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Correlation between sperm kinetics and *in vitro* fertilization potential of girolando bulls.
Correlação entre a cinética espermática e o potencial da fertilização *in vitro* de touros Girolando.

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Abstract

The present study to evaluate the correlation between semen kinetics parameters analyzed using Computer-assisted Semen Analysis (CASA) and *in vitro* embryo production (IVEP) in Girolando breed bulls. First, semen samples from five Girolando bulls were used for *in vitro* embryo production; cleavage and blastocyst rates were assessed. Subsequently, three semen samples from each bull (from the same batch used for fertilization) were analyzed using CASA. The averages for the parameters examined for each bull were correlated with cleavage and blastocyst rates using principal component analysis. The first component (PC1 – 70%) demonstrates that curvilinear velocity (VCL) and average path velocity (VAP) had higher correlation with *in vitro* embryo production.
Keywords: Andrology. Bovine. CASA. Reprodution.

Resumo

Objetivou-se avaliar a correlação entre os parâmetros cinéticos do sêmen analisados pelo *Computer-assisted Semen Analysis* (CASA) com a produção *in vitro* (PIV) de embriões na raça Girolando. Inicialmente, Amostras de sêmen congelado de cinco touros foram utilizadas para produção de embriões *in* vitro, avaliando taxas de clivagem e blastocistos. Posteriormente, três doses de sêmen de cada touro (mesma partida utilizada na fertilização) foram analisadas no CASA. A média dos parâmetros analisados de cada touro foi correlacionada com as taxas de clivagem e blastocisto usando análise de componentes principais. Nesta análise, o primeiro componente (PC1 – 70%) demonstrou que a velocidade curvilínea (VCL) e a velocidade média do percurso (VAP) possuem maior correlação com a PIV de embriões.
Palavras-chave: Andrologia. Bovino. CASA. Reprodução.

Introduction

The sperm samples that are used in fertilization strongly influence *in vitro* embryo production (IVP) results (MORTIMER, 1997, p. 419). The effect in bulls has generally been reported to be a cause of variation in rates of development. The ability to select sperm samples with improved *in vitro* performance based on sperm features would be a useful tool for improving embryo production yields (SIQUEIRA et al., 2018, p. 8).

Semen analysis has been used for more than half a century now for estimate the fertilization potential of a semen sample (GRAHAM; MOCÉ, 2005, p. 493). Computer-assisted semen analysis (CASA) provides an objective evaluation of sperm motility more rapidly and accurately than traditional methods such as visual observation (MORTIMER, 2000, p. 520; NAGY et al., 2015, p. 371). The efficiency of CASA enables it to set a standard for semen analysis in laboratories, reducing bias and inaccuracy (EHLERS et al., 2011, p. 451; AMAN; WABERISKY, 2014, p. 13; MORTIMER et al., 2015, p. 546).

Some sperm motility parameters may be predictors of the *in vitro* potential of semen samples (AMAN; WABERISKI, 2014, p. 12). Evaluation of these parameters in combination with ultrastructural analysis (GU et al., 2019, p. 4-6), genomic analysis (TAYLOR et al., 2018, p. 7), and *in vitro* insemination analysis (PUGLISI et al., 2012, p. 22) among others, prior to use of cryopreserved/thawed semen in IVP, may help to shed further light on the role of semen in embryo development.

In the present study, groups of *in vitro* matured oocytes were inseminated with five different semen samples from 5/8 Girolando bulls (crossbred; *Bos indicus*, Gyr x *Bos Taurus*, Hollstein) with a view to ascertaining the IVP outcome for each individual sample. The samples then underwent CASA to ascertain the sperm motility parameters for further correlation with embryo production and cleavage rates. In view of the scarcity of data on reproduction in the Girolando breed, and the importance of this breed to Brazilian dairy industry (CANAZA-CAYO et al., 2016, p. 113), the present study may help to establish a method for predicting fertilization rates using CASA data, thereby saving time and money in the selection of bulls for reproduction, enhancing pregnancy rates in cows.

Material and methods

Ethics statement

The present study was approved by the Ethic Committee for Animal Experimentation, CEUA, of Federal Rural University of Pernambuco (UFRPE), under license number: 090/2017.

The culture medium was purchased from the Gene Up biotechnology company (Presidente Prudente-SP-Brazil), unless otherwise stated. The frozen semen from five Girolando bulls was prepared at the SEMBRA® company (Barretos-SP-Brazil), in 0,25 ml straws (30 x 10^6/ml sperm) and was purchased for the experiment. These semen samples were used for *in vitro* embryo production. Three semen samples doses from each bull were also analyzed at the Andrology Laboratory of the Department of Veterinary Medicine, at the Federal Rural University of Pernambuco, making a total of 15 analyses. Semen samples were taken from same batch of bulls in order to minimize differences.

In vitro embryo production (IVEP)

Bovine ovaries were collected from a commercial slaughterhouse immediately after slaughter. These were transported in thermal bottles containing saline solution and antibiotics (0.9% NaCl and 30 mg/mL of gentamicin) at 37 °C to the laboratory. The ovaries were rinsed three times in phosphate-buffered saline solution - PBS (pH 7.0-7.2) and kept in a water bath at 37 °C.

The cumulus-oocyte complexes (COCs) were aspirated from antral follicles of 3-5 mm in diameter. The COCs were selected under a stereomicroscope (SMZ-745, Nikon, Tokyo, Japan); according to the number of layers of cumulus cells (>3 layers) homogeneity and color of cytoplasm (GONÇALVES et al., 2007). Each group of 15-20 COCs was then allowed to undergo in vitro maturation (IVM) in a 100 µL droplet of IVM medium [TCM-199, 0.2 mM sodium pyruvate, 10% FBS , 0,1 mM cysteamine, 10 ng/mL epidermal growth factor (EGF), 20 µg/mL FSH/LH, 1 µg/mL estradiol benzoate (E2)] under mineral oil for 24 hours at 38.5 ˚C in an incubator (Water-Jacketed CO_2 Incubators, Thermo Scientific Forma® Series II, USA/CANADA),under 5% CO_2 air and saturation humidity.

Semen straws were thawed in a warm bath (Semen Defrost, WTA/Biodux, São Paulo, Brazil) for in vitro fertilization at 36.5 ˚C for 30 seconds. Samples were subsequently selected on a discontinuous Percoll gradient (45 - 90%) and each group of in vitro matured COCs was placed in 30 µl of IVF-TALP medium [Tyrode's solution with albumin, lactate and pyruvate (TALP), supplemented with 50 mg/ml heparin] covered with mineral oil, inseminated with 2x106 sperm/ml and incubated for 18 h under the same conditions used for IVM. At 18 h post-insemination (hpi) the groups of presumptive zygotes (PZs) were transferred to an in vitro culture (IVC) in 100 µl of synthetic oviductal fluid (SOF) supplemented with 5% fetal bovine serum (FBS) and incubated until 168 hpi.

Computer assisted sperm analysis (CASA)

Frozen semen straws from each bull were thawed in a warm bath at 37 Cº and incubated for 5 minutes after dilution in a warm Tris buffer (v/v). Aliquots of 5µl were placed on previous warmed slides (37 Cº) and covered with coverslips to taking photos using a camera (Basler Vision Technologies A312FC) attached to a phase contrast microscope. A minimum 2000 spermatozoa in five non-consecutive fields were randomly chosen for analysis. The parameters analyzed using Sperm Class Analyzer – SCA™ software v. 5.1 (Microptics, S.L., Barcelona, Spain) were: progressive motility (PM, %), linearity (LIN, %), straightness (STR, %), wobble (WOB, %), curvilinear velocity (VCL, µm/s), straight line velocity (VSL, µm/s) and average path velocity (VAP, µm/s), amplitude of lateral head (ALH, µm), and beat cross frequently (BCF, hz). The settings for these parameters were: 50 frames s^{-1}; 20-90 μm^2 for the head area and VCL > 10 $\mu m/s^{-1}$ to classify spermatozoa as motile.

Experimental design

Five bulls from the SEMBRA® Company (Barretos-SP-BR) provided the semen samples. These were represented by the letter “B” followed by a number (1-5) representing each bull: B1, B2, B3, B4 and B5. The experiment was conducted in two steps. First, the frozen semen samples were thawed and used to fertilize oocytes (n= 715) acquired from a commercial slaughterhouse. At

least three fertilizations were performed for each bull. Then, on day 2 and day 7 after IVF, cleavage and blastocyst rates were observed, respectively. In the second step, each semen sample from the same batch of fertilized oocytes underwent CASA in triplicate to analyze the sperm kinetics parameters of each bull (total of 15 analyses). The average of the three results from each bull was correlated with cleavage and blastocyst rates.

Statistical Analysis

The cleavage and blastocyst rates of the bulls were compared with the Kruskal-Wallis Test (P value<0.05 for a significant difference), and CASA data were compared using the Tukey test (P value<0.05). Sperm kinetics parameters were correlated with the cleavage and blastocyst rates of all bulls using the Pearson correlation ($r=\pm1$, $P<0.05$) and the importance of each sperm kinetics parameter for IVEP outcome was analyzed using principal component analysis (PCA) by way of the PRINCOMP procedure. This generates a set of variables identified by PRIN representing by the overall effect of all nine parameters acquired using CASA analysis. The nine parameters were weighted according to the sum of the original values. The values obtained are represented by the covariance matrix with the eigenvalue as axis' length and the eigenvectors as axis' directions. The statistical analyses were conducted using SAS University (2020).

Results

In vitro embryo production (IVEP)

Individual bulls differenced from one another in terms of embryo production. Bulls B2 and B3 had the highest cleavage rates (P<0.05) and B1 the lowest (P<0.05), with no significant difference among the other bulls. B2 had the highest blastocyst rate (P<0.05) and B1 the lowest (P<0.05), with no significant difference among the other bulls (Table 1).

Table 1 - *In vitro* embryo production (IVEP)

Bull	**Total fertilized oocytes (n)**	**Cleavage % (n)**	**Blastocyst % (n)**
B1	111	51.35% (57) c	10.81% (12) c
B2	118	72.03% (85) a	44.91% (53) a
B3	207	68.11% (141) b	29.47% (61) b

B4	145	72.41% (105) a	33.79% (49) b
B5	134	67.16% (90) b	30.6% (41) b

[abc] Different letters indicate significant difference (P<0.05)

Correlation between sperm kinetics parameter and cleavage/blastocyst rates

The progressive motility rate, VSL, VAP, WOB and ALH did not exhibit any correlation with cleavage or blastocyst rates in any of the bulls evaluated (Table 2). VCL was positively correlated (r= 0.99, P< 0.05) with cleavage rate in B2, and LIN showed a negative correlation (r= -0.99, P< 00.5) with blastocyst rate only in B1. STR had the same negative correlation with cleavage and blastocyst rates in B3 (r= -0.99, P< 0.05), while BCF showed the same positive correlation with cleavage and blastocyst rates in B4 (r=0.99, P< 0.05) (Table 2).

Table 2 - Correlation (r=) between sperm kinetics parameters and IVEP results

		Sperm kinetics parameters								
Bull Semen		VCL (µm/s)	VSL (µm/s)	VAP (µm/s)	LIN (%)	STR (%)	WOB (%)	PM (%)	ALH (µm)	BCF (Hz)
B1	**Cleavage rate (%)**	0.80	0.53	-0.54	0.96	0.61	0.94	-0.41	-0.36	0.86
	Blastocyst rate (%)	0.67	0.68	-0.69	0.99*	0.75	0.98	-0.23	-0.53	0.75
B2	**Cleavage rate (%)**	0.99*	0.99	0.97	0.77	0.41	0.11	0.77	0.47	0.55
	Blastocyst rate (%)	-0.45	0.37	-0.66	0.15	0.57	0.80	-0.93	0.52	0.44
B3	**Cleavage rate (%)**	0.70	0.90	0.95	0.96	-0.99*	0.18	0.93	-0.18	0.57
	Blastocyst rate (%)	0.70	0.90	0.95	0.96	-0.99*	0.18	0.93	-0.18	0.57

B4	**Cleavage rate (%)**	-0.07	0.46	-0.43	0.84	-0.55	0.92	-0.48	0.90	0.99*
	Blastocyst rate (%)	-0.07	0.46	-0.43	0.84	-0.55	0.92	-0.48	0.90	0.99*
B5	**Cleavage rate (%)**	-0.78	0.59	-0.60	0.82	0.64	0.83	0.97	-0.88	-0.81
	Blastocyst rate (%)	-0.78	0.59	-0.60	0.82	0.64	0.83	0.97	-0.88	-0.81

* Indicates significant difference (P<0,05); PM: progressive motility, LIN: linearity, STR: straightness and WOB: wobble, VCL: curvilinear velocity, VSL: straight line velocity, VAP: average path velocity, ALH: amplitude of lateral head, BCF: beat cross frequency.

Importance of each sperm kinetics parameter for IVEP outcomes

The eigenvalues that represent the length of the axis' for principal components and the eigenvectors that represent directions of the axis' for principal components were obtained by principal component analysis (Table 3). These values indicate that the first principal component (PC1) represents 70% of the cumulative variation of the data analyzed, while, together with the second component (PC2), it represent up to 97.73%, and with the third component (PC3) 99.80%. This demonstrates the importance of the first three principal components.

Table 3 - Eigenvalues of covariance matrix of grouped variables and proportion of database analysis

PC	Eigen-value	Difference	Proportion	Cumulative
1	244,48	149,50	0,70	0,70*
2	94,98	86,30	0,27	0,97*
3	8,68	8,18	0,02	1,00*
4	0,50	0,34	0,00	1,00
5	0,16	0,13	0,00	1,00
6	0,03	0,02	0,00	1,00
7	0,01	0,00	0,00	1,00
8	0,01	0,00	0,00	1,00
9	0,00	0,00	0,00	1,00

*Variables responsible for greater proportion of variability in database analysis. PC= Principal component

Analysis of the first principal component (PC1), representing the general effect of all CASA parameters on cleavage and blastocyst rates, reveals certain predominance of the effects of parameters attributable to those with a higher weighting in the matrix; namely VCL (0.711), VAP (0.304), and VSL (0,073). This indicates the importance of these for IVEP outcome, as PRIN1 represents 70% of cumulative variability (Table 4). With the second principal component (PC2), the

greater portion of the effect is produced by VCL (0.36), VSL (0.49), VAP (0.41), LIN (0.46) and STR (0.41) parameters. The third principal component (PC3) was predominantly affected by the WOB (0.66) parameter. Since others components had 0% of cumulative variability they were not considered in the analysis.

Table 4 - Eigenvectors of covariance matrix demonstrating correlation values for variables (PC) and sperm parameters under analysis.

	PC1	**PC2**	**PC3**	**PC4**	**PC5**	**PC6**	**PC7**	**PC8**	**PC9**
PM	-0.001	0.007	0.004	0.057	-0.031	-0.255	-0.057	-0.577	0.144
VCL	0.712	0.364	-0.111	-0.461	0.259	-0.146	0.206	0.054	0.601
VSL	0.073	0.498	-0.088	0.465	-0.397	-0.329	-0.040	0.458	0.625
VAP	0.304	0.411	0.290	0.345	-0.083	0.433	-0.257	-0.449	0.039
LIN	-0.434	0.462	0.141	-0.371	0.272	-0.210	-0.557	0.045	0.075
STR	-0.365	0.415	-0.636	-0.092	-0.081	0.293	0.363	-0.234	-0.139
WOB	-0.266	0.254	0.662	-0.032	0.116	0.085	0.614	0.073	0.008
ALH	0.049	-0.009	-0.003	-0.170	-0.049	0.688	-0.224	0.407	-0.041
BCF	-0.011	0.020	-0.183	0.519	0.819	0.047	0.011	0.117	0.067

*PM: progressive motility, LIN: linearity, STR: straightness and WOB: wobble, VCL: curvilinear velocity, VSL: straight line velocity, VAP: average path velocity, ALH: amplitude of lateral head, BCF: beat cross frequency.

Discussion

Sperm motility is one of the most important characteristics associated with the ability of spermatozoa to fertilize eggs and is indicative of their viability and structural integrity in numerous species (KATHIRAVAN et al., 2011, p. 167; DEL OLMO et al., 2013, p. 106; FERRAZ et al., 2014, p. 1070). In the present study, even though bulls had a good *in vivo* fertility history according to the SEMBRA® company, the IVP results showed significant differences ($P<0.05$) among the bulls included in this study, underlining the differences between *in vitro* and *in vivo* production. The correlation between semen parameters and cleavage/blastocyst rates may thus help to ascertain why these differences may occur.

Semen parameters showed varying degrees of correlation with IVEP results. In the present study, some parameters correlated negatively (STR), and others positively (LIN and BCF). However, these results were specific to each bull, and do not indicate a general overall common to all the bulls. Nagy et al. (2015, p. 374-376) found that VAP is the most useful semen kinetics parameter with clinical importance for the prediction of bull fertility in artificial insemination. Hirano et al. (2001, p. 217) also found that VCL was an important parameter that could predict whether *in vitro* fertilization was successful or not in humans. In this study, VAP did not exhibit a significant difference ($P>0.05$) between the semen with the highest IVEP results, semen of lowest IVEP results and other groups ($P>0.05$). There was thus no correlation between VAP with IVEP results for any group. These results highlight individual differences among animals in order to correlate *in vitro* embryo production with semen parameters. Based on this analysis, individual bull semen parameter/IVEP correlation could be unable to identify possible fertility predictors (TOLEDO-ALVORADO et al., 2017, p. 8225; MUUTTORANTA et al., 2019, p. 8188).

However, the principal component analysis sheds new light on the data, as all the parameters and their results can be combined into components determining the weight of each parameter in IVEP production (HONGYU et al., 2016, p. 84). Agarwal et al. (2003, p. 345) have reported results indicating that human semen assessment can be reduced to fewer variables using principal components to analyze fertility based on the CASA outcome. This study showed that VCL, VSL and VAP featured heavily in the covariance matrix in terms of correlation with IVEP production. These results thus underline the importance of sperm velocity in enhancing fertility rates as reported by Del Olmo et al. (2013, p. 106-107) in rams. The results for the second principal component showed the importance of STR and LIN. This was expected, since VSL, VCP and VAP are used to calculate these parameters (MORTIMER et al., 2000, p. 519) and may thus also play a role in fertility.

Li et al. (2014, p. 79) found a positive correlation between high progressive motility and *in vitro* embryo production in Holstein bulls showing that PM could be a possible predictors. This differs from the findings of our study. The covariance matrix results show a low influence of PM on cleavage and blastocyst rates, when all principal components are analyzed. Even in individual bulls, PM exhibit great variability, with negative correlation in some bulls (B1 and B4) and moderate correlation in others (B2- $r < 0.90$). This underlines the differences among breeds and the influence of other factors, such as seminal plasma components and concentration of ejaculate (LI et al., 2014, p. 78). The correlations between cleavage and blastocyst rates and sperm parameters were similar in all bulls. These results were not expected by the authors since blastocyst rates had more influence on oocyte maturation (citoplasmatic and nuclear) making them more dependent on oocyte quality than cleavage rate (LONERGAN; FAIR, 2015, p. 258).

Various authors have used genomic traits and flow cytometry to predict fertility in females, including sperm analysis, but the results do not suggest that these are good predictive tools in mammals (AMANN; DEJARNETTE, 2012, p. 811; OKANO et al., 2019, p. 609). The female tract may select the numbers of spermatozoa and this does not appear in analysis. Other variables may also affect the fertility rate including the nutritional status of females, the technician who performed the AI, and environmental factors (CHRISTENSEN et al., 2005, p. 104; BROEKHUIJSE et al., 2011, p. 1480-1481). In principal component analysis, however, factors such as nutrition and the environment may be transformed into linear variables correlated with semen parameters components, producing a covariance matrix indicating their importance for fertility rates. It is therefore suggested that further studies must be conducted to apply this methodology to *in vivo* fertility.

Conclusion

Analysis of sperm kinetics parameters as predictors of the *in vitro* potential of semen samples using Computer-Assisted Sperm Analysis (CASA) is an interesting tool that may shed further light on the role of sperm in *in vitro* embryo development. VCL, VSL and VAP, which are regarded as principal components may be able to predict *in vitro* embryo production potential (PC1- 70%), followed by LIN and STR, regarded as secondary principal components (PC2- 27%).

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