University of São Paulo
Luiz de Queiroz College of Agriculture

Strategies to predict and minimize differences in field fertility in bulls

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Dissertation presented to obtain the degree of Master in Science. Area: Animal Science and Pastures

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2021

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versão revisada de acordo com a resolução CoPGr 6018 de 2011

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DEDICATION

I dedicate to God and my family, the basis of my life.

“*Motivation is a door that opens from the inside*”

Mario Sergio Cortella

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SUMMARY

RESUMO

Estratégias para predizer e minimizar diferenças de fertilidade a campo de touros

Foram realizados dois estudos objetivando ajustar protocolos de inseminação artificial em tempo fixo (IATF) para incrementar a prenhez por IA (P/IA) de touros Nelore de menor fertilidade e predizer a fertilidade a campo de touros Holandês. No primeiro estudo, vacas Nelore (n = 1133) foram submetidas a um protocolo de IATF, iniciando no Dia -10, com a inserção de um dispositivo intravaginal de progesterona (P4, 1,0 g) e aplicação de benzoato de estradiol (BE, 2,0 mg). No Dia -2, o dispositivo de P4 foi removido e as vacas receberam cloprostenol sódico (PGF, 0,5 mg), eCG (300 UI) e cipionato de estradiol (CE, 1,0 mg). No Dia -0,5 ou 0 as vacas foram submetidas aos tratamentos com GnRH (G-16 = GnRH 16 h antes da IA ou G0 = GnRH na AI). No Dia 0 as vacas foram inseminadas e distribuídas no tratamento de categoria de fertilidade de touros [A = uma dose de sêmen de touro de fertilidade mais alta (n = 3); B1 = uma dose de sêmen de touro de fertilidade mais baixa (n = 3); B2 = duas doses de sêmen de touro B (n = 3)]. Além disso, foi realizado o teste de ligação espermática em agregados de células do oviduto. Foi utilizado sêmen de touros A (n = 3) e B (n = 3) da raça Nelore. Para isso, foram coletados tratos reprodutivos de vacas em abatedouro e utilizou-se a região do istmo do oviduto para coletar as células no laboratório. As células foram cultivadas por 24 h para a formação de agregados celulares, seguindo por coincubação com espermatozoides por 36 h. A motilidade espermática foi avaliada pelo sistema CASA e a integridade das membranas foi avaliada por microscopia de fluorescência e citometria de fluxo. A fertilidade dos touros A e B foi semelhante ($P > 0,10$), independente do ajuste do momento da ovulação com GnRH e do número de doses de sêmen. Entretanto, apesar de a P/IA das vacas inseminadas com touros A ter sido similar independente do ECC das vacas, vacas de ECC < 3,0 tiveram menor P/IA quando inseminadas com touros B [ECC < 3,0: 50,5% (166/329); ECC ≥ 3,0: 60,6% (254/419); $P < 0,05$]. O número de espermatozoides ligados por mm de agregado celular não diferiu em 0,5 h ($P = 0,10$), mas em 12 h, 24 h e 36 h houve maior número de espermatozoides/mm de touros A ($P > 0,05$). Espermatozoides A tiveram maior motilidade total e progressiva do que espermatozoides B. As características de membranas analisadas não diferiram entre os touros. No segundo estudo, foi utilizado sêmen de touros A (n = 3) e B (n = 4) da raça Holandês. Avaliou-se o número de espermatozoides/mm de agregado celular. Além disso, a motilidade espermática foi avaliada pelo CASA e a integridade das membranas espermáticas por citometria de fluxo. Com 0,5 h de coincubação houve tendência para maior número de espermatozoides A ligados por mm de agregado, e a partir de 12 h de coincubação a ligação de espermatozoides nos agregados foi maior para A. Com 24 h e 36 h o número de espermatozoides ligados por agregado foi mantido, sendo maior para espermatozoides de touros A, e com 36 h houve uma alta correlação entre a fertilidade de campo e o número de espermatozoides ligados por mm de agregado (r = 0,89). Dos padrões de motilidade avaliados pelo CASA, apenas a velocidade retilínea foi maior para touros A. Das características de membranas espermáticas analisadas, touros B tiveram maior porcentagem de células com membranas plasmática e acrossomal íntegras (80,3% vs. 74,5%; $P = 0,01$) e maior potencial mitocondrial de células com membrana plasmática íntegra ($P = 0,004$).

Sendo assim, o ECC das vacas, tempo de ovulação e características espermáticas influenciaram a fertilidade a campo de touros. Associadas às análises convencionais de motilidade e integridade de membranas espermáticas, a menor capacidade de se ligar às células do oviduto pode ser a causa da menor fertilidade de touros B, possibilitando o uso da técnica pela indústria como uma nova estratégia de predição da fertilidade a campo de touros.

Palavras-chave: IATF, Sêmen, Espermatozoide, GnRH, Oviduto, Prenhez, Bovino

ABSTRACT

Strategies to predict and minimize differences in field fertility in bulls

Two studies were carried out aiming to adjust timed-artificial insemination (TAI) protocols to increase pregnancy per AI (P/AI) of Nelore bulls of lower fertility and to predict the field fertility of Holstein bulls. In the first study, Nelore cows (n = 1133) underwent a TAI protocol, starting on Day -10, with insertion of an intravaginal progesterone device (P4, 1.0 g) and treatment with estradiol benzoate (EB, 2.0 mg). On Day -2, the P4 device was removed, and cows received cloprostenol sodium (PGF, 0.5 mg), eCG (300 IU) and estradiol cypionate (EC, 1.0 mg). On Day -0.5 or 0 cows were subjected to GnRH treatments (G-16 = GnRH 16 h before AI or G0 = GnRH at AI). On Day 0 cows were inseminated and assigned to the fertility category bull treatment (H = one dose of higher fertility bull semen [n = 3]; L1 = one dose of lower fertility bull semen [n = 3]); L2 = two doses of semen from bull L [n = 3]). In addition, the sperm binding test was performed on oviduct cell aggregates. Semen from H (n = 3) and L (n = 3) Nelore bulls was used. For this, reproductive tracts of cows in slaughterhouse were collected and the region of the isthmus of the oviduct was used to collect the cells in the laboratory. Cells were cultured for 24 h to form cell explants, followed by co-incubation with sperm for 36 h. In addition, sperm motility was assessed by the CASA system and the integrity of membranes was assessed by fluorescence microscopy and flow cytometry. The fertility of bulls H and L was similar ($P > 0.10$), regardless of the adjustment of time of ovulation with GnRH and number of semen doses. However, despite P/AI of cows inseminated with H bulls was similar regardless of the BCS, cows with BCS < 3.0 had lower P/AI when inseminated with L bulls (BCS < 3.0: 50.5% [166/329]; BCS ≥ 3.0: 60.6% [254/419]; $P < 0.05$). The number of sperm bound per mm of cell explant did not differ at 0.5 h ($P = 0.10$), but at 12 h, 24 h and 36 h there was a greater number of sperm/mm of H bulls ($P > 0.05$). H sperm had greater total and progressive motility than L sperm. The membrane characteristics analyzed did not differ between H and L groups. In the second study, semen from H (n = 3) and L (n = 4) Holstein bulls was used. The number of sperm/mm of cell aggregate was evaluated. In addition, sperm motility was assessed by CASA and the integrity of sperm membranes by flow cytometry. With 0.5 h of co-incubation there was a tendency for a greater number of H sperm bound per mm of aggregate, and from 12 h of co-incubation on the binding of sperm in aggregates was greater for H. With 24 h and 36 h the number of spermatozoa bound per aggregate was maintained, being greater for sperm from bulls H, and at 36 h there was a high correlation between field fertility and the number of sperm bound per mm of explant (r = 0.89). Out of the motility patterns evaluated by CASA, only the straight velocity was higher for bulls H. Out of the sperm membrane characteristics analyzed, L bulls had the greatest percentage of cells with intact plasma and acrosomal membranes (80.3% vs. 74.5%; $P = 0.01$) and greater mitochondrial potential of cells with an intact plasma membrane ($P = 0.004$). Thus, the BCS of cows, timing of ovulation and sperm characteristics influenced field fertility of bulls. Combined with conventional analyzes of sperm motility and membrane integrity, the lower ability to bind to oviduct cells may be the cause of

lower fertility in L bulls, allowing the industry to use this technique as a new strategy for predicting field fertility of bulls.

Keywords: TAI, Semen, Sperm, GnRH, Oviduct, Pregnancy, Cattle

1. INTRODUCTION

Artificial insemination (AI) has the potential to promote genetic improvement and reproductive efficiency, increasing the productivity of beef and dairy farms [1]. With the advancement of research, hormonal protocols capable of synchronizing the emergence, growth and ovulation of a dominant follicle were developed [2], enabling timed-AI (TAI). Through TAI, it is possible to carry out the breeding season in beef cattle or the distribution of births throughout the year in dairy farms, with fertility similar or superior to AI [3]. However, for the success of TAI bull fertility is extremely important, and there are still no reliable tools capable of predicting male fertility before the bull is tested in the field [4,5].

After 8 to 12 h of AI, a functional sperm reservoir is formed in the oviduct [6], with only sperm capable to bind to oviduct epithelial cells (OEC) to maintain their viability (i.e., ability to fertilize the oocyte) [7,8]. Only tens to hundreds of spermatozoa have the potential to bind to OEC [9,10], as they are non-hyperactivated [11,12] and have intact membranes [13]. However, conventional analyzes performed by the industry of sperm motility and membrane integrity do not have the potential to predict field fertility of bulls [14]. Several studies have investigated fertility markers in bull sperm, however the difference in fertility is multifactorial and may be related to characteristics of sperm cells, seminal plasma, and even embryonic development [15–17].

With the functional formation of the spermatic reservoir in the oviduct, the mechanisms involved in ovulation trigger sperm release and hyperactivation so that fertilization occurs in the oviduct [18]. Thus, insemination with hyperactivated sperm, which has a low potential to form the functional reservoir, can result in good pregnancy per AI (P/AI) when performed close to the time of ovulation [19].

Thus, based on the hypothesis that semen from higher fertility bulls (HF) have a greater capacity to form the functional sperm reservoir in the oviduct, we conducted *in vivo* and *in vitro* studies comparing sires with retrospect of having lower (LF) or higher (HF) field fertility. The aim of the first study was to adjust the timing of ovulation in cows and to inseminate with one or two straws of semen from LF bulls in *Bos indicus* cows, and to compare conventional sperm analysis and the *in vitro* sperm binding to the explants of oviduct cells of HF vs. LF sires. The aim of the second study was to evaluate sperm characteristics associated with field fertility in Holstein bulls by means of evaluating: 1) motility and integrity of sperm membranes, and 2) *in vitro* sperm binding to the explants of oviduct cells.

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2. STRATEGIES TO IMPROVE FERTILITY OF LOWER FERTILITY SIRES DURING TIMED-AI IN *BOS INDICUS*

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Abstract

The objective of the study was to investigate sperm characteristics associated with the difference of field fertility of sires and to promote adjustments in the timed-AI (TAI) protocol to benefit *Bos indicus* sires of lower (**L**) fertility. Nelore cows (n = 1,133) were subjected to GnRH treatments (**G-16** = GnRH 16 h before AI or **G0** = GnRH at AI) and sire fertility treatment (**H** = single semen dose of higher fertility [**H**] sires [n = 3]; **L1** = single semen dose of L sire [n = 3]; **L2** = two semen doses of L sire). Cows were randomly distributed in one of six treatments: **G-16H** (n = 203), **G-16L1** (n = 187), **G-16L2** (n = 174), **G0H** (n = 182), **G0L1** (n = 198), or **G0L2** (n = 189). Cows were submitted to the following protocol (Day -10: P4 device [1.0 g] and 2.0 mg estradiol benzoate; Day -2: P4 withdrawal, 0.5 mg cloprostenol sodium, 300 IU eCG, and 1.0 mg estradiol cypionate), and TAI was performed 48 h after P4 device removal. The same batches of semen were used to assess the sperm motility by CASA, integrity of membranes by fluorescence microscopy and flow cytometry, and sperm binding to explant cells. The fertility of **H** and **L** sires was similar ($P = 0.80$), regardless of time of administration of GnRH ($P = 0.56$). However, there was a tendency for interaction ($P = 0.10$) between GnRH treatment and BCS, with the **G-16** benefiting cows with BCS ≥ 3.0 and/or harming cows with BCS < 3.0 (BCS ≥ 3.0: 62.2 [194/312], BCS < 3.0: 51.2 [129/252]; $P \leq 0.05$). Furthermore, cows with BCS < 3.0 tended to have lower P/AI ($P = 0.10$) when inseminated with **L** sires (H: 57.9 [95/164] vs. **L**: 50.5% [166/329]). The P/AI of cows inseminated with **H** sires was maintained independent of the BCS of cows ($P > 0.1$), however P/AI of **L** sires was lower in cows with BCS < 3.0 (50.5 [166/329] vs. 60.6% [254/419], respectively; $P \leq 0.05$). **H** sperm had greater total motility ($P = 0.02$) and progressive motility ($P = 0.001$) than **L** sperm. The characteristics of sperm membranes were similar between **H** and **L**. At 12, 24, and 36 h of co-culture with oviduct cell explants the number of sperm/mm was greater for **H** ($P < 0.05$). The fertility of bulls is multifactorial, influenced by the BCS of the cows, timing of ovulation and sperm characteristics. **H** bulls maintain good fertility regardless of the BCS of the cows, have sperm with greater progressive motility and greater sperm viability *in vitro*. These results suggest that sperm

viability analysis, especially the sperm binding to explant cells, could be a reliable test to predict sire fertility.

Keywords: Bull fertility; GnRH; Ovulation; Pregnancy; CASA

2.1. Introduction

Fertility (i.e., ability to produce an offspring) is an important characteristic that supports domestic animal production [1,2]. However, despite both male and female fertility contributes to reproductive performance and efficiency, animal selection programs have been more focused on females than males [3,4]. As a consequence, the individual effect of sire on pregnancy per artificial insemination (P/AI) and/or pregnancy loss has been reported in beef [1,5,6] and dairy cattle [7,8]. Therefore, considering that P/AI to the first service is one of the main indicators that contribute to economic viability of AI programs [9,10], use of higher fertility sires, associated with a proper hormonal synchronization protocol [11,12], is an essential strategy to achieve higher reproductive efficiency, consequently reducing the involuntary culling on the herd [13,14].

Researchers and the AI industry are focused on selecting and maximizing sire fertility [4,15,16]. There are several factors involved with male fertility, including compensable and non-compensable factors, such as the number of sperm cells needed to achieve maximum fertility [17,18]. The high potential of the mitochondrial membrane and the integrity of the plasma and acrosomal membranes of sperm cells, evaluated by fluorescence microscopy, were related to high fertility characteristic of sires, as well as the low production of reactive oxygen species (ROS), assessed by flow cytometry [19,20]. The destabilization of the plasma membrane of sperm cells can lead to malfunction, reduced fertilization capacity, and changes in motility [21,22]. However, sperm classified as acceptable in these techniques do not necessarily ensure high P/AI in the field [23,24]. As described by Zoca et al. [25], fertility of some sires was improved by increasing semen concentration, whereas fertility of other sires was maintained.

The exposure of sperm cells to the female reproductive tract is necessary for the capacitation process [18]. In addition, a stock of sperm cells is formed in the oviduct [26], and lower fertility sires may have a lower capacity of maintaining sperm cells alive for extended periods (i.e., lower longevity/viability of sperm cells) [27,28]. Therefore, adjustment of ovulation time in relation to AI could favor lower fertility sires [29,30]. In the same way, several studies demonstrated a beneficial effect of the administration of GnRH analogues at

the time of AI in beef cows that did not express estrus in estradiol (E2)/progesterone (P4)-based protocols and/or cows with lower body condition score (BCS) [30–34]. After the LH peak, ovulation of the dominant follicle occurs in ~ 28 h, with the greatest P/AI obtained when AI occurs 12 to 16 h before ovulation [35,36]. Additionally, controlling the time of ovulation at the end of synchronization protocols may improve fertility outcomes [37,38].

This study aimed to evaluate strategies to optimize the time of ovulation in a TAI protocol in order to improve P/AI of lower fertility sires during TAI in *Bos indicus* cows. The strategies were: 1. Adjusting the timing of ovulation induced by GnRH; and 2. Using simultaneously two semen doses to increase sperm number at AI. The second objective was to evaluate the conventional sperm analysis and sperm binding to oviduct cell explants. Our hypothesis was that sperm from bulls with lower field fertility have shorter longevity in the female reproductive tract, with their P/AI increased with the anticipation of the treatment with GnRH in relation to TAI, less sperm bound to oviduct cell explants over time, and the conventional analysis of motility and integrity of sperm membranes are similar between bulls of lower or higher field fertility.

2.2. Material and methods

The experiment was performed according to The Animal Research Ethics Committee of "Luiz de Queiroz" College of Agriculture of the University of São Paulo (ESALQ/USP) and all animal procedures were previously approved (Protocol CEUA # 2017.5.1618.11.9).

2.2.1. Location

The field experiment was performed at Marajá Farm, located in Pirapemas, MA, Brazil. Cows were kept on pasture (*Panicum maximum*, cv. Mombaça), supplemented with mineral salt, and had *ad libitum* access to water. The mineral salt of the primiparous cows received an increase of 10% of corn.

The laboratorial part of the experiment was performed at the Laboratory of Teaching and Research in Reproductive Pathology (LEPPaR) and the Laboratory of Semen Biotechnology and Andrology (LBSA), School of Veterinary Medicine and Animal Science (FMVZ/USP), located in Pirassununga, SP, Brazil.

2.2.2. Experimental design

A total of 1,133 lactating Nelore cows (*Bos indicus*; multiparous [n = 800] and primiparous [n = 333]) with an average BCS of 2.9 ± 0.4 (scale 1-5 points, using 0.25 increments [39]) were randomly assigned to one of six treatment groups (Fig. 1). All cows were submitted to a synchronization protocol based on insertion of a 1.0 g intravaginal P4 device (Sincrogest; Ourofino, Cravinhos, Brazil) and administration of 2.0 mg estradiol benzoate im (EB; Sincrodiol; Ourofino) on Day -10. On Day -2, the P4 device was removed, and cows received 1.0 mg estradiol cypionate (EC; SincroCP; Ourofino), 0.5 mg cloprostenol sodium (PGF; Sincrocio; Ourofino) and 300 IU eCG (SincroeCG; Ourofino). In addition, cows had the base of their tail painted with a tail chalk, on Day -2, for estrus detection at AI. On Day -0.5, cows were randomized to receive 10.5 μg buserelin acetate im (GnRH; Gonaxal, Biogénesis Bagó, Curitiba, Brazil) approximately 16 h before TAI (**G-16**) or concomitant with TAI, on Day 0 (**G0**). Additionally, on Day 0, cows were distributed to receive a single dose of semen (semen straw with a volume of 0.25 mL containing an average of 7.9 x 10^6 motile sperm per dose) of higher fertility sires (**H**; Concept Plus program; Alta Genetics, Uberaba, Brazil), or a single (**L1**; semen straw with a volume of 0.25 mL containing an average of 5.6 x 10^6 motile sperm cells per dose) or double (**L2**; two semen straws with a volume of 0.25 mL each containing an average of 5.6 x 10^6 motilesperm cells per straw) semen doses of lower fertility sires (Alta Genetics). Therefore, treatments were: **G-16H** (GnRH on Day -0.5 and TAI with a single semen dose of H sires; n = 203), **G-16L1** (GnRH on Day -0.5 and TAI with a single semen dose of L sires; n = 187), **G-16L2** (GnRH on Day -0.5 and TAI with double semen doses of L sires; n = 174), **G0H** (GnRH on Day 0 and TAI with a single semen dose of H sires; n = 182), **G0L1** (GnRH on Day 0 and TAI with a single semen dose of L sires; n = 198), and **G0L2** (GnRH on Day 0 and TAI with double semen doses of L sires; n = 189). On Day 22 of the study, all cows were submitted to an early resynchronization program, based on administration of 2.0 mg EB im and insertion of a P4 device. Eight d later (Day 30), pregnancy diagnosis was performed, and non-pregnant cows received the same hormonal treatments used on Day -2 (P4 device removal, PGF, eCG and EC), followed by the same randomized experimental treatments (**G0** or **G-16**, and **H**, **L1** or **L2**). In cows diagnosed pregnant on Day 30, the P4 device was removed, and no other treatment was applied. Thus, first (n = 880) and second service cows (n = 253) were considered in this experiment.

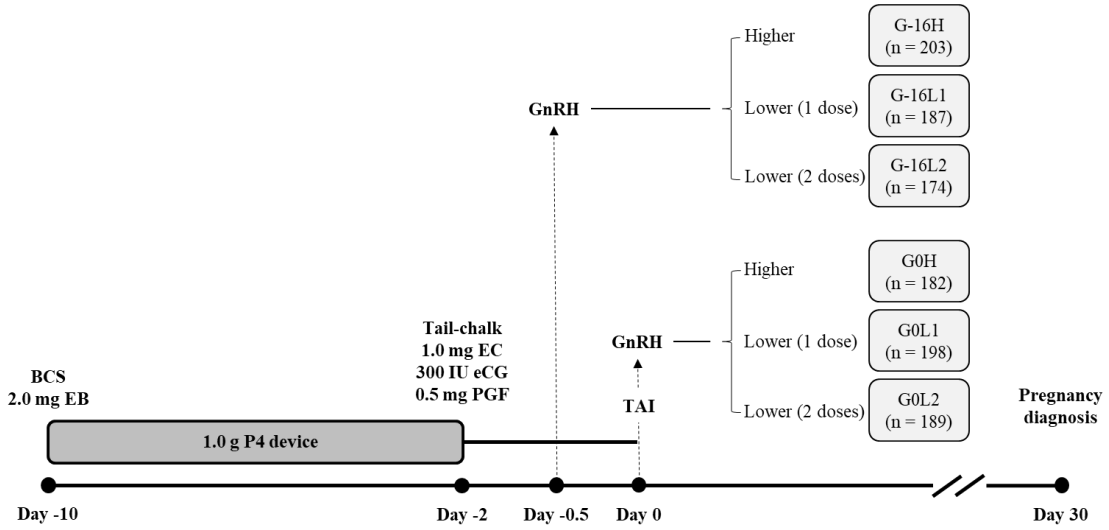

Fig. 1. Experimental design using *Bos indicus* cows. BCS = body condition score evaluation; Day -10 = beginning of protocol at a random day of the estrous cycle with insertion of P4 (progesterone intravaginal device) and admnistration of EB (estradiol benzoate); Day -2 = P4 removal and admnistration of EC (estradiol cypionate), eCG (equine chorionic gonadotropin), and PGF (cloprostenol sodium); Day 0 = timed-artificial insemination (TAI); Day 30 = Pregnancy diagnosis; Tail-chalk on Day -2 for estrus detection (Day -0,5 and Day 0); GnRH = gonadotropin-releasing hormone – 10 µg Buserelin acetate (**G-16** = GnRH on D-0,5 [~16 h before TAI]; **G0** = GnRH on D0); **H** = one dose of a higher fertility sire; **L1** = one dose of a lower fertility sire; **L2** = two doses of a lower fertility sire.

2.2.3. Classification of higher and lower fertility sires

For this experiment, commercial batches of cryopreserved semen from six Nelore sires with known outcomes of field fertility were used. Retrospective data of sire fertility were obtained in partnership with Alta Genetics (Uberaba, Brazil), through the database of the Concept Plus Beef program, composed of data from more than 6 million insemination. This program evaluates the performance of several sires used in TAI programs carried out in herds by technicians, classifying them as of higher, intermediate, or lower fertility. For this experiment, the number of batches of cryopreserved semen (**L** = 3 sires [P/AI = 45%]; **H** = 3 sires [P/AI = 55%]) were: three of the bull A (**H**), three of the bull B (**H**), two of the bull C (**H**), three of the bull D (**L**), three of the bull E (**L**), and three of the bull F (**L**).

2.2.4. Ultrasound examinations

Pregnancy diagnosis was performed on Day 30 using a B mode transrectal ultrasound with a 7.5 MHz linear transducer (Infinit 1V, Ultramedic, Shenzhen, China) and only cows containing an embryo with heartbeat were considered pregnant.

2.2.5. Sperm concentration

Sperm concentration was evaluated using the Neubauer chamber technique using a dilution (1:100) in a buffered saline solution containing 4% formaldehyde. For this, 10 μL of semen were added to 990 μL of this solution, and sperm count was performed at 400 x magnification in phase contrast microscopy (Model 80i, Nikon, Tokyo, Japan).

2.2.6. Assessment of sperm morphology

The sperm sample was fixed in a buffered saline solution containing 4% formaldehyde previously heated to 37 °C. For evaluation of sperm morphology, 200 cells were analyzed by the wet chamber technique, under differential interference contrast microscopy (DIC, model 80i, Nikon, Tokyo, Japan) with 1,000 x magnification. Cells were classified according to Blom [40].

2.2.7. Computer-assisted assessment of sperm motility

Motility characteristics were evaluated using the Hamilton Thorne Research Motility Analyzer (HTM-IVOS- Ultimate, Hamilton Thorne Biosciences, Bevely, MA, USA). The set up was previously adjusted for the analysis of bovine sperm in the IDENT option. The setting has been preset to a number of frames of 30, frames per second of 60 Hz, minimum contrast of 80, minimum cell size of 5 pixels, contrast to static cells of 30, average path velocity cutoff (defining slow cells) of 40 μm/s, minimum average path velocity (defining progressive cells) of 30 μm/s, straight-line velocity cutoff (defining slow cells) of 15 μm/s, cell size of 6 pixels, cell intensity of 75, static head size of 0.60–3.40, static head intensity of 0.75–1.70, static elongation of 8–92, magnification of 1.86 x, video frequency of 60, illumination intensity of 2241, and temperature of 37 °C. Five fields were selected for each sample. For the evaluation,

10 μL of the sperm sample diluted to 10 x 10^6 sperm/mL in TALP sperm medium (4.2 mg/mL of sodium chloride, 1.87 mg/mL of potassium chloride, 2.1 mg/mL of sodium bicarbonate sodium, 50 μg/mL of sodium phosphate, 290 μg/mL of calcium chloride monohydrate, 80 μg/mL of magnesium chloride hexahydrate, 6.5 mg/mL of Hepes) supplemented with albumin, sodium lactate, pyruvate sodium and penicillin-streptomycin [41] were inserted into the Makler chamber (Selfi-Medical Instruments, Haifa, Israel) that was used for the evaluation. The following variables were analyzed: total motility (%), progressive motility (%), average path velocity (VAP, μm/s), straight-line velocity (VSL, μm/s), curvilinear velocity (VCL, μm/s), amplitude of lateral head displacement (ALH, μm), beat/cross frequency (BCF, Hz), straightness (STR, %), and linearity (LIN, %).

2.2.8. Evaluation of mitochondrial function, plasma membranes and acrosomal membranes by fluorescence microscopy

For the analysis under fluorescence microscopy, an epifluorescence microscope (model 80i, Nikon, Tokyo, Japan) was used at 1000 x magnification, using the triple filter (D/F/R, C58420) presenting the UV- sets 2E/C (340-380 nm excitation and 435-485 nm emission), B-2E/C (465-495 nm excitation and 515-555 nm emission) and G-2E/C (540-525 nm excitation and 605 emission -655 nm). For evaluation of the mitochondrial membrane potential and the integrity of plasmatic and acrosomal membranes, sperm concentration was adjusted to 25 x 10^6 sperm/mL in TALP sperm medium. Then, to the volume of 150 μL of diluted semen, 2 μL of Hoestch 33342 (H342, 0.5 mg/mL, Molecular Probes, H1399), 2 μL of propidium iodide (PI, 0.5 mg/mL in Dulbecco's PBS, Molecular Probes, P3566), 6 μL of tetraethylbenzimidazolylcarbocyanine iodide (JC-1, 153 μM in dimethyl sulfoxide, Molecular Probes, T3168) and 20 μL of fluorescein-conjugated *Pisum sativum* agglutinin (FITC- PSA, 100 μg/mL in Dulbecco's PBS). After 8 min of incubation at 37 ºC, 7 μL of the sample were put on a slide, and 200 cells were classified according to the fluorescence emitted into: intact plasma membrane, intact acrosome, and high mitochondrial function (IPIAH), adapted from Celeghini [42].

2.2.9. Evaluation of mitochondrial membrane potential, lipid peroxidation, and fluidity of the membranes by flow cytometer

Flow cytometry analyzes were performed with the Accuri C6 flow cytometer (Becton, Dickinson and Company, San Jose, CA), using a technique adapted from Díaz et al. [43]. To determine the particle size and dispersion property, the samples were stained with 150 µL of SYTO-59 (S59; 5 nM, S10341) and incubated for 10 min at 37 °C. Afterwards, the samples were evaluated with a 675/25 nm bandpass filter, determining the characteristics of the nucleus of cells, stained with S59. An acquisition rate of approximately 600 to 1,000 events/sec, acquiring 10,000 positive S59 events per assay. The samples were excited by an argon laser at 488 nm and by a red laser at 640 nm. After the incubation period, all samples were diluted with 150 µL of TALP sperm medium to obtain a concentration of 2.5 x 10^6 sperm/mL for analysis in the flow cytometer.

To evaluate mitochondrial membrane potential, in an aliquot of 150 µL semen (5 x 10^6 sperm/mL), 1 µL of PI, 1 µL of JC-1, and 3 µL of S59 were added and incubated for 10 min at 37 °C and later analyzed. For evaluation of lipid peroxidation of viable cells (PI negative), 1 µL of probe C11-BODIPY $^{581/591}$ (BP; 1mg/mL, D-3861) was added in an aliquot of 150 µL of semen (5 x 10^6 sperm/mL) and incubated for 20 min at 37 °C. After 20 min, 1 µL of PI and 3 µL of S59 were added. After 10 min of incubation the samples were analyzed. To assess the fluidity of the membranes, semen was diluted in TALP sperm medium to a concentration of 5 x 10^6 sperm/mL, and 1 µL of Yo-Pro-1 (YP; 7,5 nM) was added and incubated for 10 min. After incubation, 1 µL of S59 was added and incubated for 10 min. After incubation, 2 µL of merocyanine 540 (M540; 810 µM) was added and incubated for 70 s and later analyzed. The number of batches of cryopreserved semen analyzed by flow cytometry were: two of the bull A (**H**), two of the bull B (**H**), two of the bull C (**H**), three of the bull D (**L**), three of the bull E (**L**), and two of the bull F (**L**).

2.2.10. Evaluation of sperm binding to explant cells

Reproductive tracts of cows were collected in a slaughterhouse and transported to the laboratory at 35 ºC in saline solution (NaCl 0.9%), supplemented with penicillin G (100 IU/mL) and streptomycin sulfate (50 µg/mL). The isthmus region of the oviduct was collected, using the oviduct contralateral to the corpus luteum (CL), considering up to 4 cm from the uterotubal junction. The lumen of eight to 12 oviducts was washed twice with 1 mL of TALP medium (TCM-199 with Hank's salts, supplemented with 10% fetal bovine serum, 50 mg/L gentamicin sulfate and 25 µg/mL of amphotericin B; Vitrocell, Campinas, Brazil) in

a 15 ml conical bottom tube. The conical bottom tube with cells was incubated at 35 ºC with 5% CO2 for 50 min, being washed twice with 5 mL of TALP medium, disaggregating the cells at each wash with an automatic pipette of 1,000 µL, followed by cultivation for 24 h in a 100 mm petri dish supplemented with 25 mL of TALP medium, changing 50% of the medium 6 to 8 h after the start of culture.

A Petri dish was prepared for each bull with 160 explants distributed in 12 drops of 30 µL of sp-TALP medium (Botupharma, Botucatu, Brazil) under silicone oil, evaluating one bull of each treatment in each repetition. Three semen straws from three different lots were used, and 250 µL of the sperm cell pool were washed with 1 mL of synthetic oviductal fluid (SOF; Botupharma), centrifuging at 700 x g for 5 min. After washing, the supernatant was removed, and the pellet was resuspended in SOF medium. The number of sperm was counted in a Neubauer chamber to accurately use 1 x 10^5 motile sperm/mL per drop containing explants (0 h). Three drops from the incubation plate were removed at each time of evaluation (0.5 h, 12 h, 24 h, and 36 h), transferring the sperm-explant complex into a 30 µL drop of sp-TALP medium on a glass slide, discarding the explants after analysis. For each sperm-explant complex, two photographs were taken using the EUREKAM 1.3 image capture system (BEL Engineering, Monza, Italy). The two photographs were used to count the number of bound sperm and one to measure the perimeter of the explant with ImageJ 1.3 software (National Institute of Health, Bethesda, USA). The number of bound sperm was divided by the perimeter of each explant to obtain the number of bound sperm per mm of explant (sperm/mm), adapted from Kadirvel et al. [44].

2.2.11. Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC).

The analyses of binomial variables (expression of estrus and P/AI) were performed using the GLIMMIX procedure fitting a binomial distribution with the Link Logit function. The analysis was performed as a 2x3 factorial design, in which the model for expression of estrus included effects of GnRH treatment (at TAI and 16 h before), semen treatment (higher, lower, and two doses of lower fertility), the interaction between them, BCS class (< 3.0 and ≥ 3.0), and parity. The model for P/AI on 30 d after TAI included effects of GnRH and semen treatments and their interactions, expression of estrus (with and without estrus expression up to Day 0) and BCS class. The interactions between treatments and the described variables

were also evaluated. The SLICE command in the LSMEANS was used to interpret significant interactions.

For CASA analysis, all data were tested for normality of studentized residuals using the UNIVARIATE procedure of SAS according to the Shapiro-Wilk test. The homogeneity of variances was evaluated with the Levene test using the Hovtest and Welsh methods. The analyses were performed using the GLIMMIX procedure fitting a Gaussian distribution.

The number of sperm/mm followed a Gamma distribution, so the analyses were performed using the GLIMMIX fitting a Gamma distribution. The final model included the effect of fertility, hour, the interaction between them, and OEC explant perimeter as a covariate.

Tukey honest significant difference post hoc test was performed to determine differences. Values are presented as means ± standard error of the mean (SEM) (continuous variables) or as percentage (%; binomial variables). Significant differences were declared when $P \leq 0.05$, whereas tendencies were considered when $0.05 < P \leq 0.10$.

2.3. Results

Expression of estrus did not differ between G-16 and G0 treatments on Day -0.5 (14.6 [82/562] vs. 12.9% [72/558]; $P = 0.35$), nor on Day 0 (76.9 [433/562] vs. 81.0% [452/558]; $P = 0.11$). There was an effect of BCS class (< 3.0 or ≥ 3.0) at the beginning of the protocol on expression of estrus, in which cows with higher BCS presented a greater incidence of estrus than cows with lower BCS (82.6% [523/640] vs. 74.1% [361/493]; $P < 0.01$).

Despite cows that expressed estrus had greater P/AI (estrus = 58.4 [516/884] vs. no estrus = 49.2% [116/236]; $P = 0.04$), P/AI was not affected by the time of GnRH administration nor by fertility of the sires or by the number of semen doses (Fig. 2).

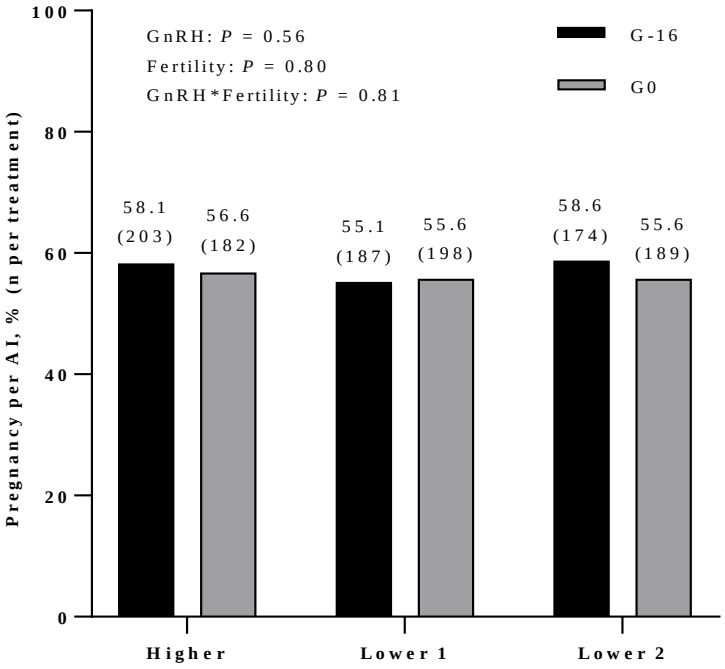

Fig. 2. Pregnancy per AI (%, n) 30 d after AI of Nelore (*Bos indicus*) cows submitted to an E2/P4-based TAI protocol, with GnRH administration 16 h prior to AI (G16) or at the time of AI (G0), and inseminated with a single semen dose of higher fertility sires, or with one or two semen doses of lower fertility sires.

Although there was no effect of GnRH treatment on P/AI, when cows were separated in BCS classes, a tendency for interaction ($P = 0.10$) was detected between the time of GnRH administration and BCS class. Cows in the G0 group had similar P/AI regardless of BCS class, whereas, in the G-16 group, cows with BCS ≥ 3.0 had greater P/AI than cows with BCS < 3.0 (Fig. 3).

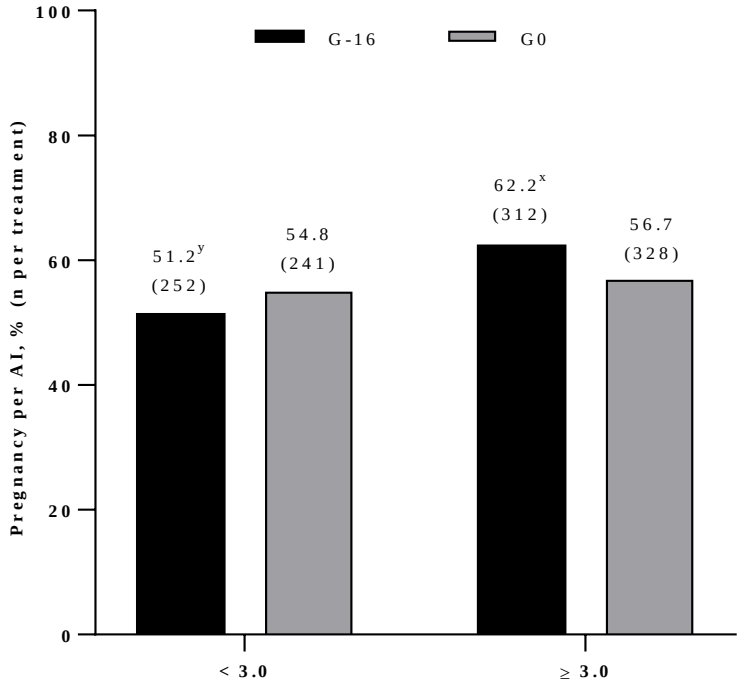

Fig. 3. Pregnancy per AI (%, n) 30 d after AI of Nelore (*Bos indicus*) cows submitted to an E2/P4-based TAI protocol, with GnRH administration 16 h prior to AI (G16) or at the time of AI (G0), according to their BCS class. There was a tendency for an interaction between GnRH treatments and BCS class ($P = 0.10$). $^{x,y}P \leq 0.05$.

Interestingly, there was a tendency for interaction between BCS class and sire fertility regardless of number of semen doses ($P = 0.07$). Cows with BCS < 3.0 inseminated with higher fertility sires tended ($P = 0.10$) to have greater P/AI than those inseminated with lower fertility sires, whereas no differences were found in cows with BCS ≥ 3.0. In addition, within the group inseminated with lower fertility sires, cows with BCS ≥ 3.0 had 10 percentual points greater P/AI than cows with BCS < 3.0 ($P \leq 0.05$; Fig. 4).

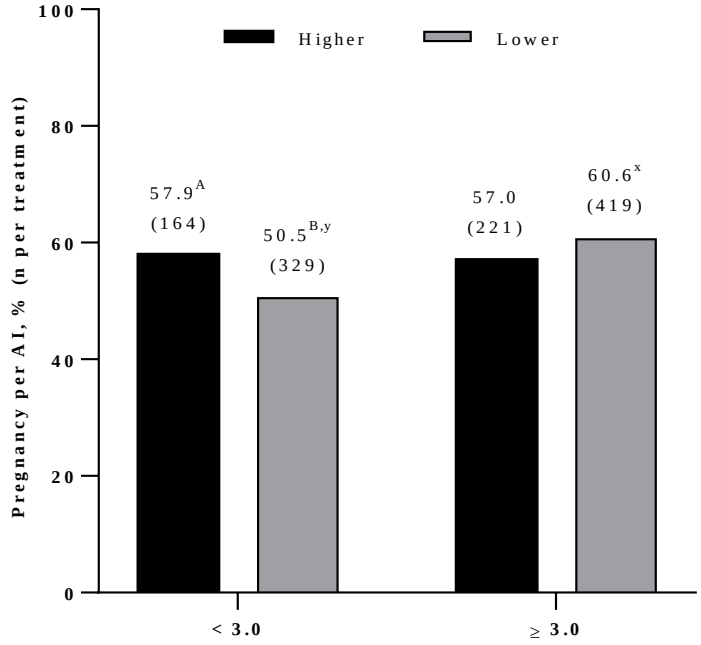

Fig. 4. Pregnancy per AI (%, n) 30 d after AI of Nelore (*Bos indicus*) cows submitted to an E2/P4-based TAI protocol inseminated with higher or lower (with one or two doses) fertility sires, according to their BCS class. There was a tendency for an interaction between fertility of sires and BCS class ($P = 0.07$).
[A,B]Indicate the effect of sire fertility within BCS class < 3.0 ($P = 0.10$).
[x,y]Indicate the effect of BCS class within Lower fertility sires ($P \leq 0.05$).

The major defects (**H** = 3.9 ± 1.5 vs. **L** = 3.8 ± 1.3; $P = 0.94$), minor defects (**H** = 3.1 ± 0.8 vs. **L** = 3.9 ± 1.3; $P = 0.62$) and total defects (**H** = 7.0 ± 1.5 vs. **L** = 7.6 ± 2.4; $P = 0.84$) were similar between the fertility categories of bulls.

The sperm motility data obtained using CASA are shown in Table 1. **H** sperm had higher total motility ($P = 0.02$) and progressive motility ($P = 0.001$) than **L** sperm. In addition, there was a lower heterogeneity of progressive motility of **H** sperm compared to **L** sperm (Fig. 5). Also, the Straight-line velocity (VSL), Beat/cross frequency (BCF), Straightness (STR), and Linearity (LIN) were higher for **H** sperm.

Table 1. Means ± standard error of computer-assisted semen analysis of sperm motility in higher vs. lower fertility sires.

Variables	Higher Fertility (n = 8)	Lower Fertility (n = 9)	*P*
Total motility (%)	69.3 ± 2.9	53.3 ± 4.8	0.02
Progressive motility (%)	55.5 ± 2.5	36.6 ± 4.0	0.001
Average path velocity (VAP; µm/s)	119.3 ± 3.9	114.5 ± 2.9	0.33
Straight-line velocity (VSL; µm/s)	92.6 ± 3.4	78.7 ± 1.9	0.002
Curvilinear velocity (VCL; µm/s)	219.6 ± 6.7	220.2 ± 8.6	0.98
Ampl. of lateral head displacement (ALH; µm)	9.2 ± 0.1	8.9 ± 0.2	0.12
Beat/cross frequency (BCF; Hz)	32.6 ± 1.6	28.8 ± 1.0	0.05
Straightness (STR; %)	78.1 ± 2.3	70.2 ± 1.9	0.02
Linearity (LIN; %)	45.0 ± 2.0	39.4 ± 1.4	0.03

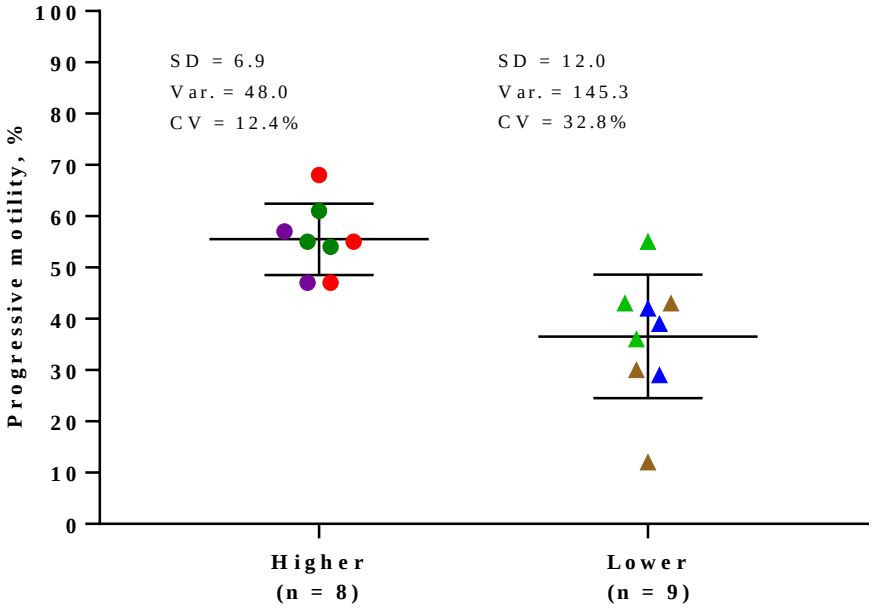

Fig. 5. Dispersion of progressive motility (%) assessed by computer-assisted semen analysis. Circles represent higher fertility sires and triangles represent lower fertility sires. SD = standard deviation; Var. = variance; CV = coefficient of variation.
Similar colors indicate the progressive motility of each batch of the same sire.

The percentage of sperm with intact plasma membrane did not differ (*P* = 0.53) between **H** and **L** sires (49.7 vs. 45.7%, respectively). Furthermore, the percentage of sperm cells with intact acrosome was similar between **H** and **L** sires (95.1 vs. 94.6%, respectively; *P* = 0.87). However, the percentage of sperm with greater mitochondrial function was extremely high when compared to the values described in the literature for cryopreserved bovine semen (**H** = 98.8% and **L** = 98.5%; *P* = 0.55), so we disregarded the mitochondrial analysis.

Flow cytometry analysis results are presented as the arbitrary units (a.u.). There was no difference between groups in sperm with intact plasma membrane and high mitochondrial membrane potential (**H** = 3,537.8 ± 2,128.8 a.u. vs. **L** = 3,023.6 ± 835.8 a.u.; *P* = 0.64), lipid peroxidation (**H** = 816.8 ± 72.8 a.u. vs. **L** = 930.4 ± 33.1 a.u.; *P* = 0.14), and fluidity of the membranes (**H** = 17,679.7 ± 1,193.6 a.u. vs. **L** = 15,855.9 ± 1,386.7 a.u.; *P* = 0.35).

The numbers of sperm bound per mm of explant are distributed into eight groups, considering the bull fertility (**H** and **L**) at each assessment time (0.5, 12, 24, and 36 h; Fig. 6). At 0.5 h of co-incubation there was a tendency (*P* = 0.10) towards greater number of sperm/mm in **L**. However, at 12, 24, and 36 h there was a greater number of sperm/mm for **H** (*P* < 0.05). Sperm from **H** bulls had a distinct pattern of binding to oviduct cell explants than sperm form **L**, with greater number of sperm/mm in 12 h when compared to 0.5 h, decreasing in 24 and 36 h. On the other hand, **L** bulls had greater number of sperm/mm in 0.5 h and decreased in 12 and 24 h, when it stabilized and was similar to 36 h.

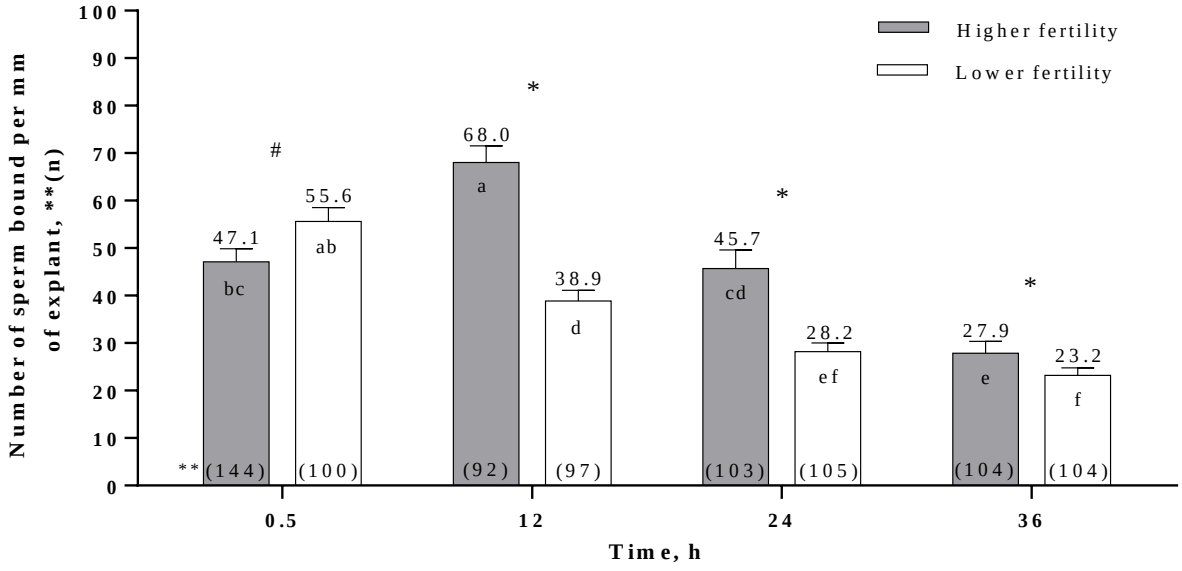

Fig. 6. Number of sperm bound per mm of oviduct cell explant after 0.5 h, 12 h, 24 h, and 36 h of co-incubation. Semen was from Nelore bulls with higher (**H**, n = 3) or lower (**L**, n = 3) field fertility.
[a,b,c,d,e,f]Letters indicate differences among the comparisons of the eight groups (each treatment [H and L] at time point of evaluation [0.5, 12, 24 and 36 h]). Within each time point, the differences between H and L are indicated separately ([#]$P \geq 0.05$; [*]$P < 0.05$).
[**]Symbol represents the number of explants in each group.

2.4. Discussion

Our study aimed to investigate strategies to improve P/AI of **L** sires in TAI programs and to investigate for sperm patterns associated with field fertility of sires. Our hypothesis was that sperm from **L** sires have shorter longevity in the female reproductive tract, fewer sperm binding to explants of oviduct cells and the P/AI of **L** sires is increased with the anticipation of GnRH in relation to TAI. Although P/AI had no major statistical differences between GnRH treatments or sire treatments (Fig. 2), the BCS of the cows seems to have influenced some of the outcomes of P/AI.

Despite cows with higher BCS (≥ 3.0) expressing more estrus, P/AI was similar to cows with lower BCS (< 3.0). This result may be explained by a potential positive effect of GnRH in increasing fertility regardless of the time of administration. All cows in this study received EC as an ovulation inducer and GnRH to increase ovulation synchrony [37,45,46]. After administration of 1.0 mg EC, ovulation occurs between 60 and 72 h, and GnRH has the potential to favor fertility [47–49]. During a recent trial performed by our laboratory [12], cows were submitted to protocols based on 7 d of P4 device and cows with BCS ≥ 3.0 had a larger ($P < 0.01$) diameter of the dominant follicle (DF) at the moment of device removal than cows with BCS < 3.0 (9.5 ± 0.09 [n = 290] vs. 9.0 ± 0.09 mm [n = 248]; respectively), but the diameter of the DF did not differ at the time of AI. This demonstrates that cows with a lower BCS may have a delayed LH surge in comparison with cows with higher BCS. Thus, the lower P/AI of cows with BCS < 3.0 (Fig. 3) in G-16 group may be associated with the induction of ovulation of a less steroidogenic follicle and/or less capacity to respond to the peak of LH (i.e., LH receptors in the granulosa cells) [50]. In the same way, Rodrigues et al. [30] demonstrated that Nelore cows that express estrus, in addition to greater P/AI, also had bigger DF at the time of TAI, greater area of corpus luteum and more circulating P4 17 d after ovulation. Furthermore, authors demonstrated that the cows that did not have estrus expression had greater P/AI ($P < 0.05$) if treated with GnRH at the time of AI (GnRH: 45.9% [216/470]; No GnRH: 36.2% [169/467]), but ovulation rate and circulating P4 did not differ in these cows, showing the positive effect of GnRH on fertility in adjusting the time of ovulation in relation to the TAI.

The expression of estrus is related to fertility and one of the reasons for this is modulation of the uterine environment [51,52]. Estradiol secreted by the DF, as well as EC administration alters expression of uterine genes and receptors related to early pregnancy, such as insulin and IGF-1 receptors [48,53,54]. At the time of estrus, there is a decrease in uterine pH, reducing sperm metabolism, which increases again when approaching ovulation, helping in sperm hyperactivation and capacitation, consequently spermatozoa that

hyperactivate later has greater longevity [55–57]. Therefore, fertility of cows with smaller DF and lower BCS can be increased when using non-hyperactivated sperm. This characteristic has been associated with the semen of greater fertility of sires used for TAI, decreasing this effect when inseminating by estrus detection, due to the shorter interval between insemination and ovulation [57]. This information supports our findings related to interaction of cows with BCS < 3.0 having lower P/AI when insemination occurred with **L** sires (Fig. 4), regardless of the number of doses. However, cows with BCS ≥ 3.0 had similar P/AI if inseminated with **H** or **L** fertility sires.

The assessment of sperm motility by the CASA system guarantees the measurement of specific patterns not evaluated by subjective analysis [58]. However, there are different methods of analyzing CASA systems, making it impossible to compare the results between them [59]. Furthermore, in each system, some factors can influence the results, such as the diluent, the pH, and the sperm concentration of the sample [59,60]. Morrel et al. [61], showed a positive correlation of 57% between the sperm oscillation index and fertility, as well as Farrell and others [62], which found a positive correlation between motility patterns and field fertility.

The evaluation of the sperm population with high mitochondrial potential and integrity of plasma and acrosomal membranes [42], by fluorescent probes, showed a correlation with sire fertility [24]. Sperm motility can be influenced by membrane integrity [63]. However, Sellem et al. [19] found no association between field fertility and plasma membrane integrity, assessed by flow cytometry. In a study by Hidalgo et al. [64], sperm from **H** field fertility sires had lower plasma membrane integrity than higher fertility sires (78.8 vs. 83.2%, respectively; $P = 0.03$), contrasting with data from our study that found no effect, but the progressive motility (Table 1) of **H** was superior to **L** sires ($P = 0.007$), as found in our study, in addition to greater variance in progressive motility of **L** sires (Fig. 5).

We hypothesized that the lower longevity of sperm in the female reproductive tract may be related to the lower field fertility of **L** bulls, and we used an *in vitro* assay to mimic sperm longevity. Within 0.5 h of co-incubation there was a tendency for a greater number of sperm bound per mm of oviduct cell explant of **L** bulls ($P = 0.10$; Fig. 6), however, at 12, 24, and 36 h there was a greater number of sperm/mm for **H** ($P < 0.05$), demonstrating that these evaluation moments (12, 24, and 36 h) were efficient in discriminating the **H** and **L** bulls used in our study. Saraf et al. [65], found after 1 h of co-incubation of sperm from buffalo bulls, an $R^2 = 0.47$ between the sperm number/mm and field fertility. In this same sense, Carvalho et al. [28], used sperm binding in explants to assess the longevity of sexed or non-sexed sperm,

evaluating binding at 0.5 and 24 h. As a result, the number of sperm/mm in 0.5 h was similar, however after 24 h, the non-sexed sperm had a greater number of sperm/mm (23.6 vs. 6.7; $P < 0.05$), demonstrating that the lowest P/AI of sexed sperm may be related to lower sperm longevity in the female reproductive tract.

Although the findings of conventional laboratory analyses of sperm motility and membrane integrity are very important as a screening test for sperm quality, no parameters directly related to field fertility were found. However, possibly, the results of conventional analyzes can help to understand the results of the sperm binding to explants. Although the mitochondrial membrane potential did not differ between **H** and **L** ($P = 0.64$), **H** sperm had some motility characteristics superior to **L**. Recent studies demonstrate that bovine sperm can utilize other energy sources in addition to mitochondrial oxidative phosphorylation [66,67]. This may indicate that **H** sperm has a greater ability to use other energy sources in addition to mitochondrial oxidative phosphorylation, consequently with a greater ability to maintain sperm binding to explants over time.

Despite the fertility of sires was similar in this study, the fertility of some sires can be increased by increasing the number of sperm per AI. This characteristic has been classified as compensable by Saack et al. [9]. Although, Zoca and others [25] evaluated the effect of semen dosage (10, 20 and 40 x 10^6 sperm per dose) from five Angus sires, finding a difference in fertility between sires, but that was not matched with the increase in the dose of sperm. As such, Den Daas et al. [68] found no correlation between the maximum number of sperm per dose (17.3 x 10^6) and the highest rates of non-return to estrus after 56 d of AI. Thus, there is a difference in fertility between sires, being maintained after determining the minimum sperm concentration for each animal to reach the maximum of its fertility [9,25,69].

2.5. Conclusion

Our data indicate that the BCS of cows can be used for decision making regarding the fertility of the semen that can be used. Cows with lower BCS have greater P/AI when inseminated with semen from higher fertility sires. Furthermore, regardless of administration time (16 h before or at TAI), the GnRH treatment may have been responsible for the lack of difference in P/AI between the field fertility of sires, but more studies are needed to understand this effect. The fertility of sires is multifactorial and the association of sperm motility analysis, membrane integrity, and sperm viability analysis, especially sperm binding

to explant cells, could be a possible test of sire fertility to be used by the industry for sire selection.

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3. ABILITY OF SPERMATOZOA TO BIND TO OVIDUCT CELL EXPLANTS AS A PREDICTOR OF BULL FIELD FERTILITY

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Abstract

There is no laboratory evaluation effectively capable of predicting field fertility of sires, and sperm viability in the female's reproductive tract may be a factor related to the greater pregnancy per AI (P/AI) of some bulls. This study evaluated the number of sperm of Holstein bulls bound to oviduct epithelial cell explants (OEC) at 0.5, 12, 24, and 36 h of coincubation. Other analyzes of semen quality, such as sperm motility, assessed by computer-assisted semen analysis (CASA), and membrane integrity, evaluated by flow cytometry. were performed as well. Based on the fertility of 1,833 Holstein cows from a commercial dairy farm, sires were classified as of higher (n = 3, **HF**, P/AI = 34.2%) or lower fertility (n = 4, **LF**, P/AI = 20.6%). For the analysis of sperm bound to OEC, reproductive tracts of cows were collected in a slaughterhouse, transported to the laboratory at 35 °C, and the oviduct isthmus region was dissected, and the cells from the lumen were collected. Cells were cultured for 24 h in TALP medium to form the explants. For the analysis of each sire, 160 explants were selected and distributed in 12 droplets of sp-TALP medium, adding $1x10^5$ motile spermatozoa/mL per drop. Regarding CASA, only straight-line velocity was lower for **LF** (**HF**: 94.7 ± 3.1%, **LF**: 80.9 ± 3.1%). The percentage of cells with intact plasma membrane and intact acrosome was greater for **LF**, as well as **LF** sperm had greater mitochondrial membrane potential. The **HF** sires tended to have more sperm per mm of OEC than **LF** at 0.5 h of co-incubation (59.1 vs. 54.0), and at 12 h there was a greater ($P < 0.05$) number of **HF** sperm (**HF**: 36.6, **LF**: 28.6). At 24 and 36 h, the number of spermatozoa bound to OEC was similar within the fertility groups, but also greater for **HF** than **LF** (**HF** 24 h: 24.0, **LF** 24 h:15.3, **HF** 36 h: 26.0, **LF** 36 h: 12.1**)**. Field fertility was highly correlated (r = 0.89), especially, with the number of sperm bound per mm of OEC at 36 h of co-incubation. In conclusion, the assessment of motility and membrane integrity were not able to identify the difference between the fertility of bulls. In contrast, more sperm from sires with greater field fertility were bound to OEC at all times evaluated *in vitro* than **LF** sires, suggesting the potential of this assay to predict field fertility. Moreover, these results suggest that, at least some **LF** sires, may have fertility impaired due to the lower capacity to bind to oviduct cells *in vivo*.

Keywords: Sire, Semen, Artificial insemination, *in vitro*, Cattle

3.1. Introduction

Artificial insemination (AI) has the potential to promote genetic improvement and reproductive efficiency, increasing the productivity of dairy herds. With the advancement of research, hormonal protocols capable of synchronizing the emergence, growth and ovulation of a dominant follicle were developed (Pursley et al., 1995), enabling timed AI (TAI), which, in general, contributes to a greater 21 d pregnancy rate (PR21), due to increased service rate and, potentially, increased pregnancy per AI (P/AI) compared to AI to estrus in dairy cattle (Batista et al., 2016; Consentini et al., 2021). Moreover, TAI-based reproductive programs have the potential to decrease the incidence of postpartum metabolic diseases by anticipating the time of the first postpartum AI (Middleton et al., 2019). However, the window of time for ovulation in relation to AI in TAI protocols is relatively wide and it may be related to suboptimal fertility depending on the sire used for AI (Pancarci et al., 2002; Consentini et al., 2021). Besides, there is still no tool capable of predicting with a relatively high accuracy bull field fertility.

The US Department of Agriculture (USDA) is responsible for evaluating the sire conception rate (SCR), an index used to classify bulls according to field fertility. This information is combined with other assessments of sires, such as genomics to predict different productive characteristics, working as criteria for choosing the semen of the sires that will be used in the farm. However, to increase SCR accuracy, a large number of AI must be performed in many farms, and after obtaining the P/AI results, the bull is classified according to its fertility (McWhorter et al., 2020). Although SCR analysis is accurate, it is costly, time-consuming and not applicable when breeding with young sires. Therefore, development of new tools to predict with more accuracy the field fertility of bulls are warranted.

The causes of variation in fertility are multifactorial and involve not only sperm traits, but also the maintenance of embryo development (Ortega et al., 2018; O'Callaghan et al., 2021). Conventional analyzes of sperm membrane motility and integrity have low relationship with sire field fertility, being the possible explanation of lower fertility only in few individuals (Cornwell et al., 2006). There are several sperm laboratory analyzes with the potential to determine the fertility of bulls. However, there is no punctual analysis with high accuracy, so that the more characteristics evaluated, the greater the odds of detecting the causes of lower fertility in some individuals (Graham and Mocé, 2005).

Only tens to hundreds of sperms contained in a semen straw can reach the oviduct and remain bound to cells until ovulation takes place (Sostaric et al., 2008). This population of

sperm has intact membranes and a non-hyperactive motility pattern, in addition to cell receptors that enable the binding to oviduct epithelial cells (OEC) (Fazeli et al., 1999; Gualtieri and Talevi, 2000; Leemans et al., 2014). This functional sperm reservoir in the oviduct allows the maintenance of sperm energy, favoring greater viability (i.e., sperm capacity to remain viable for fertilization) in the female's reproductive tract (Boquest et al., 1999). Thus, understanding the sperm population with the ability to bind and maintain the functional reservoir may be the key to determining the difference in fertility between sires (Saint-Dizier et al., 2020).

The aim of this study was to evaluate the sperm characteristics associated with field fertility in Holstein bulls by means of *in vitro* sperm binding to the explants of oviduct cells. Our hypothesis was that, due to a lower capacity to bind to oviduct cells or, more specifically, due to a potential shorter survival time in the female reproductive tract, less sperm from bulls with lower field fertility would be bound to OEC explants over time, and the motility and integrity of sperm membranes would not differ between sires with lower or higher field fertility.

3.2. Material and methods

The experiment was performed at the Laboratory of Teaching and Research in Reproductive Pathology and the Laboratory of Semen Biotechnology and Andrology from the School of Veterinary Medicine and Animal Science of University of São Paulo (FMVZ/USP), located in Pirassununga, SP, Brazil. All the procedures were approved by The Animal Research Ethics Committee of "Luiz de Queiroz" College of Agriculture of the University of São Paulo (ESALQ/USP), in Piracicaba, SP, Brazil (Protocol CEUA # 2017.5.1618.11.9).

3.2.1. Bull fertility

Fertility data of seven Holstein sires were retrospectively obtained from 1,833 TAI performed during a 2-year period in a commercial dairy herd (Tainá Farm, São Pedro, SP) with 300 lactating cows, managed in free-stall barns, milked three times a day, and with an average daily milk production of 30 kg. The sires were classified as of higher fertility (**HF**, n = 3; 35.0% [362/1,034]) or of lower fertility (**LF**, n = 4; 21.4% [171/799]). The P/AI and the number of batches of cryopreserved semen (CRV Lagoa) per bull in 0.25 mL straws containing average of 5.9 x 10^6 motile sperm per straw were: bull A **HF** (36.8% [25/68]; n =

4), bull B **HF** (35.8% [290/809]; n = 4), bull C **HF** (29.9% [47/157]; n = 4), bull D **LF** (21.7% [103/475]; n = 4), bull E **LF** (21.5% [42/195]; n = 3), bull F **LF** (21.1% [19/90]; n = 2), and bull G **LF** (17.9% [7/39]; n = 3).

3.2.2. Assessment of sperm morphology

The sperm sample was fixed in a buffered saline solution containing 4% formaldehyde previously heated to 37 °C. For evaluation of sperm morphology, 200 cells were analyzed by the wet chamber technique, under differential interference contrast microscopy (DIC, model 80i, Nikon, Tokyo, Japan) with 1,000 x magnification. Cells were classified according to Blom (1973).

3.2.3. Computer-assisted assessment of sperm motility

Motility traits were evaluated using the Hamilton Thorne Research Motility Analyzer (HTM-IVOS- Ultimate, Hamilton Thorne Biosciences). The set up was previously adjusted for the analysis of bovine sperm in the IDENT option. For the evaluation, it was used 10 µL of the sperm sample diluted to 10 x 10^6 sperm/mL in TALP sperm medium (4.2 mg/mL of sodium chloride, 1.87 mg/mL of potassium chloride, 2.1 mg/mL of sodium bicarbonate, 50 µg/mL of sodium phosphate, 290 µg/mL of calcium chloride monohydrate, 80 µg/mL of magnesium chloride hexahydrate, 6.5 mg/mL of Hepes) supplemented with albumin, sodium lactate, sodium pyruvate, and penicillin-streptomycin was added into the Makler chamber (Selfi-Medical Instruments) used for the evaluation. The setting has been preset to a number of frames of 30, frames per second of 60 Hz, minimum contrast of 80, minimum cell size of 5 pixels, contrast to static cells of 30, average path velocity cutoff (defining slow cells) of 40 µm/s, minimum average path velocity (defining progressive cells) of 30 µm/s, straight-line velocity cutoff (defining slow cells) of 15 µm/s, cell size of 6 pixels, cell intensity of 75, static head size of 0.60–3.40, static head intensity of 0.75–1.70, static elongation of 8–92, magnification of 1.86 x, video frequency of 60, illumination intensity of 2241, and temperature of 37 °C. Five fields were selected for each sample, and the following variables were analyzed: total motility (%); progressive motility (%); average path velocity (VAP, µm/s); straight-line velocity (VSL, µm/s); curvilinear velocity (VCL, µm/s); amplitude of

lateral head displacement (ALH, μm); beat/cross frequency (BCF, Hz); straightness (STR, %); linearity (LIN, %).

3.2.4. Integrity of sperm membranes by flow cytometry

Flow cytometry analyses were performed according to Díaz et al. (2017) with an Accuri C6 flow cytometer (Becton, Dickinson and Company). Samples were stained with 150 μL SYTO-59 (S59; 5 nM, S10341) and incubated for 10 min at 37 °C. After incubation, the samples were evaluated with a 675/25 nm bandpass filter. S59 is permeable to the plasma membrane and stain the DNA of the sperm cells. Thus, particles with different size and scatter properties were excluded from the analysis. Spectral overlap of staining in the flow cytometer analyzes was compensated when necessary. Samples were processed through the instrument at an acquisition rate of approximately 600 to 1,000 events/sec, acquiring 10,000 positive S59 events per assay. The cells were excited by an argon laser at 488 nm and by a red laser at 640 nm.

For the analysis of plasma and acrosomal membrane integrity, an aliquot of 150 μL of semen with 5 x 10^6 sperm/mL was deposited in a microtube with 3 μL of S59, 1 μL of propidium iodide (PI; 0.5 mg/mL), 1 μL of fluorescein isothiocyanate – *Pisum sativum agglutinin* (FITC-PNA, 100 μL/mL), and incubated for 10 min at 37 °C. After incubation, 150 μL of TALP sperm medium was added to dilute the sample for analysis in flow cytometry. To evaluate the mitochondrial membrane potential, in an aliquot of 150 μL semen (5 x 10^6 sperm/mL), 1 μL of 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide (JC-1; 153 μM, T-3168), 1 μL of PI and 3 μL of S59 were added, and incubated for 10 min at 37 °C. After incubation, the sample was diluted with 150 μL of TALP sperm medium to obtain a concentration of 2.5 x 10^6 sperm/mL for analysis. For evaluation of lipid peroxidation of viable cells (PI negative), 1 μL of probe C11-BODIPY $^{581/591}$ (BP; 1mg/mL, D-3861) was added to an aliquot of 150 μL of semen (5 x 10^6 sperm/mL) and incubated for 20 min at 37 °C. After 20 min, 1 μL of PI and 3 μL of S59 were added. After 10 min of incubation, 150 μL of TALP sperm medium were added to dilute the sample for analysis in flow cytometry. To assess the fluidity of the membranes, the semen was diluted in TALP sperm medium to a concentration of 5 x 10^6 sperm/mL, and 1 μL of Yo-Pro-1 (YP; 7,5 nM) was added and incubated for 10 min. After incubation, 1 μL of S59 was added and incubated for 10 min. After incubation, 2 μL of merocyanine 540 (M540; 810 μM) was added and

incubated for 70 s. Then, the dilution was made in 150 μL in TALP sperm medium and the samples were analyzed by flow cytometry.

3.2.5. Collection and culture of oviduct cells

Eight to 12 reproductive tracts of cows were collected in a slaughterhouse per session of *in vitro* analyses. The tracts were transported at 35 ºC in saline solution (NaCl 0.9%), supplemented with penicillin G (100 U/mL) and streptomycin sulfate (50 μg/mL). In the laboratory, the oviducts whose ovaries did not have a corpus luteum (CL) were dissected from the adjacent structures, and only the isthmus region was used, considering up to 4 cm from the uterotubal junction. The oviduct lumen was washed twice with 1 mL of TALP medium (TCM-199 with salts of Hank's, supplemented with 10% fetal bovine serum, 50 mg/L gentamicin sulfate, and 25 μg/mL amphotericin B; Vitrocell) in a 15 mL conical bottom tube. The conical bottom tube with cells was incubated at 35º C with 5% CO_2 for 50 min, being washed twice with 5 mL of TALP medium, performing cell de-aggregated at each wash with an automatic pipette of 1000 μL. Finally, the cells were transferred to a 100 mm petri dish, supplemented with 25 mL of TALP medium and kept in culture for approximately 24 h to form the cell explants, changing 50% of the medium 6 to 8 h after the onset of culture, according to Kadirvel et al. (2012).

3.2.6. Evaluation of the number of sperm bound per mm of explant

In each replicate, one sire from each treatment was evaluated, using for each bull 160 explants distributed in 12 droplets of 30 μL of sp-TALP medium (Botupharma) under silicone oil. A sperm pool for each bull was produced using three straws of three different batches, and 250 μL of the sperm cell pool were washed with 1 mL of synthetic oviductal fluid (SOF; Botupharma), centrifuging at 700 x g for 5 min. After washing, the supernatant was removed, and the pellet was resuspended in SOF medium.

The number of spermatozoa was counted in a Neubauer chamber to accurately use 1 x 10^5 motile spermatozoa/mL per drop containing explants (0 h). The evaluation of the number of sperm bound to the explant was performed at 0.5 h, 12 h, 24 h, and 36 h, removing three drops from the incubation plate at each evaluation time, discarding the explants after analysis. The sperm cell-explant complex was transferred to a 30 μL drop of sp-TALP medium on a

glass slide. Using the EUREKAM 1.3 image capture system (BEL Engineering), two photographs of each explant were taken to count the number of sperm bound and to measure the explant perimeter with the ImageJ 1.3 software (National Institute of Health). The number of sperm bound per mm (sperm/mm) of explant was calculated by dividing the number of sperm bound by the perimeter of explant.

3.2.7. Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, Version 9.4 for Windows SAS Institute Inc., Cary, NC).

For CASA analysis, all data were tested for normality of studentized residuals using the UNIVARIATE procedure of SAS according to the Shapiro-Wilk test. The homogeneity of variances was evaluated with the Levene test using the Hovtest and Welsh methods. The analyses were performed using the GLIMMIX procedure fitting a Gaussian distribution, and the option ddfm = kenwardroger was included in the model statement to adjust the degrees of freedom for variances.

The number of sperm/mm followed a Gamma distribution, so the analyses were performed using the GLIMMIX procedure fitting a Gamma distribution. The final model included the effect of fertility, hour, the interaction between them, and OEC explant perimeter as a covariate.

Tukey honest significant difference post hoc test was performed to determine differences. Values are presented as means ± standard error of the mean (SEM) (continuous variables) or as percentage (%; binomial variables). Significant differences were declared when $P \leq 0.05$, whereas tendencies were considered when $0.5 < P \leq 0.10$.

Correlation coefficient between field P/AI and sperm/mm at 0.5 h, 12 h, 24 h, and 36 h was calculated using the PROC CORR procedure of SAS, while the intercept and slope of the linear equation were obtained using the option solution in the GLIMMIX procedure.

3.3. Results

The major defects (**HF** = 8.8 ± 1.6 vs. **LF** = 5.5 ± 1.0; $P = 0.08$), minor defects (**HF** = 4.3 ± 0.8 vs. **LF** = 3.7 ± 0.6; $P = 0.52$) and total defects (**HF** = 13.1 ± 2.2 vs. **LF** = 9.1 ± 1.3; $P = 0.13$) were similar between the fertility categories of bulls.

There was no difference between **HF** and **LF** sires for the sperm motility variables assessed by the computer-assisted semen analysis (Table 2), apart from VSL, which was greater in **HF** than **LF** bulls (94.7 ± 3.1 vs. 80.9 ± 3.1, respectively; *P* = 0.005). Total motility, progressive motility, VAP, VCL, ALH, BCF, STR, and LIN were similar (*P* > 0.10) between **HF** and **LF** bulls.

Table 1. Means ± standard error of computer-assisted semen analysis of sperm motility variables in higher and lower field fertility sires.

Variables	Higher fertility (n = 3 sires)	Lower fertility (n = 4 sires)	*P*
Total motility (%)	72.9 ± 2.6	69.2 ± 3.9	0.43
Progressive motility (%)	57.8 ± 3.4	50.6 ± 4.4	0.20
Average path velocity (VAP; μm/s)	124.3 ± 3.8	113.7 ± 5.9	0.15
Straight-line velocity (VSL; μm/s)	94.7 ± 3.1	80.9 ± 3.1	0.005
Curvilinear velocity (VCL; μm/s)	234.8 ± 10.6	214.6 ± 17.6	0.34
Ampl. of lateral head displacement (ALH; μm)	9.5 ± 0.5	8.8 ± 0.8	0.47
Beat/cross frequency (BCF; Hz)	31.6 ± 1.7	30.3 ± 1.1	0.52
Straightness (STR; %)	76.4 ± 1.8	74.3 ± 2.8	0.54
Linearity (LIN; %)	43.4 ± 2.2	45.3 ± 3.7	0.67

The integrity of plasma membrane (P) and acrosome (A) considered cells with intact (I) or damaged (D) structures expressed as a percentage of four cell groups. The IPIA cell group was higher for **LF** (80.3% vs. 74.5%; *P* = 0.01), which also had lower IPDA (**HF** = 1.3% vs. **LF** = 0.6%; *P* = 0.001). However, the DPIA [**HF** = 18.5% vs. **LF** = 14.6; *P* = 0.09], and DPDA [**HF** = 5.6% vs. **LF** = 4.5%]; *P* = 0.47) were similar.

These results of the analysis by flow cytometry were presented with the arbitrary units (a.u.). The population of cells with intact plasma membrane and high mitochondrial membrane potential was lower for **HF** (1,060.3 ± 206.2 a.u. vs. 3,285.2 ± 766.1 a.u.; *P* = 0.004). There was no difference in sperm with lipid peroxidation (**HF** = 762.5 ± 45.1 a.u. vs. **LF** = 879.0 ± 47.7 a.u.; *P* = 0.09), and fluidity of the membranes (**HF** = 14,109.2 ± 1911.9 a.u. vs. **LF** = 15,734.5 ± 1071.6 a.u.; *P* = 0.47).

When evaluating the number of sperm bound per mm of explant (Figure 1) within each co-incubation period, there was a tendency for greater number of sperm/mm in **HF** at 0.5

h (59.1 vs. 54.0, $P = 0.08$). At 12, 24 and 36 h, the number of sperm/mm were greater for **HF** compared to **LF**.

To better understand and compare the number of sperm/mm of OEC explant between **HF** and **LF** throughout time, we performed an analysis considering eight groups (each treatment [**HF** and **LF**] at each hour [0.5, 12, 24 and 36 h]; Figure 1). The **HF** at 0.5 h had a greater number of sperm/mm than the **HF** at 12 h, which was greater than the number of sperm/mm of the **HF** at 24 and 36 h, but the number of sperm/mm did not differ between these two later times. The comparison among times for the **LF** sires presented a similar pattern as the **HF** (Figure 1).

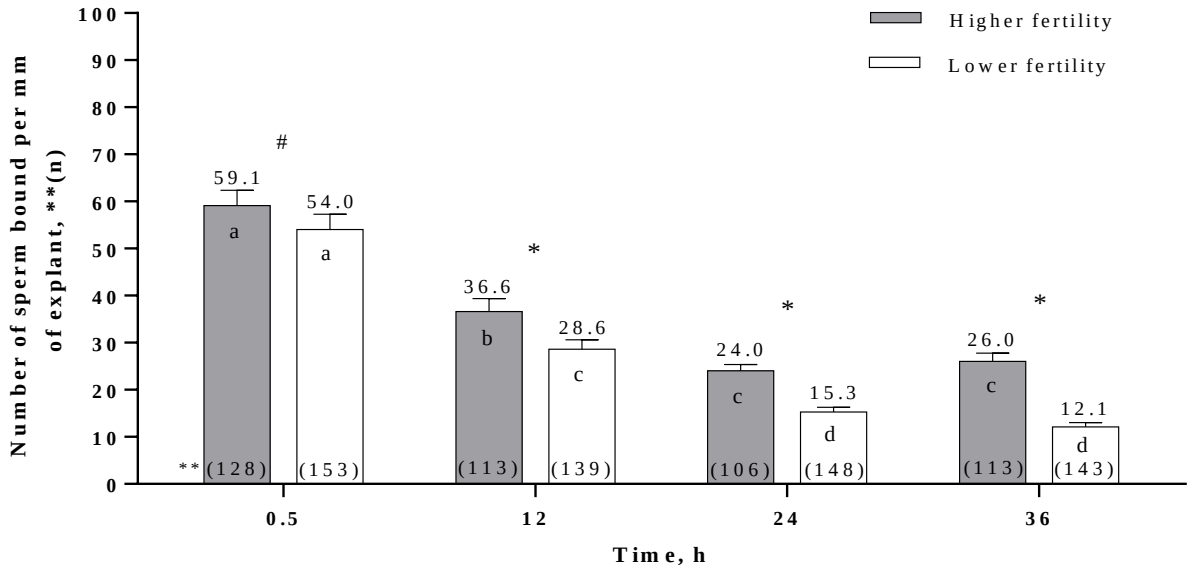

Figure 1. Number of sperm bound per mm of explant after 0.5 h, 12 h, 24 h, and 36 h of co-incubation. Semen was from Holstein bulls with higher (**HF**, n = 3) or lower (**LF**, n = 4) field fertility. [a,b,c,d]Letters indicate differences among the comparisons of the eight groups (each treatment [**HF** and **LF**] at each hour [0.5, 12, 24 and 36 h]). Within each hour, the differences between **HF** and **LF** are indicated separately ([#]$0.5 < P \leq 0.10$; [*]$P \leq 0.05$). [**]Represents the number of explants in each group.

There was no correlation between the number of sperm/mm and field fertility of bulls at 0.5 h (r = 0.06, $P = 0.90$; Figure 2) or 12 h (r = 0.31, $P = 0.49$; Figure 3). However, within 24 h of co-incubation (Figure 4) there was a r = 0.77 ($P = 0.04$) and there was a high correlation (r = 0.89, $P = 0.007$) between field P/AI of Holstein bulls and the number of sperm bound to explants at 36 h of co-incubation (Figure 5).

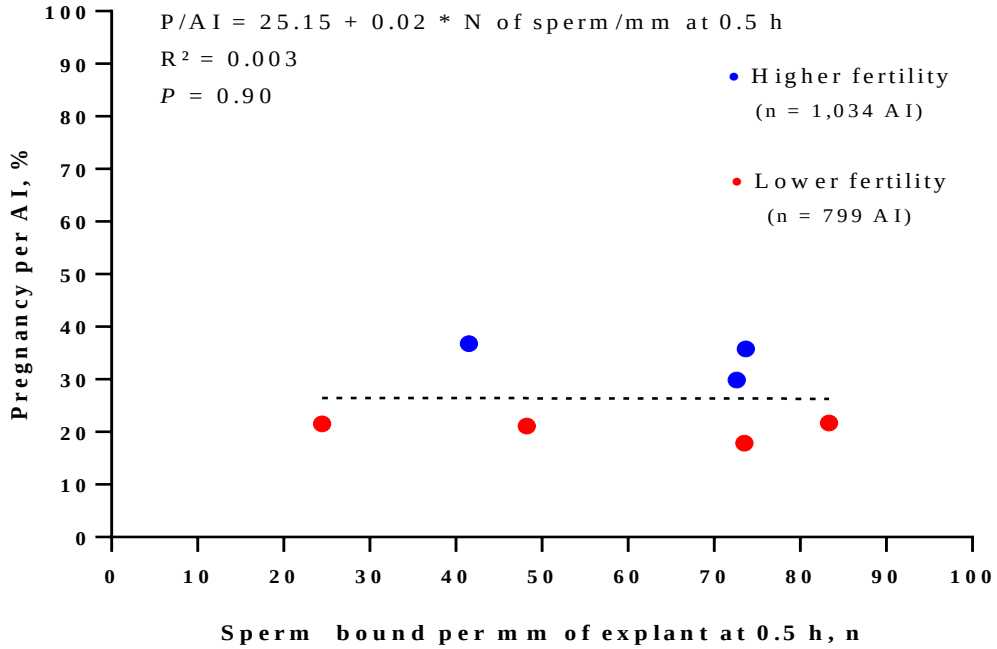

Figure 2. Relationship between field pregnancy per artificial insemination (P/AI) of higher (**HF**, n = 3 sires) and lower (**LF**, n = 4 sires) fertility Holstein sires and the number of sperm bound per mm of explants at 0.5 h of co-incubation.
Red circles represent **LF**, and blue circles represent **HF** sires.

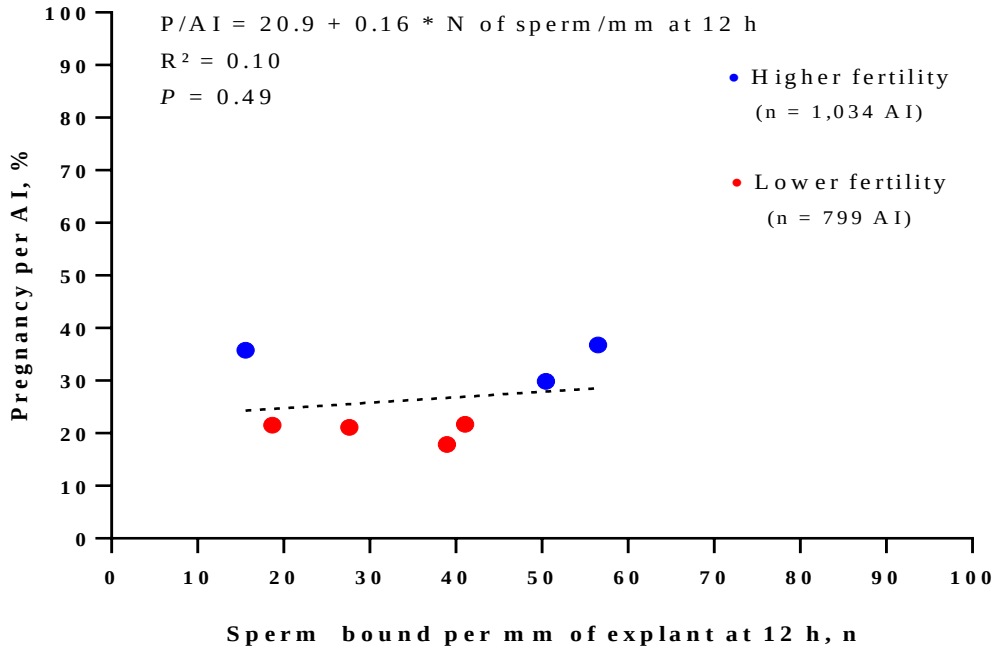

Figure 3. Relationship between field pregnancy per artificial insemination (P/AI) of higher (**HF**, n = 3 sires) and lower (**LF**, n = 4 sires) fertility Holstein sires and the number of sperm bound per mm of explants after 12 h of co-incubation.
Red circles represent **LF**, and blue circles represent **HF** sires.

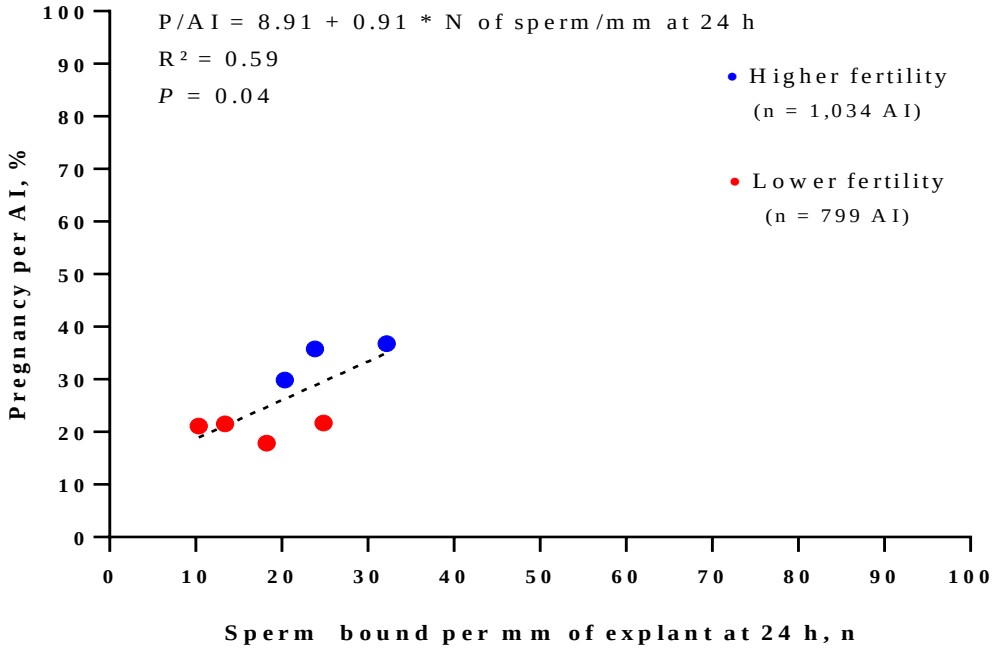

Figure 4. Relationship between field pregnancy per artificial insemination (P/AI) of higher (**HF**, n = 3 sires) and lower (**LF**, n = 4 sires) fertility Holstein sires and the number of sperm bound per mm of explants after 24 h of co-incubation.
Red circles represent **LF,** and blue circles represent **HF** sires.

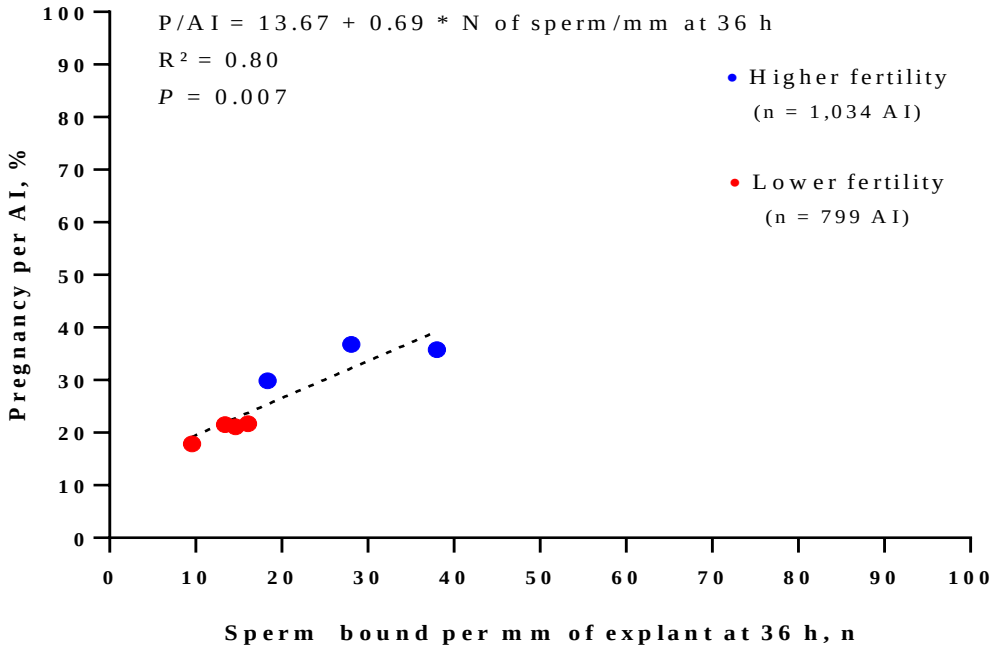

Figure 5. Relationship between field pregnancy per artificial insemination (P/AI) of higher (**HF**, n = 3 sires) and lower (**LF**, n = 4 sires) fertility Holstein sires and the number of sperm bound per mm of explants after 36 h of co-incubation.
Red circles represent **LF,** and blue circles represent **HF** sires.

The change in % of sperm bound to explant over time, considering 0.5 h as 100% is represented in Figure 3. This descriptive analysis represents how sperm bound to explant behaves throughout time of *in vitro* co-incubation. With 24 h and 36 h of co-incubation, sperm from **HF** bulls maintained 40.6% and 44.0%, respectively, of the percentual of sperm bound to explants. However, sperm from **LF** bulls decrease to 28.3% and 22.2% binding to explants at 24 h and 36 h of co-incubation, respectively.

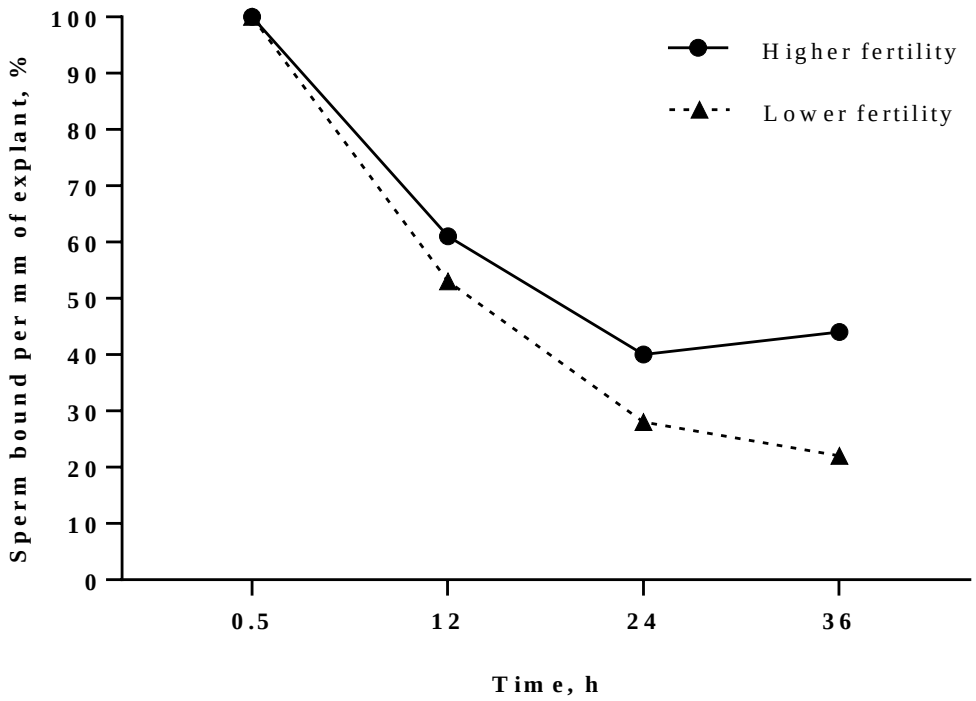

Figure 6. Sperm from Holstein bulls with higher (**HF**, n = 3 sires) or lower (**LF**, n = 4 sires) field pregnancy per artificial insemination (P/AI) bound per mm of oviduct epithelial cell explant after 0.5 h, 12 h, 24 h, and 36 h of co-incubation. The number of sperm bound to explants per treatment (**HF** or **LF**) at 0.5 h of co-incubation was considered 100% to calculate the relative percentage drop in sperm number/mm of explant in each hour of co-incubation.

3.4. Discussion

The fertility of sires in breeding programs influences to the productivity and profitability of the production system (Davis and White, 2020). Several characteristics of sperm cells and seminal plasma have been studied as possible fertility trackers, such as morphology, motility, membrane integrity, and biomarker molecules (Fair and Lonergan, 2018; Moraes et al., 2021). Despite that, there are no laboratory tests effectively capable of predicting field fertility, which, associated with genomic evaluation, can help in the early selection of bulls. Although the commercial semen undergoes rigorous analyzes that demand

quality standards by the industry, these factors are not able to identify sires with superior fertility (Vincent et al., 2012). Based on that, our study aimed to evaluate the conventional analyzes of membrane integrity and sperm motility of Holstein bulls of higher or lower field fertility, and the sperm capacity to bind and remain bound to OEC explants.

The P/AI of the cows affects the financial return of the dairy farms. Recently, Pacheco et al. (2021) identified a 20% difference in the fertility of the Brown Swiss bull from **HF** or **LF**. In agreement with other studies, they found a 10 to 15% difference in P/AI of Jersey and Holstein bulls from **HF** or **LF** (Peñagaricano et al., 2012; Rezende et al., 2018). In the financial analysis performed by Dolecheck et al. (2016), the total costs of nonpregnant cows raise at an increasing rate from 60 d in milk (DIM) to 250 DIM, and with 300 DIM the cost ranges from US$0.31 to US$0.41 per day open. Delay in conception does not exploit the maximum productive potential of the herd's lactation curve, causing cows to reach the transition period with higher BCS, predisposing cows to metabolic diseases such as ketosis, resulting in delayed conception in the next lactation, lower fertility and increases the number of nonpregnant cows in the herd (Middleton et al., 2019; Consentini et al., 2021).

After formation of the functional sperm reservoir in the oviduct, intrinsic characteristics of sperm and female's reproductive tract (i.e., fluid, hormone concentrations, and OEC receptors) influence the lifespan of male gametes until ovulation. Only 20 to 50% of sperm from cryopreserved semen can bind to oviduct cells, which are the motile and noncapacitated spermatozoa with intact acrosome. However, we did not detect motility alterations (Table 1) related to hyperactivation or other motility patterns described as responsible for the difference in fertility between bulls (Pfeifer et al., 2019).

In the study by Saraf et al. (2018), sperm binding to oviduct cells after 1 h of co-incubation was evaluated as a predictor of fertility in buffalo bulls with higher, intermediary, or lower fertility, with a greater number of sperm binding to explants for higher fertility bulls compared to intermediary or lower fertility, which did not differ between each other. Furthermore, after 1 h of co-incubation, a $R^2 = 0.47$ was found for the relationship between field fertility and number of sperm bound to explants. We detected a tendency ($P = 0.08$) to greater number of sperm of **HF** binding to explants at 0.5 h, and at the other time points (12, 24, and 36 h), **HF** bulls had greater numbers of sperm bound to explants (Figure 1). Moreover, a unique aspect of our study was to detect that sperm stay bound to explants up to 36 h of co-incubation, and the number of sperm per mm at 24 h and 36 h had a high correlation (r = 0,77 and r = 0.89, respectively) with individual bull field fertility (Figure 4 and 5).

After 8-12 h of AI, a functional sperm reservoir is formed in the isthmus region of the oviduct (Wilmut and Hunter, 1984). Only tens to hundreds of sperms in the *in vivo* or *in vitro* milieu can remain bound to oviduct cells, prolonging their viability until ovulation. This binding does not depend on the sperm's previous interaction with uterine cells or fluids, as demonstrated by a study that has deposited the sperm directly in the oviduct and observed sperm bound to oviduct cells *in vivo* (Lefebvre et al., 1995). In the *in vitro* production of embryos, sperm with alterations in membrane integrity and surface receptors, that are important for *in vivo* fertility in TAI or superovulation, are not necessary to promote fertilization (Baba et al., 2002). Furthermore, co-incubating sperm with oviduct cells prior to *in vitro* fertilization (IVF) selects for better quality sperm (i.e., fertilizing capacity), such as increasing blastocyst rate and decreasing problems such as polyspermy (Gualtieri and Talevi, 2003; Lamy et al., 2017). These aspects support our idea of relating sperm binding to oviduct cells with field fertility (Saint-Dizier et al., 2020). Our hypothesis that less sperm from bulls with lower field fertility would be bound to OEC explants over time was partially accepted, since even at 0.5 h of co-incubation there was already a tendency for less sperm bound to the explants in the **LF** group. This difference between groups, in fact increased over time (0.5 h = 8.3%, 12 h = 21.9%, 24 h = 36.3%, and 36 h = 53.5%) as seen in Figure 1 and 6. This indicates that the difference in fertility between bulls can be more evident in TAI protocols with greater dispersion of ovulation.

The role of mitochondria in the energetic support of bull sperm has not yet been fully elucidated. Laboratory studies have shown that glycolysis can be an exclusive pathway for energy production for sperm motility in the absence of other metabolic pathways (Krzyzosiak et al., 1999). Our finding of greater mitochondrial membrane potential for post-thaw **LF** sperm may indicate greater use of mitochondrial oxidative phosphorylation (OXPHOS) in **LF** when compared to **HF**, as the motility patterns assessed by CASA were similar. In the same sense, due to the potential greater use of OXPHOS for energy in **LF**, the depletion of this pathway may be related to the lower sperm binding in OEC explants over time. However, more studies should be conducted to understand the relationship between the maintenance pathways of sperm metabolism and the functional sperm reservoir.

The ideal time to perform insemination is approximately 12 to 16 h before ovulation (Pursley et al., 1998), thus bulls with greater fertility may have sperm with the ability to remain viable for a longer time until ovulation. However, there are TAI protocols that promote more dispersed ovulation, such as the Cosynch protocol, in which GnRH is administered at the time of AI. In the Cosynch protocol, cows that have a dominant follicle (>

10 mm) have the potential to respond to GnRH and ovulate 28 h after TAI, but cows that expressed estrus before AI will ovulate sooner, not requiring high longevity sperm. Another ovulation induction strategy at the end of the protocol is to administer estradiol cypionate (EC), however this results in greater dispersion of ovulation when compared to GnRH (Pancarci et al., 2002; Souza et al., 2009). In our study, the fertility of bulls was determined in cows undergoing ovulation induction with EC, so the larger ovulation window may have contributed to selecting bulls with sperm with higher (HF) or lower (LF) viability in the reproductive tract of the female. Greater synchrony of ovulation and insemination at the optimal time can minimize the fertility gap between bulls. In the study of Pfeifer et al. (2019), beef cows inseminated with semen with hyperactive motility profile had lower P/AI with later times of ovulation after FTAI, and when AI was performed with non-hyperactive sperm, P/AI was similar when ovulation occurred later or at earlier times. In the same sense, Macmillan and Watson (1975) demonstrated that the difference in fertility between the evaluated sires was not detectable when insemination occurred closer to ovulation.

After ovulation, several mechanisms stimulate the release of sperm from the functional reservoir (Leemans et al., 2014), with sperm capacitation occurring, and allowing fertilization in the ampulla region of the oviduct. In the recent study by O'callaghan et al. (2021), the number of accessory sperm in *in vivo* produced embryos was greater ($P < 0.05$) for semen from **HF** bulls than **LF** bulls (**HF**: 2.9 ± 1.03, **LF**: 0.34 ± 0.13). Fertilization rate of superovulated or single ovulated cows may be limited by the number of accessory sperm, as they were found in smaller quantities in unfertilized oocytes when compared to viable embryos (Saacke et al., 1998).

3.5. Conclusion

In conclusion, the conventional assessments of motility and membrane integrity were not able to identify differences between semen of bulls with distinct field fertility. In contrast, more sperm from sires with greater field fertility were bound to oviduct explants at all times evaluated *in vitro* than **LF** sires, suggesting the potential of this assay to predict field fertility. Moreover, these results suggest that, at least some **LF** sires, may have fertility impaired due to the lower capacity to bind to oviduct cells *in vivo*. Therefore, combined with other sperm analysis tools and genomic testing, counting the number of sperm bound to explants has the potential to be used by the industry for early selection of bulls and to improve the efficiency of reproductive programs.

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4. FINAL CONSIDERATIONS

The present study aimed to evaluate strategies to increase P/AI of LF sires and to predict field fertility of bulls. The first study evaluated the effect of inseminating with two doses of semen from LF bulls and adjusting the timing of ovulation with GnRH in Nelore. Regardless of strategy, P/AI was similar, however, cows with BCS < 3.0 had lower P/AI when inseminated with LF bulls. The sperm bound per mm of cell explant was greater for HF sires at 12 h, 24 h, and 36 h, proving to be efficient in detecting differences in the fertility of bulls. In this study, sperm motility characteristics evaluated by CASA indicated that HF bulls had greater sperm motility and progressive motility. The integrity of membranes was similar between HF and LF sires. The second study evaluated sperm binding to oviduct cell explants, motility, and sperm membrane integrity as predictors of fertility in bulls. The evaluation of sperm binding to explants at 24 h and 36 h of co-incubation showed a high correlation with field fertility, however, sperm motility and membranes characteristics were not able to identify the difference between field fertility of bulls. Thus, the sperm binding to explants has the potential to be used by the industry as a predictor of field fertility of bulls.