. The percentage (%) and the standard deviation of the mean (± SD) are based on six experimental repeats

* Seven days post-in vitro fertilization-in vitro production of embryos (IVPE)

** Pregnancy diagnosis after 30 days post-fixed-time artificial insemination (FTAI)

![Fig. 1 Degree of association between in vitro production of embryos (IVPE) and pregnancy by fixed-time artificial insemination (FTAI) using A Nelore and B Brangus bull semen. The Spearman’s correlation test \((rs)\) was used to assess the correlation](image)
bull, environmental factors and even to semen manipulation (cryopreservation, storage, transport). (Bhave et al. 2020; Fuerst-Waltl et al. 2006; Lymberopoulos and Khalifa 2010; Ugur et al. 2019; Westfalewicz et al. 2019). Bulls and their semen undergo various evaluation processes (for motility, viability, membrane integrity, morphology, capacitation, and acrosome reaction for example) before being marketed in specialized artificial insemination centers (Dias et al. 2009; Freitas et al. 2009; Maziero et al. 2009). Therefore, the variability in semen fecundity between bulls selected by the artificial insemination centers should be able to be estimated prior to the application of the semen in the field. However, the results of such evaluations do not always correlate with semen fecundity results using IVPE or artificial insemination programs (Kumaresan et al. 2017; Morrell et al. 2018; Sudano et al. 2011). It is of fundamental importance that the fertility of bull semen that is intended for artificial insemination or IVPE programs can be better predicted using more reliable tests such as those performed in this study. These tests should be implemented in addition to the main evaluation methods that are already employed at the artificial insemination centers (Kumaresan et al. 2017; Morrell et al. 2018; Sudano 2011).

The inaccuracies in traditional semen evaluation procedures can generate significant variation in the fertility rates achieved in artificial insemination and in IVPE programs in cattle. This leads to losses that could be attenuated (de Arruda et al. 2015; Maziero et al. 2009; Thundathil et al. 2016).

In recent decades, several laboratory methods have been developed in order to provide more accurate analyses of semen composition and structural integrity, with the aim of improving field performance (de Arruda et al. 2015; Khalil et al. 2019; Kipper et al. 2017; Maziero et al. 2009). These methods are indispensable but do not yet effectively predict semen fecundity. However, the association of these methods with in vitro embryo production may be essential for providing an estimate of the pregnancy rate for each semen batch. This is supported by the results of this study. The rate of blastocyst production (IVPE) and pregnancy (FTA1) varied among the semen in this study. However, most of semen had a good fertility performance, with values in line with those found in the literature and foreseen for these biotechnologies (Crites et al. 2018; Cunha et al. 2019; Franco et al. 2018; Sovernigo et al. 2017). Nevertheless, the evaluations performed in the insemination centers are indispensable, but more tests are essential for predicting semen fertility. These additional tests could include the IVPE performed in this study. The use of these additional tests could increase the reproductive efficiency of cattle and reduce the costs involved with management and resynchronization.

Comparing the rate of blastocyst production (IVPE) with pregnancy (FTA1) for both breeds (Nelore or Brangus) of bull (semen) evaluated in this study, there was a significant association between the variables. This indicates that increases in the rate of blastocyst production via IVPE for a given semen, correlates with increases in the rate of pregnancy via FTAI. The correlation of a lot/set of semen having a good performance in IVPE and FTAI suggests that IVPE can be used as an efficient tool to evaluate bull semen fecundity after tests carried out by the specialized artificial insemination centers. The IVPE technique could be used to provide an estimate of the pregnancy rates for each semen. This could be used to validate semen fertility in preparation for artificial insemination and other assisted reproduction biotechnologies.

The results of the present study indicate that IVPE may be used in addition to conventional semen analyses, to evaluate and validate the semen fertility of bulls from artificial insemination centers. Such a method could benefit biotechnologies (Crites et al. 2018; Cunha et al. 2019; Franco et al. 2018; Sovernigo et al. 2017). The correlation of a lot/set of semen having a good performance in IVPE and FTAI suggests that IVPE can be used as an efficient tool to evaluate bull semen fecundity after tests carried out by the specialized artificial insemination centers. The IVPE technique could be used to provide an estimate of the pregnancy rates for each semen. This could be used to validate semen fertility in preparation for artificial insemination and other assisted reproduction biotechnologies.

The results of the present study indicate that IVPE may be used in addition to conventional semen analyses, to evaluate and validate the semen fertility of bulls from artificial insemination centers. Such a method could benefit biotechnological productivity by promoting predictability and increasing pregnancy rates through artificial insemination.

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Code availability  Not applicable.

Declarations

Ethics approval  The experimental design involving animals were in accordance with the ethical standards Pitágoras Unopar University ethics committee under the protocol 007/20.

Consent for publication  Not applicable.

Consent to participate  Not applicable.

Conflicts of interest  The authors declare that they have no conflict of interest.

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