



Influence of embryonic morphological development stage on the pregnancy rate of recipients of *in vitro*-produced bovine embryos. Influência do estágio de desenvolvimento morfológico embrionário sobre a taxa de prenhez de receptoras de embrião bovino produzidos *in vitro*.

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Abstract

Embryo transfer (ET) is a global biotechnology whose main objective is to produce a large number of genetically superior offspring per female in a breeding season. However, in order to achieve satisfactory results, factors such as ambient temperature, embryo quality, and developmental stage must be considered. Thus, the present study aimed to assess the influence of the morphological embryonic development stage on the conception rate of recipients of bovine embryos. To that end, 2,797 embryo transfers were evaluated from selected farms in Rondônia state, Brazil, which use this biotechnology as a tool for multiplying superior genetics. The project was developed in partnership with a laboratory renowned for producing and transferring *in vitro*-produced embryos, thereby enabling research and data analysis. The embryos were produced *in vitro* and evaluated on the seventh day of production. After evaluation, they were packaged and transferred fresh. The results indicate that more advanced embryos, such as expanded blastocysts (BX) and blastocysts (BL), resulted in higher pregnancy rates. As such, these two developmental stages should preferably be transferred into a PIVE program to improve pregnancy rates.

Keywords: *In vitro* fertilization. Reproduction. Embryo transfer.

Resumo

A transferência de embriões (TE) é uma biotecnologia mundialmente difundida, com o objetivo principal de produzir um número elevado de descendentes geneticamente superiores por fêmea dentro de uma estação de monta. No entanto, para que se obtenha resultados satisfatórios alguns fatores devem ser considerados, como a temperatura ambiente, qualidade e estágio de desenvolvimento do embrião. Diante disso, o presente trabalho teve como objetivo avaliar a influência que a classificação do desenvolvimento morfológico do embrião possui sobre a taxa de prenhez de receptoras de embriões bovino. Para tanto, foram avaliadas 2.797 transferências de embrião de fazendas do estado de Rondônia que utilizam essa biotecnologia como ferramenta para multiplicação de genética superior. O projeto foi desenvolvido em parceria com um laboratório que é referência em produção e transferência de embriões produzidos *in vitro*, viabilizando o desenvolvimento da pesquisa e análise dos dados. Os embriões foram produzidos *in vitro* e avaliados no sétimo de produção, após a avaliação, foram envasados e transferidos a fresco. Os resultados indicam que os embriões em estágios mais avançados, como blastocisto expandido (BX) e blastocisto (BL), apresentaram maiores taxas de gestação e dessa maneira, esses dois estágios dedesenvolvimento devem ser preferencialmente transferidos em programa de PIVE como forma de melhorar os índices de prenhez.

Palavras-chave: Fertilização *in vitro*. Reprodução. Transferência de embriões.



Introduction

The productivity of cattle herds has increased significantly due to breeding and genetic selection, aimed at maximizing productive characteristics by implementing and refining animal reproduction biotechnologies (MELLO et al., 2016). As such, the growing application of biotechniques such as fixed-time artificial insemination (FTAI), embryo transfer (ET), and *in vitro* fertilization (IVF) of embryos optimizes the reproductive potential of female bovines and optimizes the reproductive potential and profitability indicators in both the beef and dairy cattle sectors (SCANAVEZ et al., 2013).

In vitro production of bovine embryos is an important biotechnological tool in assisted reproduction, significantly improving reproductive efficiency and producing genetically superior animals, contributing to the growth of Brazilian cattle productivity (LEAL et al., 2021).

IVF is the interaction between sperm and oocytes outside the female's reproductive tract under laboratory conditions, resulting in the formation of a new individual. The goal is to generate viable embryos from healthy females with high genetic value, or to obtain offspring from females unable to reproduce using conventional techniques (SOUZA; ABADE, 2018; GRÁZIA; SANTOS, 2021). The process involves collecting oocytes using ultrasound-guided follicular aspiration (OPU), followed by *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* cultivation (IVC) of zygotes and embryos outside the female's uterus until the blastocyst stage, when the embryos can be cryopreserved and/or transferred to synchronized recipient females (SOUZA-FABJAN et al., 2014; MELLO et al., 2016). The main advantage of IVF is the significant increase in the number of offspring per female. Silva et al. (2015) reported that each donor can produce around 50 pregnancies per year, whereas only one calf per year would be physiologically possible. Additionally, this technique allows the use of females with reproductive issues, senile, or pre-pubertal animals, providing an alternative to optimize herd standardization with genetically superior animals and preventing the early culling of high-genetic-value females (SCANAVEZ et al., 2013; BARUSELLI et al., 2019).

However, variability in the success of *in vitro* embryo transfers is one of the major obstacles to its expansion. To achieve satisfactory pregnancy rates using IVF, several factors and their influence on the technique's outcomes must be considered (JELONSCHEK et al., 2018). Among these are the quality of the corpus luteum, synchronization of donors and recipients, donor breed, embryo quality, sire effects, semen quality and viability, heat stress, time of the year, previous embryo transfers to the recipient, as well as their body condition and nutritional status (ANDRADE et al., 2012; MELLO et al., 2016; BECHER et al., 2018).

Thus, the present study aimed to assess the influence of factors such as morphological evaluation, developmental stage, and quality of transferred embryos on the pregnancy rate of recipients that received *in vitro*-produced bovine embryos from Nelore cattle.

Material and methods

This study was conducted using retrospective data from 2,797 *in vitro*-produced bovine embryo transfers. The data were provided by a private company specializing in animal reproduction, located in the municipality of Ariquemes, Rondônia State. The study included data obtained from October 2023 to January 2024, from eight beef cattle farms in the municipalities of Cacoal, Ariquemes, Jaru, and Ouro Preto.

Follicular aspirations were performed using portable ultrasound equipment with a 7.5 MHz sectorial transducer adapted for intravaginal examination and a biopsy needle guide. Disposable needles (20 G) were connected to a vacuum system with a pressure of approximately 50 mmHg or equivalent to a flow rate of 11 ml per minute. The follicular fluid and cumulus-oocyte complexes (COC) were aspirated into a 50 ml tube containing 10 ml of Dulbecco's PBS (DPBS), supplemented with 10% fetal bovine serum (Nutricell) and 100 IU of sodium heparin (Liquemine, Roche, Basel, Switzerland), heated to 36°C. All recovered oocytes were stored in cryotubes identified with buffered medium and transported to the laboratory within 3 to 12 hours at 37°C.

***In vitro* embryo production**

The *in vitro* maturation of COCs was carried out in TCM 199 medium (Gibco) added with 10% fetal bovine serum, FSH, LH, estrogen, pyruvate, and antibiotics, in an incubator at 38.8°C with 5% CO₂ in atmospheric air, and 95% humidity for 24 hours. The oocytes from each donor were placed in separate drops in groups of 1 to 30 per drop. The matured COCs were fertilized using proven bull semen. The sperm were prepared using the Percoll gradient method (Invitrogen). Fertilization was performed in IVF medium containing heparin, for approximately 18-22 hours, under the same atmospheric conditions as maturation. Presumed zygotes were cultured using CR4aa containing bovine fetal serum and BSA. The embryos were co-cultured with granulosa cells under the same conditions as fertilization. On the seventh day of culture, the blastocysts were assessed and classified according to their developmental stage (morula, early blastocyst, blastocyst, expanded blastocyst, and hatched blastocyst).

Embryo transfers

A total of 2,797 Nelore and crossbred (F1 Angus) recipient females over 24 months old (body condition score (BCS) of 3 ± 5 on a scale of 1-5) (PAZZIM, 2021) from the herds of multiple farms applying ET with reproductive biotechnology were used. The animals were raised on Brachiaria pastures under an extensive production system, with natural shading and water and mineral supplements provided ad libitum. All animals underwent sanitary management to immunize them against IBR, BVD, and leptospirosis. They were later submitted to the fixed-time embryo transfer (FTET) protocol. The selected animals underwent a synchronization protocol for the estrous cycle in the recipient females. The start was designated day zero, with the insertion of a 1g progesterone intravaginal device and intramuscular application of 2mg of estradiol benzoate. On the eighth day, the device was removed, and an intramuscular injection of 300 IU of equine chorionic gonadotropin (eCG), 0.5mg of prostaglandin F₂ α (PGF₂ α), and 2mL of estradiol cypionate was applied. Embryo transfer was carried out on day 17. The females' reproductive system was evaluated via ultrasonography in order to determine the presence of a corpus luteum and the side on which ovulation occurred, with those showing a corpus luteum considered ready to receive an embryo.

Data were collected on the timing of embryo transfer to the recipients; the developmental stage of the embryos (morula, early blastocyst, blastocyst, expanded blastocyst), as well as the ovary ipsilateral to the corpus luteum (left or right) and size of the corpus luteum (3: small, 2: medium, or 1: large). The data were collected, organized, tabulated in spreadsheets, and analyzed. The conception rate was calculated considering the effects of the timing of embryo transfer, embryonic development phase, embryo quality and processing, the ovary ipsilateral to the corpus luteum, and the size of the

corpus luteum. The size of the corpus luteum was also assessed according to its occurrence in the left or right ovary. Pregnancy diagnosis was performed 28 days after embryo transfer, which corresponds to 35 days after *in vitro* fertilization, using ultrasonography. Pregnancy was confirmed based on the location and presence of a viable fetus, while the absence indicated a negative pregnancy (empty).

Results and discussion

The embryo transfers resulted in a 38.43% pregnancy rate, with a total of 1,075 pregnancies out of 2,797 transfers at 35 days of gestation. This result is similar to that reported by Cochito (2013) and Morais (2013), where pregnancy rates for embryo transfer were around 30%. However, some authors have found higher conception rates, such as Oliveira et al. (2017), who assessed 443 animals and obtained rates of 44.46%. Table 1 shows the value of each category assessed.

Table 1 - Embryo classification and conception rate.

Embryo classification	Total analyzed	Positive pregnancies	Non-pregnancies	% of pregnancies
Morula (MC)	54	11	43	20.37%
Initial blastocyst (IB)	346	114	232	32.90%
Blastocyst (BL)	1339	520	619	38.80%
Expanded blastocyst (BX)	1055	430	625	40.70%
Total	2.797	1075	1722	38.40%

In a study conducted by Florentino (2011), 5,403 transfers were performed, resulting in a pregnancy rate of 34.33%. The embryonic stage with the highest rate was BX (38.2%), similar to the present study, where BX achieved the most significant result (40.7%). Considering the stage of embryonic development, Bi had a pregnancy rate of 32.9%, which is consistent with the expected results, but is one of the lowest pregnancy rates achieved in this study. Cochito (2013) reported a pregnancy rate of 28.1% for Gir embryos classified in the same developmental stage, which agrees with the rates found here.

Ortlibas et al. (2015) reported a very low pregnancy rate using Bi, with only 19.57% of pregnancies following Bi transfers; however, the low number of embryos transferred at this stage may have influenced the results. By contrast, Peixoto et al. (2007) conducted a logistic regression analysis of factors affecting the pregnancy rates of zebu embryos and observed that, in general, the highest rates were obtained when using early blastocysts and morulae. However, these authors obtained embryos from a multiple ovulation program, not through PIVE.

Embryos classified as BX and BL had pregnancy rates greater than the expected 30%, achieving 40.7 and 38.8%, respectively. These stages of embryonic development obtained higher pregnancy rates. For 1,055 transferred embryos at the BX stage and 1,339 at the BL stage, there were 430 and 520 pregnancies, respectively. Cochito (2013) reported a pregnancy rate of 54.2% for BX, which is higher than that obtained in the present study. On the other hand, Ortlibas et al. (2015)

reported a gestation rate of 33.53% for the same embryonic stage, which is lower than that observed here. However, Zaniboni et al. (2009) e Colombo et al. (2010) reported lower pregnancy rates (around 30%) for BL and BX embryos transferred.

In a study conducted by (MELO et al., 2023) analyzing embryo transfer in different categories, heifers and cows obtained results of 52.63% and 47.06% respectively, a result higher than that found in the present study. These results can be linked to the total number of animals evaluated in the research. Regarding the embryonic classification evaluated, the blastocyst (BL) reached 45.45% and expanded blastocyst (BX) 50.75%, results above the average, which can again be associated with the number of animals and a more controlled transfer environment. showing that there is better reproductive efficiency on the property than compared to other studies, which may have possibly occurred since this work used data from a single farm that has very efficient nutritional and sanitary control.

According to Lobato et al. (2019), they concluded that embryos in more advanced stages of development result in better reproductive efficiency per TE in Girolando cows, which is consistent with the results obtained in the present study. However, they also concluded that the category of the recipient influences P/TE and that heifers should be prioritized when choosing the recipient. The authors justify the possibility of the category of the recipient influencing P/TE, considering that cows and heifers have different hormone production. Regarding the stage of embryo development, all were fertilized on the same day, and those that developed more quickly probably have a greater capacity for implantation and maintenance of pregnancy compared to those that developed more slowly.

In a study conducted by (GRÁZIA; SANTOS, 2021), it was observed that more advanced stages (hatched blastocyst and expanded blastocyst) presented a higher pregnancy rate compared to less developed stages (blastocyst and early blastocyst). The results of the present study were different from the study by Colombo et al. (2010), which found no difference in the pregnancy rate between the different stages of development. However, this biotechnique is influenced by several factors that interfere not only in the blastocyst production rates, but also in the pregnancy rates. Among these factors, we can highlight the nutritional status, physiological status, age and fertility that are directly related to the quality of the recipient females. In addition, environmental variables, such as climate, can influence pregnancy rates.

Conclusion

The results obtained in this study indicate that embryos in more advanced stages, such as expanded blastocyst (BX) and blastocyst (BL), resulted in higher pregnancy rates than those in less developed stages, such as morula and early blastocyst.

Conflicts of interest

There were no conflicts of interest among the authors.

Authors' contributions

Sallomão Dhankel da Silva Lima – data collection, result interpretation and writing; Lucas Winter de Almeida Soares – data collection and result interpretation; Lucas Martins Roveda – data collection; Jomel Francisco dos Santos – corrections, text revision and supervision.

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