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Effects of sperm cryopreservation extenders and estrus in pregnancy of *Bos indicus* cows submitted to TAI

Efeitos de diluentes de sêmen e do estro na prenhez de vacas *Bos indicus* submetidas à IATF

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Highlights

OPT extender tended to improve pregnancy in estrus-expressing cows.
OPT and EYTG semen extenders had different post-thaw sperm kinematics.
Cows in estrus at TAI had greater P/AI compared to cows that did not express estrus.

Abstract

The objective of this study was to evaluate the relationship between estrus behavior of cows and the semen's cryopreservation extenders on the fertility of *B. indicus* cows subjected to timed artificial insemination (TAI). In this study, 505 postpartum *B. indicus* Nelore beef cows were enrolled in a TAI program and separated into groups according to the estrus detection on the Day of the TAI procedure, into: Cows detected in estrus (E, n = 307), and cows not detected in estrus (NE, n = 198). The semen used in this study was cryopreserved with 2 different extenders: Optixcell (OPT), and the egg yolk-Tris-glycerol (EYTG), and were distributed to inseminate E and NE cows. The pregnancy per AI (P/AI) of the groups EOPT (69.5 %), and EEYTG (60.1 %) were greater (P ≤ 0.05) in comparison with groups NEOPT (46.0 %), and NEEYTG (42.9 %). The group EOPT tended (P = 0.08) to have greater P/AI in comparison with the group EEYTG. In conclusion, among cows exhibiting estrus, those inseminated with semen diluted in OPT showed a tendency toward higher pregnancy per AI compared to those inseminated with EYTG-diluted semen.

Key words: Bovine. Estrus. Pregnancy. Sperm capacitation.

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Resumo

O objetivo deste estudo foi avaliar a relação entre o comportamento de estro das vacas e os diluentes utilizados na criopreservação do sêmen sobre a fertilidade de vacas *Bos indicus* submetidas à inseminação artificial em tempo fixo (IATF). Neste estudo, 505 vacas de corte da raça Nelore (*Bos indicus*) no pós-parto foram incluídas em um protocolo de IATF e divididas em grupos de acordo com a detecção de estro no dia do procedimento: vacas detectadas em estro (E, n = 307) e vacas não detectadas em estro (NE, n = 198). O sêmen utilizado foi criopreservado com dois diluentes diferentes: Optixcell (OPT) e gema de ovo-Tris-glicerol (EYTG), sendo distribuído para a inseminação de vacas dos grupos E e NE. As taxas de prenhez por inseminação artificial (P/IA) dos grupos EOPT (69,5%) e EEYTG (60,1%) foram maiores (P ≤ 0,05) em comparação com os grupos NEOPT (46,0%) e NEEYTG (42,9%). O grupo EOPT apresentou tendência (P = 0,08) a maior P/IA em comparação ao grupo EEYTG. Em conclusão, entre as vacas que apresentaram estro, aquelas inseminadas com sêmen diluído em OPT apresentaram tendência a maiores taxas de prenhez por IA em comparação àquelas inseminadas com sêmen diluído em EYTG.

Palavras-chave: Bovinos. Capacitação espermática. Estro. Prenhez.

The fertility of TAI programs is a multifaceted trait influenced by both semen quality and individual female responses to synchronization protocols. Cows exhibiting estrus prior to TAI ovulated earlier (Oliveira et al., 2022), and had greater pregnancy per AI (P/AI) compared to those that do not (Nogueira et al., 2019; Sá et al., 2011). Previous study from our laboratory has demonstrated that the association between post-thaw sperm kinematics and ovarian responses significantly affects the fertility of cows undergoing TAI programs (Pfeifer et al., 2019).

Extensive research has investigated the efficacy of bull semen extenders in preserving sperm quality and optimizing conception rates in cattle (Verberckmoes et al., 2004; Vishwanath & Shannon, 2000). For decades, the egg yolk-Tris-glycerol (EYTG) extender has been a pivotal component semen cryopreservation due to the cryoprotective properties of its constituents. Egg yolk, rich in lipoproteins and phospholipids, stabilizes the sperm membrane, mitigating cold shock and preserving structural integrity during freezing and thawing (Pace & Graham, 1974). Furthermore, egg yolk contains hormones that may influence sperm function (Lipar et al., 1999). However, the use of EYTG is not without its limitations. Egg yolk can interfere with cellular respiration, potentially reducing sperm motility and longevity (Crespilho et al., 2012). More critically, EYTG extenders present inherent microbiological risks, potentially compromising the quality and safety of cryopreserved semen and hindering standardization efforts (Aires et al., 2003). These concerns have driven the development and evaluation of synthetic extenders as alternatives to EYTG. Among these alternatives, OPTIXcell (OPT) has emerged as a promising candidate. Studies suggest that OPT may offer superior conservation of post-thawing semen quality in mammalian species compared to

traditional egg yolk-based extenders (Ansari et al., 2016). The membrane-stabilizing properties of semen extenders, coupled with their influence on the duration of sperm capacitation (Davis, 1981), can significantly impact the fertility outcomes of timed artificial insemination (TAI) programs. While EYTG semen extender has a well-established effectiveness in sperm cryopreservation, the OPT semen extender presents an alternative with enhanced safety profiles due to reduced contamination risks and greater potential for production standardization.

The specific impact of semen extenders on estrus manifestation and subsequent fertility in cows undergoing TAI programs remains unexplored. Furthermore, the relationship between post-thaw sperm kinematics of cryopreserved semen processed with EYTG versus OPT extenders and the influence of estrus expression on fertility outcomes has yet to be thoroughly investigated.

Based on these considerations, we hypothesize that cows detected in estrus and inseminated with semen cryopreserved using OPT extender will have a greater pregnancy per AI (P/AI) compared to cows inseminated with semen frozen using EYTG extender.

All experimental procedures apresented in this manuscript were approved by the Embrapa's Committee for Ethics in Animal Experimentation (Protocol 04/2017).

This experiment was performed at a commercial beef farm located in Rondonia State, Brazil. *Bos indicus* Nelore cows (n = 505), from 45 to 100 days postpartum, with body condition scores (BCS) between 2.75 - 3.5, on a scale of 1-5, (Ayres et al., 2009) were used in this study. The cows were kept in *Brachiaria brizantha* grazing systems with free access to water and mineral salts.

This experiment was performed in 5 replicates, in which, cows received an intravaginal device (IPD; 1.9 g, CIDR®, Pfizer Animal Health, São Paulo, Brazil) and 2 mg of estradiol benzoate (EB, Gonadiol®, Zoetis, São Paulo, Brazil) intramuscularly (im) on day 0 of the TAI protocol. After the removal of the IPD on day 8, the cows were given 300 IU of equine chorionic gonadotropin (eCG, Novormon®, Syntex, Buenos Aires, Argentina), 150 µg of d-cloprostenol (analog of PGF2α; Croniben®, Biogénesis-Bagó, Curitiba, Brazil), and 1 mg of estradiol cypionate (ECP®, Pfizer Animal Health, São Paulo, Brazil) im. At the time of IPD removal, all cows were marked in the sacrocaudal region using a marking stick to identify the animals that exhibited estrus within 48 h of IPD removal. Estrus was defined in females by the visual removal of > 75% of the ink 48 hours post-IPD removal.".

The cows were divided according to estrus expression 48 hours after IPD removal, as follows: 1) Estrus (E) and Non-Estrus (NE) cows.

Two Angus bulls, from a commercial AI center, characterized by high semen quality and freezability, were selected for this experiment. The raw ejaculate of each bull was split and diluted to achieve a concentration of 22 x 10^6 sperm per 0.25 mL insemination dose in 1 of 2 extenders (EYTG or OPT). Post-thaw semen analyses were performed using computer-assisted sperm analysis (CASA; Ivos-UltiMate™; Beverly, MA, USA) to characterize the sperm kinematics of each batch, adapted from (Pfeifer et al., 2019). The following variables were used to

characterize the semen samples as hyper-activated (H+) or non-hyper-activated (H-): amplitude of lateral head displacement (ALH), linearity (LIN) and curvilinear velocity (VCL). Semen diluted in EYTG extender was considered as H-, and semen diluted in OPT extender was considered H+ (Table 1). Batches were classified as H- if they exhibited an ALH < 7.5 µm, LIN > 50%, and VCL < 160 µm/s. Conversely, H+ batches were defined by ALH ≥ 7.5 µm, LIN ≤ 50%, and VCL ≥ 160 µm/s.

Table 1
Computer-aided sperm analysis (CASA) of sperm batches with different kinematic characteristics

Bull	Extender	ALH[1] (µm)	LIN[2] (%)	VCL[3] (µm /s)	Degree of hyperactivity[4]
A	EYTG	7,4	50,2	154,2	H-
A	OPTIXcell	8,9	46,2	195,5	H+
B	EYTG	5,9	55,0	135,8	H-
B	OPTIXcell	7,6	48,3	167,8	H+

[1]ALH: Amplitude of lateral head displacement;
[2]LIN: linearity;
[3]VCL: Curvilinear velocity;
[4]Adapted from Pfeifer et al. (2019).

Ultrasonic examination (SIUI CTS-900, linear probe with 5 MHZ, Guangdong, China) was performed 30 d post-TAI to assess pregnancy status. Visualization of the embryonic vesicle and detection of the embryo were the positive criteria for determining pregnancy.

From the cows enrolled in this study, 307 were considered in estrus and 198 were not considered in estrus at TAI. The semen was distributed to inseminate the cows according to estrus detection, as follows: 1) EOPT (n = 154), 2) EEYTG (n = 153), 3) NEOPT (n = 100), and 4) NEEYTG (n = 98).

The pregnancy per AI was analyzed by logistic regression using the GLIMMIX procedure. The initial model considered group, days postpartum, estrus, BCS, bull and sperm extender. Days postpartum, bull, BCS and sperm extender had no effect on the model. Therefore, only group and estrus were kept in the final model. In all analyses, differences between groups were considered statistically significant at $P < 0.05$.

Analysis of sperm parameters revealed a significant difference in hyperactivation between the two semen extenders. Semen diluted with OPT extender exhibited a higher degree of hyperactivation compared to semen diluted with EYTG (Table 1).

The proportion of pregnant cows inseminated with OPT and EYTG semen, according to estrus expression, is presented in Figure 1. The pregnancy per AI (P/AI) of the

groups EOPT (estrus, OPT extender; 69.5 %), and EEYTG (estrus, EYTG extender; 60.1 %) were greater (P < 0.001) in comparison with groups NEOPT (no estrus, OPT extender; 46.0 %), and NEEYTG (no estrus, EYTG extender; 42.9 %). The group EOPT tended (P = 0.08) to have greater in comparison with the group EEYTG.

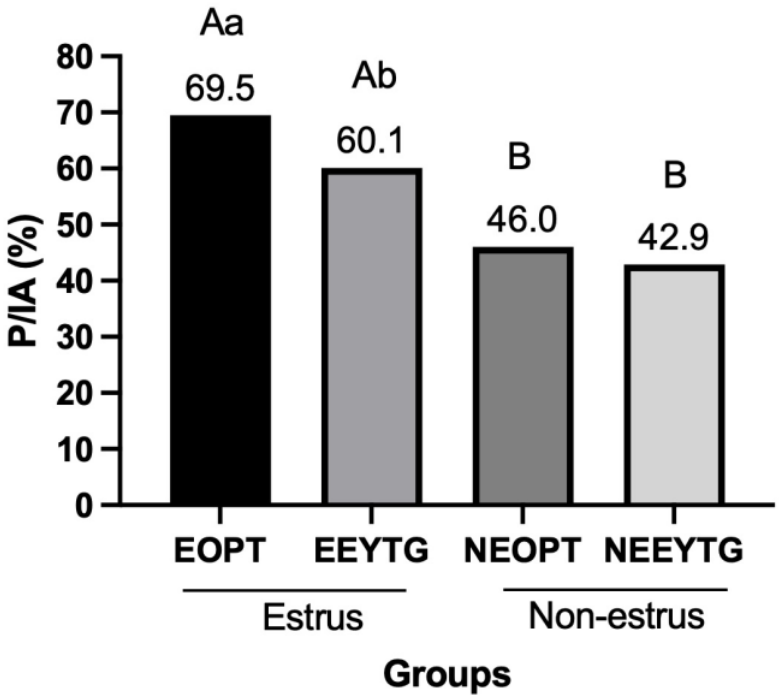

Figure 1. AI pregnancy in postpartum beef cows according to estrus expression compared to semen in different dilutors.
[AB] Different letters indicate an effect between groups (P < 0.05).
[ab] Different letters indicate a tendency between groups (P < 0.1).

Overall, cows that exhibited estrus at TAI had a significantly greater (P < 0.01) P/AI compared to cows that did not express estrus (64.8% vs. 44.4%).

The present study investigated the impact of different semen extenders and estrus expression on the fertility of cows subjected to a TAI program. Our initial hypothesis was partially confirmed. Cows that expressed estrus between PID removal and TAI and were inseminated with semen diluted in OPT extender tended to have greater P/AI compared to those cows detected in estrus and were inseminated with semen diluted in EYTG extender. To the best of our knowledge this is the first study to explore the effects of semen extender and estrus in the fertility of beef cows subjected to TAI protocols.

Previously, a study suggested that cows with delayed ovulation achieved higher pregnancy rates when inseminated with H-classified semen (Pfeifer et al., 2019). Oliveira et al. (2022) subsequently demonstrated that cows not exhibiting estrus at TAI typically experienced delayed ovulation. Based on these findings, we expected that non-estrus cows would show greater P/AI when inseminated with EYTG extender compared to OPT extender. Although our results revealed differences in sperm kinetics between the semen extenders used in the present study, the kinetics variations were less pronounced than those previously reported (Pfeifer et al., 2019).

The existing literature presents conflicting evidence regarding the superiority of OPT extender. For instance, a study conducted in buffalo demonstrated improved fertility when inseminations were performed using cryopreserved semen in OPT compared to EYTG (Ansari et al., 2016). Conversely, others indicated that OPT may not preserve the full and progressive motility of sperm for extended storage periods at room temperature compared to other diluents, potentially negatively impacting semen quality (Botta et al., 2019; Murphy et al., 2017). However, the data from the present study indicated a tendency for OPT-cryopreserved semen to increase pregnancy rates. This finding suggests a significant step towards broader adoption of this diluent for semen cryopreservation, given its potential to enhance P/AI and its reduced microbiological risk profile compared to the EYTG diluent.

Previous studies have established the importance of kinematic variables and hyperactivation attributes of frozen-thawed bull semen in predicting fertility outcomes (Schmidt & Kamp, 2004; Shojaei et al., 2012). Shojaei et al. (2012) reported a significant correlation between these kinematic variables and the hyperactivation characteristics of frozen-thawed bull semen. Moreover, Pfeifer et al. (2019) observed that cows with greater pre-ovulatory follicles were more likely to become pregnant when inseminated with hyperactivated (H+) semen. Therefore, in the present study, the elevated degree of hyperactivation observed in semen diluted in OPT extender may account for the observed tendency toward increased fertility in estrus-detected cows, in which ovulation occurs earlier in comparison with cows not detected in estrus (Oliveira et al., 2022).

This study demonstrates the critical importance of estrus expression for achieving higher pregnancy rates in cows subjected to TAI programs. Notably, among cows exhibiting estrus, those inseminated with semen diluted in OPT showed a tendency toward higher pregnancy per AI compared to those inseminated with EYTG-diluted semen. This study suggests a possible relationship between sperm extender type and fertility outcomes based on a cow's specific response to TAI protocols. While further research is needed to confirm these preliminary observations, these findings contribute to our understanding of bovine reproduction and may offer practical considerations for reproductive management in commercial cattle operations.

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