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Predicting sire fertility in artificial insemination of dairy cows by the ability of spermatozoa to bind to oviduct cell aggregates

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ABSTRACT

This study aimed to evaluate sperm morphofunctional characteristics and the ability of spermatozoa to bind to oviduct epithelial cell (OEC) aggregates as predictors of male field fertility. Based on pregnancy per artificial insemination (P/AI) data of 1,833 services in Holstein cows from a commercial dairy herd, sires were classified as of higher (HF, P/AI = 35.0% [362/1,034], n = 3) or lower (LF, P/AI = 21.4% [171/799], n = 4) fertility. Semen quality was assessed based on sperm motility (by computer-assisted sperm analysis), membrane integrity and functionality parameters (by flow cytometry), and the ability of spermatozoa to bind to OEC. For the analysis of OEC binding, the oviduct isthmus region from abattoir tracts was dissected, and cells were incubated for 24 h to allow OEC aggregate formation, followed by co-incubation with 1×10^5 motile sperm. Bound sperm were counted at 4 time points: 0.5, 12, 24, and 36 h. Statistical analyses were conducted using the GLIMMIX procedure in SAS 9.4 (SAS Institute Inc.). Sperm motility characteristics were not different between HF and LF sires, except for greater straight-line velocity in HF (HF: 94.7 ± 3.1 μm/s vs. LF: 80.9 ± 3.1 μm/s). Flow cytometry analysis was not able to explain the difference between fertility groups (P/AI). However, in the sperm binding test to OEC, the HF sires tended to have more bound sperm per millimeter of OEC than LF sires at 0.5 h of co-incubation (59.1 vs. 54.0). At 12 h (36.5 vs. 28.6), 24 h (24.0 vs. 15.3), and 36 h (26.0 vs. 12.1), HF sires had more bound sperm per millimeter of OEC than LF sires. Field fertility was positively correlated (r = 0.89) with the number of sperm bound per millimeter of OEC at 36 h of co-incubation. These results suggest that LF sires may have impaired fertility due to a reduced ability to bind to oviduct cells in vivo. In addition, the result showed the potential of sperm binding test to OEC for early evaluation or prediction of bulls' fertility, although more studies are being performed to refine the procedures during these assays, as well as to better understand other potential physiological mechanisms differing between HF and LF sires.

Key words: semen, bull, fertility, timed AI, cattle

INTRODUCTION

Artificial insemination (**AI**) and timed AI (**TAI**) are the most efficient and widely used biotechnologies to enhance genetic gain and reproductive efficiency in commercial dairy herds. Currently, TAI programs provide high service rates and increased fertility compared with programs based on AI by estrus, contributing to higher 21-d pregnancy rates (Consentini et al., 2021). Sire fertility significantly affects pregnancy per AI (**P/AI**) and contributes to variability in herd reproductive performance (Batista et al., 2016; Utt, 2016; Berry et al., 2019). Despite its importance, an accurate, large-scale method to early predict bull fertility is still lacking in the dairy industry (Vincent et al., 2012). Evaluation of sire conception rate (**SCR**), an index used by organizations such as the USDA to evaluate bull field fertility, is widely used by AI companies and incorporated into genetic evaluations released every 3 or 4 mo in the dairy industry. This index relies on performing a significant number of AI on multiple farms because the sires are classified based on P/AI results (McWhorter et al., 2020).

However, although relatively accurate, SCR analysis has limitations, such as high costs for implementation

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due to the need for large-scale insemination data collection across numerous herds, time-consuming as it requires months to generate reliable results, and most importantly, it cannot be applied to young sires that have not yet been used extensively in the field. The evaluation of bull fertility is relevant, as studies such as Pacheco et al. (2021) have shown over 20 percentage point differences in P/AI between higher fertility (**HF**) and lower fertility (**LF**) bulls in a large dataset. Therefore, the development of new methods to predict bull field fertility earlier and more accurately is desirable for the dairy industry.

Assessments of sperm morphofunctional characteristics, such as sperm motility, morphology, membrane integrity and functionality, can be applied in routine practice, as quality control of semen batches released for use in AI, and can serve as an initial screening, excluding batches with potential risk of fertility failure (Oliveira et al., 2014; Okano et al., 2019; Alves et al., 2020), but these characteristics do not always guarantee higher fertility results. This highlights the need for more biologically relevant and predictive assays to better assess male fertility potential.

Only tens to hundreds of spermatozoa in a semen straw can reach the oviduct and remain bound to oviduct epithelial cells (**OEC**) until ovulation takes place (Sostaric et al., 2008). This population of sperm has intact membranes and a nonhyperactive motility pattern, in addition to cell receptors that enable the binding to OEC to form the sperm reservoir (Fazeli et al., 1999; Gualtieri and Talevi, 2000; Leemans et al., 2014). This functional sperm reservoir, located mostly in the isthmus region of the oviduct, allows the maintenance of sperm metabolic energy and viability, regulating the timing of sperm capacitation and release at the appropriate time, thus optimizing the chances of successful fertilization (Boquest et al., 1999). Thus, understanding the sperm population with the ability to bind and maintain the functional reservoir may be critical to establishing the difference in fertility among sires (Saint-Dizier et al., 2020).

This study evaluated morphofunctional sperm characteristics and in vitro sperm binding to the aggregates of OEC of Holstein bulls classified as LF or HF based on P/AI results. The main hypotheses were (1) sperm morphofunctional characteristics, including sperm motility, morphology, plasma and acrosomal membranes integrity, mitochondrial membrane potential, lipid peroxidation, and membrane fluidity would be similar between LF and HF sires; (2) due to reduced ability to bind to oviduct cells in vitro, LF bulls would have lower number of sperm bound to OEC aggregates over time; and (3) there is a correlation between the number of sperm bound to OEC aggregates over time and P/AI.

MATERIALS AND METHODS

The experiment was performed at the University of São Paulo. All procedures were approved by the Animal Research Ethics Committee of Luiz de Queiroz College of Agriculture (ESALQ) of the University of São Paulo (Protocol CEUA #2017.5.1618.11.9). All procedures performed on the animals during the study followed the recommendations of the Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching (FASS, 1999).

Sire Fertility

Fertility data of 7 Holstein bulls were obtained from 1,833 AI performed during a 2-yr period in a commercial dairy herd (Tainá Farm, São Pedro, SP, Brazil) with 300 lactating cows, housed in freestall barns, milked thrice daily, and with the 305-d average milk production of 9,000 kg. The bulls were used contemporaneously in the insemination of lactating cows receiving their first or later services postpartum, and the cows were inseminated by estrus or submitted to TAI protocols to determine the P/AI. The TAI protocol was based on estradiol (**E2**) and progesterone and was routinely used in dairy herds (Consentini et al., 2024). The bulls were classified within 2 classes: HF (n = 3; 35.0% [362/1,034]; 63.6% higher fertility than the other bulls) or LF (n = 4; 21.4% [171/799]). The P/AI and the number of batches of cryopreserved semen (CRV Lagoa, Ribeirão Preto, Brazil) per bull, packaged in 0.25-mL straws containing an average of 5.9 $\times$ 10^6 motile sperm per straw, were as follows: bull A HF (36.8% [25/68]; 4 batches), bull B HF (35.8% [290/809]; 4 batches), bull C HF (29.9% [47/157]; 4 batches), bull D LF (21.7% [103/475]; 4 batches), bull E LF (21.5% [42/195]; 3 batches), bull F LF (21.1% [19/90]; 2 batches), and bull G LF (17.9% [7/39]; 3 batches). Thus, each bull contributed to a certain number of semen batches regardless of fertility classification, with HF bulls having a total of 12 batches from 3 sires and LF bulls having 12 batches from 4 sires. This distribution ensured a comparable representation of semen samples between fertility groups.

Sperm Morphofunctional Analysis

For sperm morphofunctional evaluations, 2 semen straws from each batch and each bull were thawed in a water bath (37°C/30 min) and homogenized. Then, semen samples were processed according to each analysis technique.

To assess sperm motility, the semen was diluted to 25 $\times$ 10^6 sperm/mL in Tyrode's albumin lactate pyruvate (**TALP**) sperm medium (4.2 mg/mL sodium chloride, 1.87 mg/mL potassium chloride, 2.1 mg/

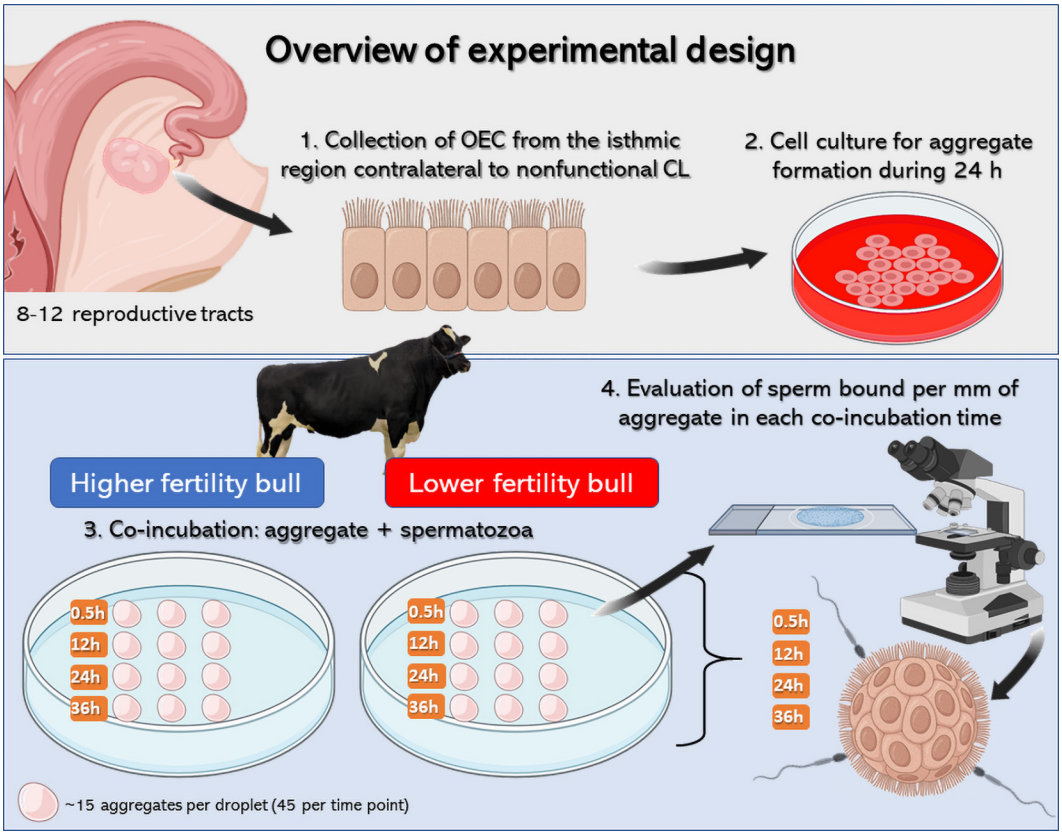

Figure 1. Overview of the experimental design. Oviductal epithelial cells (OEC) were collected from the isthmic region contralateral to the corpus luteum (CL) of 8 to 12 bovine reproductive tracts (1) and cultured for 24 h to allow the formation of aggregates (2). Aggregates were then co-incubated with sperm from bulls classified as of higher or lower field fertility (3). The experiment was conducted in 3 replicates, each including one higher-fertility and one lower-fertility bull. For each bull, a 100-mm Petri dish was prepared containing 12 droplets (3 per incubation time: 0.5, 12, 24, and 36 h), with approximately 15 aggregates per droplet (45 aggregates per time point). Sperm were thawed and washed, and the concentration used for co-incubation was 5×10^6 motile spermatozoa per milliliter. After each co-incubation time, aggregates were fixed, mounted, and evaluated under microscopy to determine the number of sperm bound per millimeter of aggregate perimeter (4).

mL sodium bicarbonate, 50 µg/mL sodium phosphate, 290 µg/mL calcium chloride monohydrate, 80 µg/mL magnesium chloride hexahydrate, 6.5 mg/mL HEPES) supplemented with albumin, sodium lactate, sodium pyruvate, and penicillin-streptomycin (Bavister et al., 1983). An aliquot of 10 µL of the sperm diluted was placed in a Makler chamber (Sefi-Medical Instruments) and evaluated using computer-assisted sperm analysis (CASA, Hamilton Thorne Research Biosciences, HTM-IVOS–Ultimate). Additional descriptive setups are provided in Supplemental Table S1 (see Notes). During the analysis, 5 fields were selected for each sample, and the following variables were analyzed: total motility (%), progressive motility (%), average path velocity (µm/s), straight-line velocity (**VSL**; µm/s), curvilinear velocity (µm/s), amplitude of lateral head displacement (µm), beat cross frequency (Hz), straightness (%), and linearity (%).

To assess sperm morphology, a semen sample was placed in a buffered saline solution containing 4% formaldehyde previously heated to 37°C. The wet chamber technique was prepared, and 200 sperm cells were analyzed under differential interference contrast microscopy (DIC, model 80i, Nikon, Tokyo, Japan) with 1,000× magnification. Sperm defects were classified as major and minor defects, according to Blom (1973).

Flow cytometry was used to assess 5 sperm parameters: plasma membrane integrity, acrosome integrity, mitochondrial membrane potential, membrane lipid peroxidation, and membrane fluidity. Analyses were performed according to Díaz et al. (2017) with an Accuri C6 flow cytometer (Becton, Dickinson and Company). For this, semen samples were diluted in TALP sperm medium (5×10^6 sperm/mL) and distributed to the 4 analysis protocols, each containing a 150-µL aliquot. In the first protocol, for analysis of the integrity of the plasma and acrosomal membranes, 3 µL of Syto-59 (S59; 5 n*M*, S10341), 1 µL of propidium iodide (**PI**, 0.5 mg/mL, P3566), and 10 µL of fluorescein isothiocyanate-conjugated peanut agglutinin (100 µL/mL) were

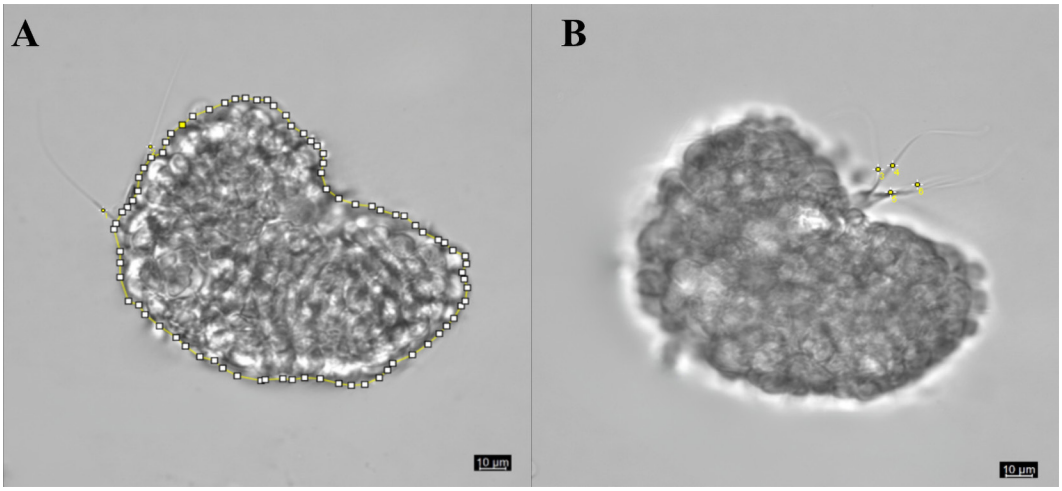

Figure 2. Representative images of sperm bound to oviductal epithelial cell (OEC) aggregates. Two focal-plane images of the same aggregate were used for analysis. In panel A, the perimeter of the aggregate was measured using ImageJ (highlighted outline), and 2 spermatozoa bound to the cilia are visible. In panel B, a different focal plane reveals 4 additional sperm bound to the same aggregate. The total number of sperm attached was divided by the perimeter (µm) to calculate the number of bound sperm per millimeter of aggregate.

added and incubated for 10 min at 37°C. The second protocol was to evaluate the mitochondrial membrane potential. For this, 1 µL of 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolcarbocyanine iodide (JC-1; 153 µ*M*, T-3168), 1 µL of PI, and 3 µL of S59 were added and incubated for 10 min at 37°C. In the third protocol, for evaluation of membrane lipid peroxidation, 1 µL of probe C11-Bodipy $^{581/591}$ (1 mg/mL, D-3861) was added and incubated for 20 min at 37°C. After 20 min, 1 µL of PI and 3 µL of S59 were added and incubated for 10 min at 37°C. Finally, in the fourth protocol, to assess the fluidity of the membranes, 1 µL of Yo-Pro-1 (YP; 7.5 n*M*) 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 µ*M*) was added and incubated for 70 s. After this step, each protocol received an additional 150 µL of TALP sperm medium to dilute the samples to a final concentration of 2.5×10^6 sperm/mL. The samples were then taken for evaluation in the flow cytometer. The results are expressed in arbitrary units. During the analysis, particles with different sizes and scatter properties were excluded. Spectral overlap of staining in the flow cytometer analyses was compensated when necessary. Samples were processed through the instrument at an acquisition rate of approximately 600 to 1,000 events/s, acquiring 10,000 events per assay, using the appropriate filters for each fluorescent probe added in each protocol. All fluorescent probes and reagents were purchased from Thermo Fisher Scientific (Waltham, MA), unless otherwise specified.

Collection and Culture of Oviduct Epithelial Cells

Eight to 12 reproductive tracts of cows were collected in a slaughterhouse for each in vitro assay, with a total of 3 replicates (Figure 1). The tracts were transported at 35°C in saline solution (NaCl 0.9%), supplemented with penicillin G (100 IU/mL) and streptomycin sulfate (50 µg/mL). In the laboratory, the oviducts contralateral to the corpus luteum (**CL**) that did not appear to be functionally active were selected based on macroscopic characteristics of Ireland et al. (1980). The oviducts were dissected from the adjacent structures, and only the isthmus region was used, considering up to 4 cm from the uterotubal junction. To collect the OEC, oviduct lumen was washed twice with 1 mL of TCM-199 medium (TCM-199 with Hanks' balanced salt solution, supplemented with 10% fetal bovine serum, 50 mg/mL gentamicin sulfate, and 25 µg/mL amphotericin B; Vitrocell, São Paulo, Brazil) in a 15-mL conical bottom tube. The supernatant was gently removed, and the cell pellet was resuspended in 5 mL of TALP medium for washing, after which the suspension was allowed to decant by gravity in 15-mL conical tubes placed in an incubator at 35°C with 5% CO_2 for 5 min. This procedure was performed twice. During each wash, cell de-aggregation was performed by gentle pipetting with a 1,000-µL automatic pipette. Finally, the cells were transferred to a 100-mm Petri dish with 25 mL of the same supplemented TCM-199 medium for cell culture for approximately 24 h to form the cell OEC aggregates, with 50% of the medium changed 6 to 8 h after the start of culture according to Kadirvel et al. (2012).

Table 1. Computer-assisted sperm analysis of motility variables of frozen-thawed semen from higher and lower field fertility Holstein sires after artificial insemination (AI)

Variable[1]	Higher fertility (n = 3 sires and 1,034 AI)	Lower fertility (n = 4 sires and 799 AI)	*P*-value
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; µm/s	124.3 ± 3.8	113.7 ± 5.9	0.15
Straight-line velocity, µm/s	94.7 ± 3.1	80.9 ± 3.1	<0.01
Curvilinear velocity, µm/s	234.8 ± 10.6	214.6 ± 17.6	0.34
Amplitude of lateral head displacement, µm	9.5 ± 0.5	8.8 ± 0.8	0.47
Beat cross frequency, Hz	31.6 ± 1.7	30.3 ± 1.1	0.52
Straightness, %	76.4 ± 1.8	74.3 ± 2.8	0.54
Linearity, %	43.4 ± 2.2	45.3 ± 3.7	0.67

[1]Values presented as mean ± SEM.

Evaluation of the Number of Sperm Bound per Millimeter of OEC Aggregate

In each replicate, one sire from each group was evaluated to determine the number of sperm bound per millimeter of OEC aggregate, using 180 aggregates per bull, distributed in 12 droplets of 30 µL of the same cell culture medium, overlaid with silicone oil. A sperm sample pool was prepared for each bull using one straw from each batch, and 250 µL of the sperm pool was washed with 1 mL of synthetic oviduct fluid (SOF; Botupharma, São Paulo, Brazil) and centrifuged at 700 × *g* for 5 min at 37°C. After washing, the supernatant was removed, and the pellet was resuspended in SOF medium.

The sperm concentration was determined using a Neubauer chamber to accurately count, then 1×10^5 motile spermatozoa/mL per drop containing OEC aggregates (0 h). To ensure consistency in OEC aggregate morphology across replicates, only aggregates with a compact, rounded structure, visible ciliary beating, and a perimeter between 100 and 200 µm were selected for analysis. Sperm and OEC aggregates were co-incubated for 0.5, 12, 24, and 36 h at 39°C in 5% CO_2 in air. After each co-incubation time, OEC aggregate-sperm complexes from 3 drops per co-incubation time were transferred to a microscope slide in a volume of 30 µL and covered with a coverslip. A total of 1,043 OEC aggregates were evaluated in this experiment, distributed across fertility groups and time points as follows: 128 (HF) and 153 (LF) aggregates at 0.5 h; 113 (HF) and 139 (LF) at 12 h; 106 (HF) and 148 (LF) at 24 h; and 113 (HF) and 143 (LF) at 36 h. Using the EUREKAM 1.3 image capture system (BEL Engineering, Monza, Italy), 2 photographs of each OEC aggregate were taken to measure the OEC aggregate perimeter and count the number of sperm bound using the ImageJ 1.3 software (National Institute of Health, Maryland). The analyses were performed by the same operator. The number of sperm bound per millimeter (sperm/mm) of the OEC aggregate was calculated by dividing the number of sperm bound by the perimeter of the OEC aggregate (Figure 2).

Statistical Analysis

Statistical analyses were performed using the Statistical Analysis System (SAS, version 9.4 for Windows, SAS Institute Inc., Cary, NC). All data of morphofunctional sperm analysis 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 per millimeter followed a gamma distribution, so the analyses were performed using the GLIMMIX procedure, fitting a gamma distribution. Each OEC aggregate was considered an experimental unit for statistical analyses. The effect of replicate (biological replicate per bull) was initially included in the model as a random factor but was removed from the final analysis due to the absence of a significant effect. Thus, the final model included the effect of fertility, hour, the interaction between them, and the OEC aggregate perimeter as a covariate. To better understand and compare the number of sperm per millimeter of OEC aggregate between HF and LF bulls over time, we also performed an analysis considering 8 groups (each treatment [HF and LF] at each time point [0.5, 12, 24, and 36 h]).

The correlation coefficient (r) and coefficient of determination (R^2) between field P/AI and sperm per millimeter at 0.5, 12, 24, and 36 h were calculated using the

PROC CORR procedure of SAS, whereas the intercept and slope of the linear equation were obtained using the option solution in the GLIMMIX procedure.

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

RESULTS

Sperm motility parameters did not differ between HF and LF bulls, except for VSL, which was higher in the HF group ($P < 0.05$; Table 1). Regarding sperm morphology, no significant differences were observed for minor defects (4.3 ± 0.8 vs. 3.7 ± 0.6; $P = 0.52$), major defects (8.8 ± 1.6 vs. 5.5 ± 1.0, $P = 0.08$), or total defects (13.1 ± 2.2 vs. 9.1 ± 1.3; $P = 0.13$) between HF and LF sires, respectively.

Sperm analysis by flow cytometry revealed no differences between fertility groups that could explain effects on fertility. It was noted that bulls classified as LF had a greater percentage of spermatozoa with intact plasma and acrosome membranes, and higher mitochondrial membrane potential. No differences were observed for membrane lipid peroxidation and membrane fluidity between fertility groups (Table 2).

When evaluating the number of sperm bound per millimeter of OEC aggregate (Figure 3) within each co-incubation period, there was a tendency for a greater number of bound sperm per millimeter in HF at 0.5 h (59.1 vs. 54.0; $P = 0.08$). At 12, 24, and 36 h, the number of sperm per millimeter was greater ($P \leq 0.001$) for HF compared with LF.

When evaluating the effects of co-incubation time (0.5, 12, 24, and 36 h) within each fertility group, both HF and LF groups had similar sperm binding at 0.5 h, which was higher than in all other groups across time points. However, at 12 h, the HF group had more bound sperm per millimeter than the LF group, and it was higher than both the HF and LF groups at 24 and 36 h. The LF group at 12 h was similar to the HF groups at 24 and 36 h. The LF group at 24 h was similar to the LF group at 36 h (Figure 3).

There was no relationship between P/AI and the number of bound sperm per millimeter of OEC aggregates at 0.5 h ($R^2 = 0.003$, $P = 0.90$; Figure 4A) or 12 h ($R^2 = 0.10$, $P = 0.49$; Figure 4B). However, at 24 h of co-incubation, there was a moderate association between fertility and sperm per millimeter ($R^2 = 0.59$, $P = 0.04$; Figure 4C), and a high relationship ($R^2 = 0.80$, $P = 0.007$) was detected between the number of sperm bound to OEC aggregates at 36 h of co-incubation and fertility (Figure 4D).

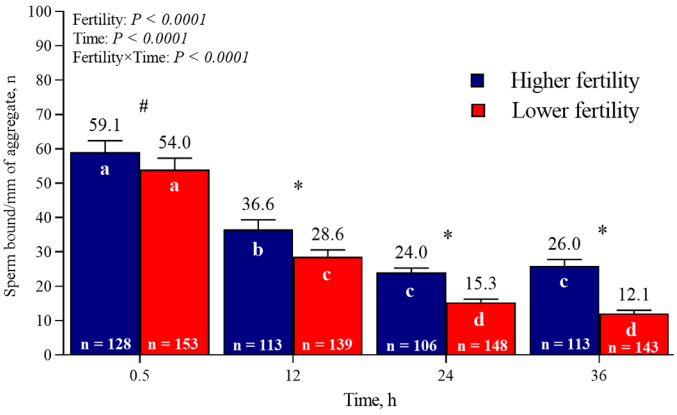

Figure 3. Number of sperm bound per millimeter of oviduct epithelial cell (OEC) aggregate after 0.5, 12, 24, and 36 h of co-incubation. The n within the columns represents the number of OEC aggregates in each group. Within each time point, differences between higher (HF) and lower (LF) field fertility bulls after artificial insemination are indicated separately (#$P = 0.08$, and *$P \leq 0.001$). Different letters (a, b, c, and d) indicate the differences among the 8 groups (each treatment [HF and LF] at each time point [0.5, 12, 24, and 36 h]). Error bars represent SEM.

Figure 5 illustrates, using the 0.5 h time point as the 100% reference, the change in the percentage of sperm bound to the OEC aggregate over time. This descriptive analysis demonstrates the dynamics of sperm binding to the OEC aggregate over time during in vitro co-incubation. At 24 and 36 h of co-incubation, sperm from HF bulls maintained 40.6% and 44.0% of the percentage of sperm bound to OEC aggregates, respectively. In contrast, sperm from LF bulls decreased to 28.3% and 22.2% binding to OEC aggregates after 24 and 36 h of co-incubation, respectively.

DISCUSSION

This study aimed to evaluate the morphofunctional sperm analysis of frozen-thawed semen from Holstein bulls of higher or lower field fertility, and the sperm capacity to bind in vitro and remain bound to OEC aggregates. Our results demonstrate a clear distinction in sperm binding capacity between bulls of HF and LF over distinct times of co-incubation. The primary results of this study were (1) the morphofunctional sperm analyses did not reveal relevant differences between HF and LF sires, (2) the number of sperm bound per millimeter of OEC aggregates was higher in HF bulls, and (3) sperm binding capacity at 36 h had a strong relationship with field fertility.

The sire's fertility in breeding programs of beef and dairy farms influences both productivity and profit-

Table 2. Evaluation of sperm membrane integrity and function by flow cytometry in frozen-thawed semen from higher and lower field fertility bulls after artificial insemination (AI); values expressed as mean ± SEM

Item	Higher fertility (n = 3 sires and 1,034 AI)	Lower fertility (n = 4 sires and 799 AI)	*P*-value
Intact plasma and acrosome membranes (%)	74.5 ± 1.8	80.3 ± 1.1	0.01
Intact plasma membrane and damaged acrosome (%)	1.3 ± 0.3	0.6 ± 0.1	0.01
Damaged plasma membrane and intact acrosome (%)	18.5 ± 1.7	14.6 ± 1.4	0.09
Damaged plasma and acrosome membranes (%)	5.6 ± 1.2	4.5 ± 0.8	0.47
Mitochondrial membrane potential (a.u.[1])	1,060.3 ± 206.2	3,285.2 ± 766.1	0.004
Membrane lipid peroxidation (a.u.)	762.5 ± 45.1	879.0 ± 47.7	0.09
Membrane fluidity (a.u.)	14,109.2 ± 1,911.9	15,734.5 ± 1,071.6	0.47

[1]Arbitrary units (a.u.).

ability (Davis and White, 2020). To better understand and predict this fertility, several characteristics of sperm cells and seminal plasma, such as morphology, motility, membrane integrity, and biomarker molecules, have been studied as possible fertility markers (Fair and Lonergan, 2018; Alves et al., 2020; Moraes et al., 2021). Although commercial semen undergoes rigorous analyses to guarantee quality standards by the industry, these analyses are not able to distinguish bulls with superior versus inferior field fertility (Vincent et al., 2012; Oliveira et al., 2014; Bollwein and Malama, 2023; Mukherjee et al., 2023). In addition, there are no laboratory tests effectively capable of predicting field fertility, which, associated with genomic evaluation, would help in the early selection of superior bulls.

Our hypothesis that sperm quality variables such as morphology, motility, and membrane integrity and functions would not differ between LF and HF bulls was supported, as most analyses did not reveal consistent differences between groups. Even when differences were observed, they did not follow the expected pattern associated with field fertility. This result suggests that, although sperm quality is essential for fertilization, the conventional parameters currently used to approve semen batches for commercialization may not be sufficient to predict field fertility outcomes. Bulls likely derive reproductive success associated also with other biological factors that contribute to their overall fertility. After the formation of the functional sperm reservoir in the oviduct, intrinsic characteristics of sperm and the female's reproductive tract (i.e., fluid, hormone concentrations, and OEC receptors) influence the lifespan of male gametes until ovulation (Suarez, 2002). A limited proportion of sperm from cryopreserved semen can bind to oviductal cells, with these sperm being motile, noncapacitated, and having intact acrosomes (Suarez, 2002). However, since only VSL was different between LF and HF, being higher in HF bulls, no alteration in motility related to hyperactivation or other motility patterns was detected. Studies have shown that higher motility is associated with increased binding of sperm to the oviductal epithelium, a key step in the fertilization process (Suarez, 2002). Conversely, several other studies did not report changes in motility as being responsible for the difference in fertility among bulls (Kastelic and Thundathil, 2008; Selvaraju et al., 2018; Pfeifer et al., 2019).

Although the percentage of spermatozoa with intact plasma membrane and intact acrosome was higher in LF bulls than in HF bulls, both groups presented values above those expected for selecting batches for use in AI. Because different factors influence sperm fertility, the lower P/AI in LF bulls suggests that, although their sperm may present integrity of the plasma and acrosome membranes, the failure in the functional capabilities required for successful fertilization has another cause (Vincent et al., 2012; Alves et al., 2020).

In addition, the mitochondrial potential was greater in LF bulls. The preferred metabolic pathway for energy production in sperm is highly species-specific (du Plessis et al., 2015). The energetic support of bull spermatozoa is primarily driven by mitochondrial oxidative phosphorylation (**OXPHOS**), although glycolysis may also contribute to ATP production (du Plessis et al., 2015; Nikitkina et al., 2022). In contrast to other species where glycolysis is essential for fertilization (e.g., mice, rats, and humans), bovine sperm predominantly rely on OXPHOS for energy production, particularly for motility and capacitation (Galantino-Homer et al., 2004). The higher mitochondrial membrane potential in the LF group, although statistically significant, does not appear to explain the difference between the high and low fertility phenotypes. However, this increase in mitochondrial membrane potential in the LF group may be responsible for the trend ($P = 0.09$) toward increased lipid peroxidation in the same group, because mitochondrial activity induces ROS production. However, because these results are presented in arbitrary units, it is difficult to establish a cutoff point for the values found and their biological effects, as opposite results were expected between the fertility groups.

Further studies should be conducted to understand the relationship between the maintenance pathways of sperm

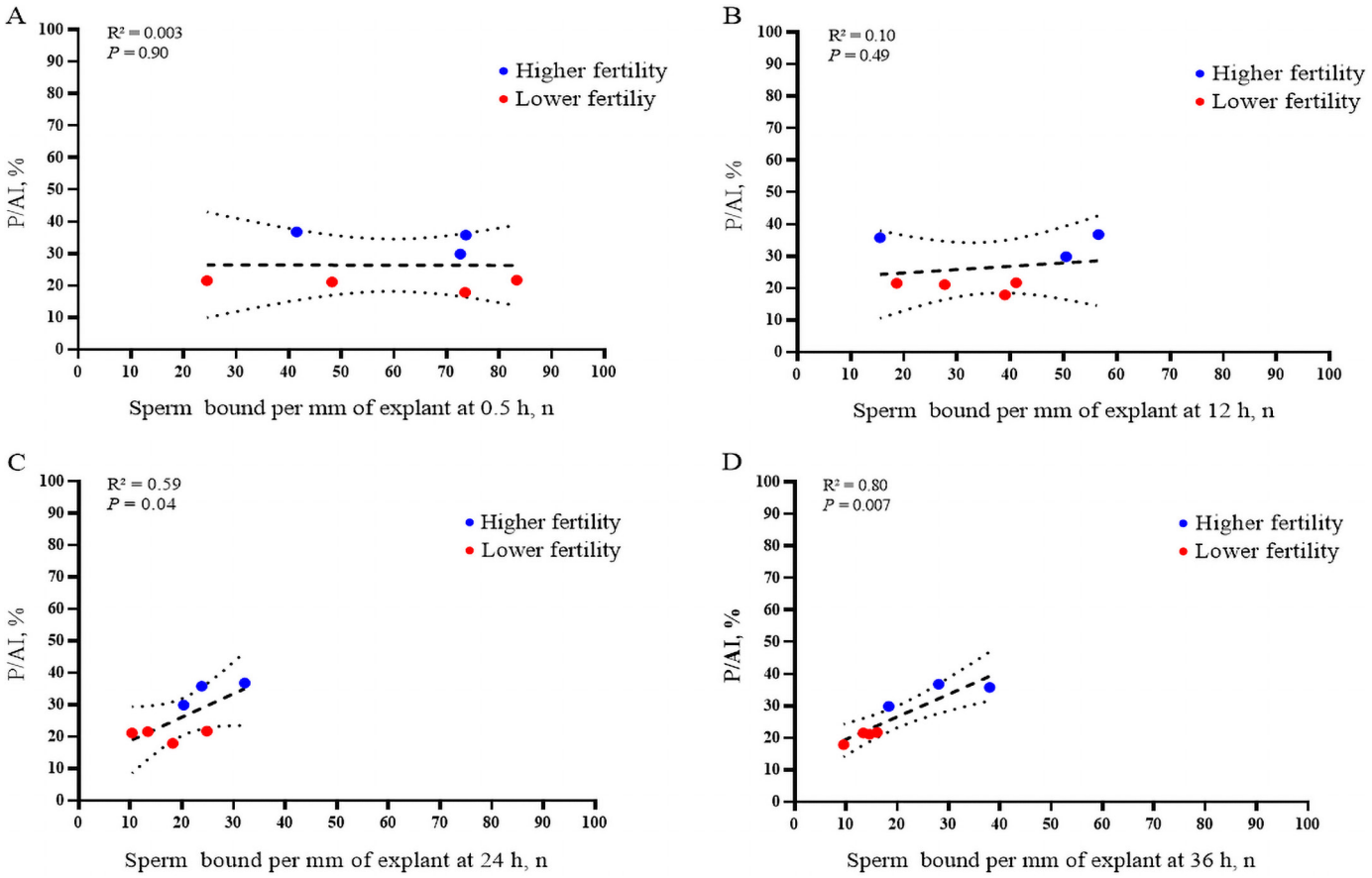

Figure 4. Relationship between field pregnancy per artificial insemination (P/AI) of higher (n = 3 sires) and lower (n = 4 sires) fertility Holstein sires and the number of sperm bound per millimeter of oviduct epithelial cell aggregates at 0.5 (A), 12 (B), 24 (C), and 36 (D) h of co-incubation. Fertility data of 7 Holstein sires were retrospectively obtained from 1,833 artificial inseminations performed during a 2-yr period in a commercial dairy herd. The dashed line shows the linear regression, and the dotted lines represent the 95% CI.

metabolism and the functional sperm reservoir. Thus, sperm quality analyses, as reported (Celeghini et al., 2008; Vincent et al., 2012; Mukherjee et al., 2023), do not provide a complete, comprehensive assessment of fertility potential. This discrepancy suggests that although these analyses may indicate overall sperm quality, they do not capture the full range of functional capabilities required for successful fertilization and pregnancy.

A previous study that attempted to correlate sperm binding per millimeter of OEC aggregate with bull fertility reported a positive correlation between the number of sperm bound to OEC aggregates after 24 h of sperm co-incubation and the nonreturn rate in cattle (De Pauw et al., 2002). Our second hypothesis, that fewer sperm from bulls with lower field fertility would bind to OEC aggregates over time, was supported. At 0.5 h of co-incubation, there was a tendency (P = 0.08) for fewer sperm to be bound to the OEC aggregate in the LF group, with this difference between groups increasing over time (0.5 h = 8.3%, 12 h = 21.9%, 24 h = 36.3%, and 36 h = 53.5% difference). Therefore, until this time, this is the first study to compare sperm from bulls with different field fertility, assessing their binding to an OEC aggregate at 4 time points.

A previous study conducted by our research group (Carvalho et al., 2018) evaluated the effect of sex-sorted sperm on sperm binding capacity to oviductal cells, considering that sexed sperm is known to have lower conception rates following AI. Ejaculates from 4 bulls were divided into non-sex-sorted and sex-sorted sperm groups, with no significant difference found in sperm binding to OEC aggregates after 30 min between groups. However, after 24 h, the sex-sorted sperm had fewer bound sperm per milliliter (6.7 ± 2.0) compared with the non-sexed sperm (23.6 ± 7.2). The results suggested that the initial binding capacity was unaffected by the sex-sorting procedure, but the ability of sex-sorted sperm to remain bound over an extended period was compromised.

A similar result was found in this study, with the LF bulls having a greater decrease in the number of sperm

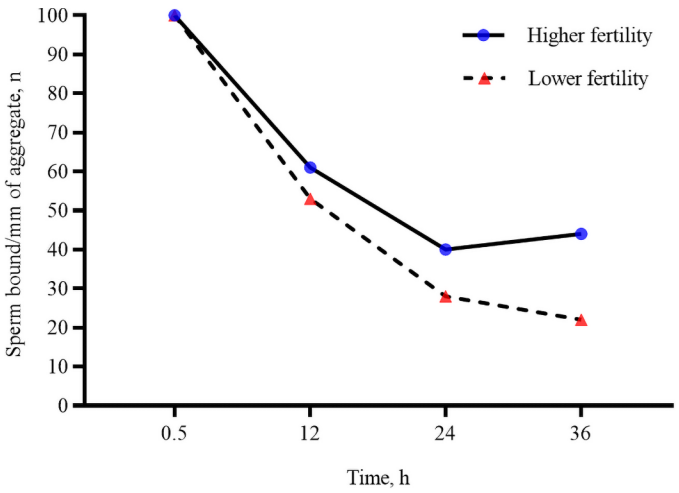

Figure 5. Sperm from Holstein bulls with higher (HF, n = 3 sires) or lower (LF, n = 4 sires) field pregnancy per artificial insemination bound per millimeter of oviduct epithelial cell (OEC) aggregate after 0.5, 12, 24, and 36 h of co-incubation. The number of sperm bound to OEC aggregates per treatment (HF or LF) at 0.5 h of co-incubation was considered 100% to calculate the relative percentage decrease in sperm number per millimeter of OEC aggregate in each hour of co-incubation.

bound as co-incubation time progressed. This result suggests that the ability to maintain sperm binding over time may play an important role in fertility, with HF bulls or non-sexed sperm having superior binding over time. The binding maintains sperm viability not only by delaying capacitation but also by providing a supportive microenvironment enriched with oviductal secretions, such as proteins, ions, and extracellular vesicles, that help to modulate sperm metabolism, motility, and function (Ferraz et al., 2020). Importantly, the oviductal epithelium is not a passive surface; it responds dynamically to the presence of sperm, constituting an active biological interaction that significantly modulates the local oviductal environment (Hunter, 2012). This regulation, initiated by sperm binding, appears to create a favorable microenvironment for the gametes while simultaneously inducing changes that prepare the oviductal milieu for successful fertilization and optimal early embryo development (Suarez, 2008).

A functional sperm reservoir is formed in the isthmus region of the bovine oviduct 8 to 12 h after AI (Wilmut and Hunter, 1984). Only tens to hundreds of sperm in the in vivo or in vitro milieu can remain bound to oviduct cells, prolonging their viability until ovulation (Gualtieri and Talevi, 2000). This binding does not depend on the sperm's previous interaction with uterine cells or fluids, as demonstrated by a study that deposited the sperm directly into the oviduct and observed sperm bound to oviduct cells in vivo (Lefebvre et al., 1995). In contrast to in vivo systems, such as TAI or superovulation protocols, where membrane integrity and functional surface receptors are essential for sperm fertility, sperm with such deficiencies can still achieve fertilization in vitro (Baba et al., 2002). In addition, co-incubating sperm with oviduct cells before in vitro fertilization selects for better sperm quality (i.e., fertilization ability), such as increasing blastocyst rate and decreasing problems as polyspermy (Gualtieri and Talevi, 2003; Lamy et al., 2017). These findings support our rationale of associating sperm binding to oviduct cells with field fertility. Although the exact stage of the estrous cycle was not controlled, only oviducts contralateral to nonfunctional CL were selected (Ireland et al., 1980), ensuring that all oviducts were collected from reproductive tracts with a standardized ovarian feature. This approach aimed to create expected homogeneity in epithelial cell characteristics across samples and minimize variability between fertility groups.

The third hypothesis, that the number of spermatozoa bound to OEC aggregates over time would be associated with retrospective data of P/AI, was confirmed, with high correlation between sperm bound and field fertility at 36 h (r = 0.89). Although 36 h does not reflect the in vivo timing of binding, it may indicate sperm resilience and sustained interaction, traits linked to fertility. Similar results were reported by Saraf et al. (2019) in buffalo, where a sperm binding test conducted after 1 h of co-incubation with OEC revealed a greater number of sperm bound and a relationship ($R^2 = 0.47$) in high-fertility buffalo compared with intermediate and low fertility. This finding highlights the potential of sperm binding assays as reliable predictors of bull fertility and suggests that, beyond bulls, this approach may also serve as a valuable predictive tool for fertility assessment in other species, emphasizing the crucial role of sperm-OEC interactions for successful fertilization. Additionally, the timing of ovulation relative to insemination plays a crucial role in determining fertilization success. The ideal time to perform insemination is approximately 12 to 16 h before ovulation (Pursley et al., 1998). However, the timing and dispersion of ovulation in TAI programs can affect fertility. There are TAI protocols that result in more variable ovulation timing, such as Co-Synch protocols, where GnRH is administered at the time of AI. In these protocols, cows with a dominant follicle (>10 mm) have the potential to respond to GnRH and ovulate approximately 28 to 30 h after TAI. However, cows expressing estrus before AI will ovulate earlier and may not require sperm with extended longevity for successful fertilization. Another ovulation induction strategy at the end of the protocol is using E2 esters such as E2 cypionate (**EC**). However, in this type of protocol, dispersion of ovulation is greater when compared with GnRH (Pancarci et al., 2002; Souza et al., 2009). In our study, the fertility

of bulls was determined in cows undergoing ovulation synchronization with EC, which induces the onset of an LH surge ~41 h after EC administration and ovulation ~31 (range of 7 to 55) h later (Sales et al., 2012), resulting in a broader ovulation window. Although our coincubation period was not designed to replicate this exact physiological timing, evaluating sperm-OEC binding for up to 36 h allowed us to assess the functional resilience of spermatozoa. This longer observation period provided insights into the ability of sperm to remain viable and maintain binding capacity over time, characteristics that may be associated with higher fertilization potential. Sperm from HF bulls remained bound for longer periods, whereas those from LF bulls had a reduced binding duration. This reduced capacity may be particularly detrimental under protocols with greater ovulation dispersion, such as those using EC, where extended sperm viability is important to ensure fertilization during the wider ovulatory window. However, further studies are needed to determine whether the predictive value of sperm-OEC binding is consistent across different reproductive strategies used under field conditions.

Fertility differences between bulls are likely multifactorial, involving not only structural and metabolic aspects but also molecular and genetic components that regulate sperm function. For example, studies have shown that altered sperm motility patterns, such as premature hyperactivation, can impair fertility when ovulation is delayed after TAI (Pfeifer et al., 2019), reinforcing the importance of temporal synchrony and sustained sperm viability. In addition, genomic studies in Holstein and Jersey bulls have identified genetic variants associated with SCR, particularly in genes related to sperm motility, acrosome reaction, chromatin remodeling, and testicular development (Peñagaricano et al., 2012; Rezende et al., 2018). These findings support the idea that sperm functional competence, as evaluated by tests such as OEC binding, may reflect deeper biological determinants of fertility and help explain the variability observed among bulls.

Considering that LF in sires results in significant economic losses, the need for a reliable fertility predictor becomes even more evident. In this context, our study highlights the strong potential of the sperm binding assay to OEC as a predictive tool for assessing bull fertility.

CONCLUSIONS

The morphofunctional sperm assessments were not able to identify major differences between the semen of bulls with distinct field fertility. However, the binding capacity of sperm to oviduct OEC aggregates was significantly greater in bulls with higher field fertility, indicating the potential of this assay as a predictor of field fertility. These results suggest that lower fertility in some bulls may be due to a reduced ability to bind to oviduct cells in vivo. Therefore, integration of this binding assay with other semen analysis tools and genomic testing could improve the early selection of bulls and ultimately improve the efficiency of reproductive programs in the dairy industry.

NOTES

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Nonstandard abbreviations used: AI = artificial insemination; CL = corpus luteum; DIC = differential interference contrast; E2 = estradiol; EC = E2 cypionate; HF = higher fertility; LF = lower fertility; OEC = oviduct epithelial cell; OXPHOS = oxidative phosphorylation; P/AI = pregnancy per artificial insemination; PI = propidium iodide; SCR = sire conception rate; SOF = synthetic oviduct fluid; TAI = timed AI; TALP = Tyrode's albumin lactate pyruvate; VSL = straight-line velocity.

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