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Oviductal cells adhesion test for bull semen quality assessment and fertility prediction of endangered breeds: proposal of a simplified method

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Abstract: Classical procedures for semen evaluation in breeding soundness examination require time and are costly. The evaluation techniques with CASA are limited due to the lack of standardization among laboratories and data analysis methods. Recent studies have contradictory conclusions on the actual predictive ability of this method alone regarding fertility. Finding a simple and objective test that can give the maximum correlation between in vitro and in vivo results has been the main focus of novel research. In this study, we aimed to develop a repeatable protocol of sperm adhesion test using oviductal explant (AOC) and the results were compared with computer-assisted semen analysis (CASA) parameters along with field fertility. After the developmental phase, AOC was applied to a group of endangered Burlina breed bulls in which effective field results are difficult to obtain due to the narrow range of the population. The AOC test was found to be proficient enough to provide a prediction on bull semen fertility. Counting the sperms adhered in three microscopic fields after a coincubation period of 20 min in phosphate buffered saline (PBS) can provide useful information on the field level of fertility. The AOC test gives additional information to complement the standard semen evaluation methods that can be applied to endangered breeds as well.

Key words: Computer-assisted semen analysis, Burlina bull, estimated relative conception rate, oviductal adhesion test, sperm quality, fertility

1. Introduction

Syngamy, the ultimate aim of the sperm, can be attained solely with the cooperation of many cells and through the synchronization system during the phases before and after fertilization [1,2]. Sperms are required to undergo significant remodeling events at the epididymis, cryopreservation, and in the female genital tract in order to be able to reach their fertilizing capacity sequentially. Just after artificial insemination (AI), sperms are then rapidly transported to the oviduct after trespassing the utero-tubal junction (UTJ) to create a sperm reservoir. At this stage, the ability of sperm to bind to the oviductal cells is determined by many factors such as lectin-like molecules, bovine seminal plasma proteins (BSP) [3–5], some polypeptides and glycoproteins secreted by oviductal epithelial cells [6–9]. More interestingly, just after the adhesion, the sperm pauses and waits for the ultimate hyperactivation goal, described by Yanagimachi [10] as a "whiplash-like beating pattern", which is a characteristic curve movement probably intercurrent by the sperm and the oviductal cells [11]. This step is highly critical for the zona pellucida (ZP) penetration [12]. This is why in vitro sperm physiological computer-assisted semen analysis CASA evaluation is considered for the expected fertility outcome.

The coincubation of sperms and explants of oviductal epithelium cells in order to calculate the number of spermatozoa adhered per unit area or adhesion index (AI) and a correlation between this index and field fertility have been reported [7]. Several regions of the oviduct (isthmus or ampulla) have been evaluated in different stages of the estrous cycle. Some of these variables (age, oviductal segment, the phase of the estrous cycle) have shown no effect on the sperm adhesion [7,13], while other studies have reported that the inclusion of the sperm is greater at the isthmus level than ampullae [14] and that the explant size may affect adhesion indices [15] and capacitation [16].

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Gualtieri and Talevi [17] have reported that: only acrosome intact sperms adhere to bovine oviductal epithelial cells; the acrosome of the sperm is preserved intact following binding; the release of sperm is probably due to changes in surface triggered by capacitation. Hereby, the adherent spermatozoa do not appear to be undergoing capacitation, are devoid of a cytoplasmic droplet, with better morphology, with low content of intracellular $Ca2^{+}$, and without an activated tyrosine phosphorylation process. The adhesion to the epithelium is certainly accepted as a means of sperm selection [13], which allows to foster and further guide the spermatozoa with good fertilization capacity. In vitro tests have shown that sperm capacitation can be facilitated by creating and optimizing a coculture of sperm and a monolayer of oviductal cells [18].

The importance of an in vitro test that can predict the fertility of bulls in relation to the nonreturn rates (NRR) has been described by De Pauw et al. [19]. The NRR defines the percentage of cows that do not come back into heat after 56 days from insemination and is vital for determining field fertility. Therefore, according to these necessities, a model to quantify the spermatozoa adherent oviductal epithelium through fluorescence microscopy has been developed and it had corroborated the hypothesis that the recovered sperm density can be used to predict the fertility of a bull in vivo. As mentioned above, the biochemical components present at the level of apical plasma membranes of the oviductal epithelial cell, able to modulate several useful steps to increase sperm fertilizing capacity, still need to be elucidated for validation.

The originality of this study is related to the introduction of a functional test to evaluate the quality of frozen-thawed sperm, identifying the limits of the classical test as kinetic parameters determined by CASA.

Therefore, our aim was to develop a simple and objective fertility test by counting the spermatozoa adhered to oviductal explant (AOC) and comparing it with CASA parameters and field fertility data in order to test its efficiency. Besides, the AOC test was proposed as a useful tool to predict the field fertility of an endangered breed, the Burlina bulls.

2. Materials and methods

The protocol was established in three phases. The first stage involved the identification of a simple and economically viable method to test the adhesion ability of bovine sperm to the explants of oviductal cells under an optical microscope. In the second phase, bull semen with proven field fertility was analyzed using the developed test and the results were compared to the parameters received by the National Italian Holstein Breeders (ANAFI). Within the third phase, the test was applied to the sperm obtained from an endangered breed, the Burlina breed bulls.

2.1. First phase

2.1.1. The collection and preparation of AOC

Oviducts used during every part of each phase were taken from a slaughterhouse, irrespective of the stage of the estrous cycle. Beef cattle and animals with apparent uterine pathologies (metritis, salpingitis) were excluded. After collection, the ovaries and relative oviducts were transported to the laboratory, where they were immersed in saline solution at room temperature (20–22 °C) within approximately 45 min.

The oviducts were classified depending on the ovarian stage and absence of anatomical abnormalities and isolated from the mesosalpinx under a biological hood. The surrounding tissues were dissected to reduce the risk of contamination by nonepithelial cells and have been engraved longitudinally with a scalpel blade (n. 11) to expose the mucosa. Explanted oviduct cells were collected from the isthmic part by cutting a mucosa portion of approximately 0.1 × 1 mm using a stereomicroscope (NIKON SA6000, Japan). Only explants with intact ciliated cylindric cellular lines were selected for testing. Portions of the mucous membrane were then placed on a glass slide and instilled with 50 µL of different medium types and incubation times in a controlled atmosphere (5% CO_2, 98% humidity) (Heraeus HERAcell, Kendro Laboratory Products, Hanau, Germany).

2.1.2. Experimental design and the preparation of stock solutions

The six protocols tested in the first phase are summarized as follows: PBS – 10 min; h-TALP – 10 min; PBS – 20 min; h-TALP – 20 min; PBS – 30 min; h-TALP – 30 min.

Phosphate buffered saline (PBS) from Sigma 10 g/L and h-TALP ("Tyrode's Albumin Lactate Pyruvate") were used as coculture mediums. The protocol for the preparation of 100 mL of solution was as follows: calcium chloride: 310 mg/L; HEPES (acid 4-2-hydroxyethyl-1-piperazinyl-ethanesulfonic): 2383 mg/L; magnesium chloride: 81.32 mg/L; potassium chloride: 230.95 mg/L; sodium bicarbonate: 2100 mg/L; sodium chloride: 5850 mg/L; L-sodium lactate (60%): 3.68 mL; sodium phosphate: 40.02 mg/L. To these, BSA (bovine serum albumin) were added: 300 mg, 1 mL sodium pyruvate, gentamicin 150 µg. All chemicals were Sigma-Aldrich compounds (Sigma-Aldrich Corp., St. Louis, MO, USA).

2.1.3. Semen assessment and evaluation

Frozen semen containing 14 million sperm in 0.5 mL straw was used. The thawing was carried out at 37 °C for 60 s and subsequently, the semen was divided into two aliquots of 0.25 mL and diluted (1:1) with the tested medium (PBS or h-TALP) maintained at 37 °C. Five µL of diluted semen containing approximately 35,000 motile sperms were dripped onto the slides of oviductal explant

cells. The slide was then incubated for the time period provided by the considered protocol. During this phase, ten semen samples were tested, each with six combinations of protocols. Subsequently, a reading of the slide using an optical microscope (Olympus CX41) and without any staining with a magnification of 400 × was performed. For counting, a readable field in which the explant cells of oviductal rectilinearly ran through the major diameter of the field was considered as visual, counting only motile sperm, still adhering to one side of the explant cell. Three optic fields were counted for each slide and the average of the three counts was recorded.

2.2. Second phase of the study

During the 2nd phase, the selection of straws was performed depending on the following collected data:

• CASA data (Hamilton-Thorne IVOS) of straws used for AI recorded as motility (MOT%), progressive motility (PROG, %), average path velocity (VAP, μm/s), VSL = straight line velocity (VSL, μm/s), straightness of track (STR, %), linearity of track (LIN, %), amplitude of the lateral head displacement (ALH, μm); curvilinear velocity (VCL, μm/s), and DANCE as VCL × ALH mean ($\mu m^2/s$).

The estimated relative conception rates (ERCR) are the bull effect on the nonreturn rate of inseminated cows expressed as the difference from the average of the percentage of general no return. This value is calculated using data derived from the functional evaluation (progeny test) available from ANAFI [20].

• ERCR index (estimated relative conceptions rates) with a value ranging from –5.00 to +5.00 for low and high fertile bulls;

• ERCR reliability index greater than 0.98;

The data were reported in a single Excel file (Microsoft Excel, version 14.6.7) and classified into the following classes of membership dependence index as ERCR1 (> 1.00), ERCR2 (–1/+1), and ERCR3 (< 1.00) for bulls with high, medium, and low fertility, respectively.

2.2.1. Adhesion tests on bulls with different classes of ERCR

Five Frisona breed bulls within each different class have been selected randomly. For each bull, three doses of semen belonging to the production lots that have contributed to the definition of ERCR were considered. Medium and incubation time were chosen depending on preliminary tests. Three doses of semen were considered and for each, after contact with three oviductal explants, the sperm adhering onto three microscopic fields were counted using the procedure in the preliminary phase (Figure 1).

2.3. Third phase adhesion tests on Burlina bulls frozen-thawed sperm

The 3rd phase considered the adhesion tests of Burlina bulls frozen-thawed sperm as an example of an endangered breed. Semen straws of eight endangered Burlina breed bulls included in the BIONET program of the RDP measure 214H WP1 were thawed and the same protocol of the Frisona bulls with ERCR was applied to evaluate their ability to adhere to oviductal cell explants. Each bull was tested 3 times using three different straws with a total of 9 tests for each bull. All Burlina bulls were selected as donors of straws because they reached the threshold of good quality classification of the semen both as fresh and postthawed semen.

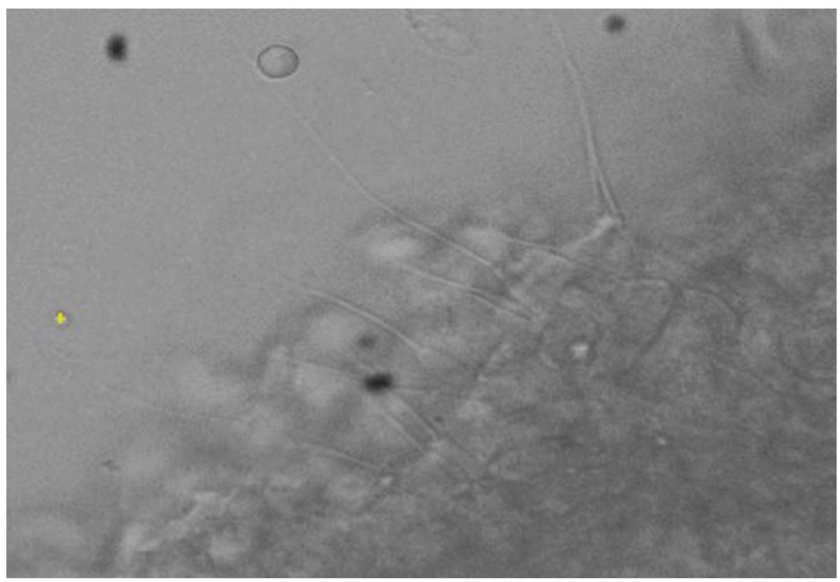

Figure 1. Bovine sperms adhered to oviductal cell explants.

2.4. Descriptive analysis and statistics

A very high level of variability was identified using 10 min of incubation. Therefore, only 20 min were considered as incubation time and PBS was considered as a medium because it was economically more sustainable. The data collected by the CASA system (MOT, PROG, VAP, VSL, ALH, LIN, and STR) associated with an individual of sperm sampling sessions were classified depending on ERCR index classes. Furthermore, the DANCE parameter was calculated in Excel by applying existing formulas from the literature [21]. The deviations from the average value were calculated in terms of the number of SD for all CASA and derived parameters. Both groups of bulls, with and without ERCR (Frisona and Burlina, respectively), were compared following the GLM procedure on the SigmaStat 2.03 software (Sigma-Aldrich Corp., St. Louis, MO, USA). ANOVA for repeated measures was applied considering the classes of ERCR index as an independent variable while the average number of adherent spermatozoa and all the CASA parameters as dependent variables. ANOVA for repeated measures was applied to compare the breeds (Frisona vs. Burlina) in terms of the number of adhered sperm considering them independent and dependent variables, respectively. $P < 0.05$ was considered the threshold of statistical significance.

3. Results

The analysis revealed that only 10 min of incubation period produced a wide variability of results both with the use of PBS and h-TALP. The use of 20 or 30 min incubation periods guaranteed mostly reliable results using the two different mediums. The values of the kinetic parameters of the three groups with different ERCR are reported in Table 1 and Figure 2. The analytical approach that took into account the number of SD from the average value identified as class 1 of ERCR has a greater positive deviation than the other two classes with regard to MOT (%) and PROG (%) parameters. Low fertility bulls (class 3) had the highest positive deviations from the mean for the parameters of VAP, VSL, STR, VCL, and DANCE (Figure 3). The number of adhered sperms was significantly different ($P < 0.001$) among the three groups (Table 1 and Figure 4). The relative standard deviations (RSD) were 8.92%, 8.04%, and 13.29% for class 1, 2, and 3, respectively.

The results of the adhesion test applied to eight Burlina bulls and run on three doses of semen for each bull are shown in Figure 5. The mean number of adhered sperm was 15.21 ± 1.14 for the Burlina breed. When the results of the adhesion test in the Burlina breed were compared with the data available for the Frisona breed, the adhesion levels of the cryopreserved sperm in this endangered species were between medium and low fertility classification.

4. Discussion

The techniques used in this study were considering the functional aspects that take place at the oviductal level and greatly influence the process of fertilization. In this study, in order to find a repeatable, easily implementable, and economically sustainable sperm adhesion test, a simple light microscope was used instead of the fluorescence one. The choice of oviductal explant use was made with the aim of maintaining the oviduct morphological characteristics as

Table 1. Values (mean ± SD) of the kinetic parameters (CASA) and oviductal cell adhesion test for 3 groups of Frisona bulls ERCR indexed.

	High ERCR	Medium ERCR	Low ERCR
Selected straws	11/1095	27/3511	8/659
MOT (%)	87.10 ± 2.74	84.69 ± 7.15	84.10 ± 5.09
PROG (%)	59.49 ± 4.07	58.44 ± 6.17	59.11 ± 3.63
VAP (μm/s)	85.63 ± 11.44	89.07 ± 9.51	90.99 ± 6.75
VSL (μm/s)	67.64 ± 6.47	70.96 ± 7.10	71.62 ± 5.58
STR (%)	73.53 ± 4.20	74.57 ± 6.64	74.83 ± 5.90
LIN (%)	44.59 ± 2.93	44.93 ± 4.49	45.27 ± 4.43
ALH (μm)	6.34 ± 0.71	6.24 ± 0.76	6.36 ± 0.53
VCL (μm/s)	152.23 ± 17.25	158.13 ± 9.02	159.10 ± 14.98
DANCE	991.23 ± 188.62	991.13 ± 153.37	1014.48 ± 145.08
Adhesion test (number of adhered sperm)	26.22 ± 2.34 *	17.91 ± 1.44**	12.64 ± 1.68**

MOT = motility; PROG = progressive motility; VAP = average path velocity; VSL = straight line velocity; STR = straightness of track; LIN= Linearity of track; ALH = amplitude of the lateral head displacement; VCL = curvilinear velocity; ERCR (estimated relative conceptions rates).

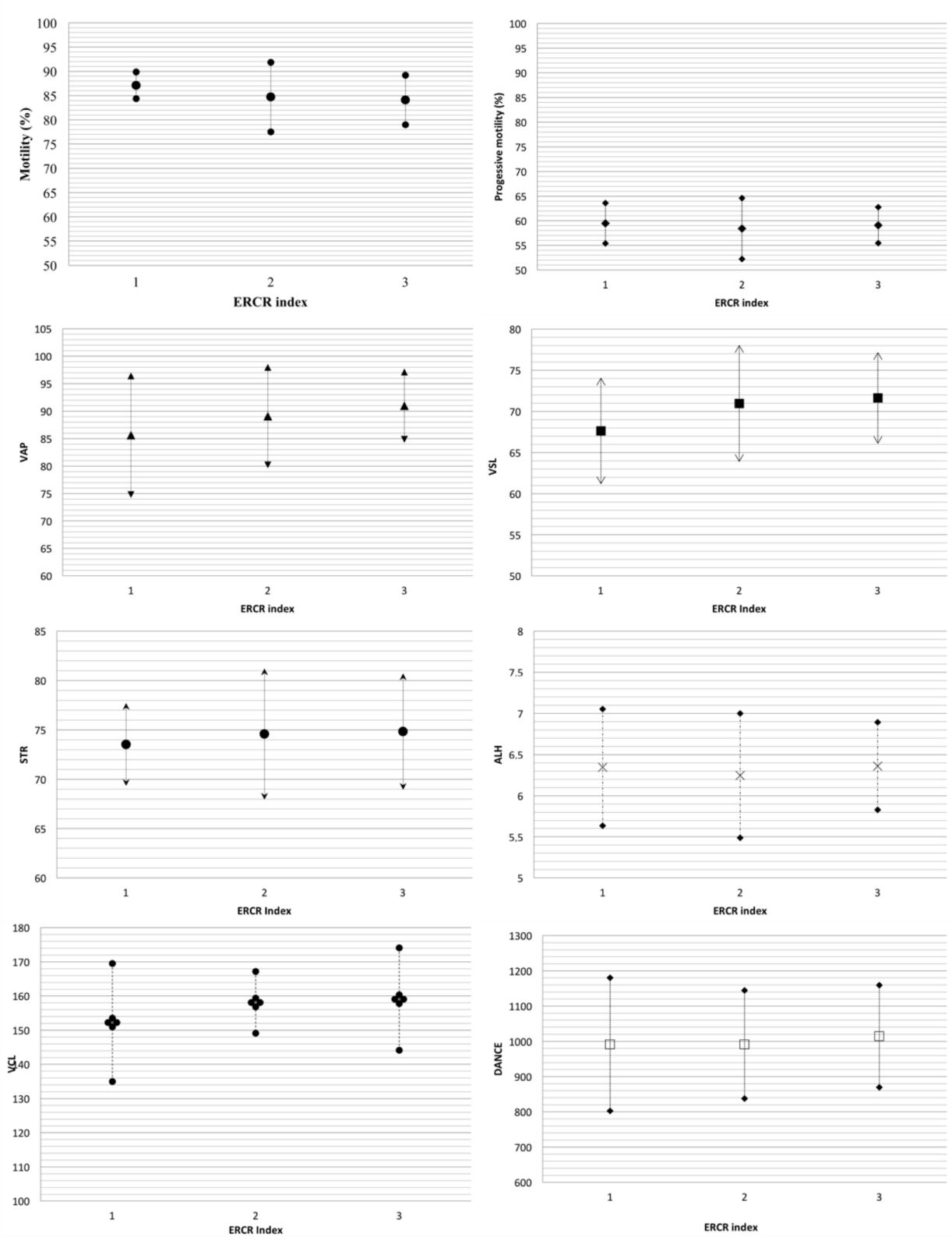

Figure 2. CASA parameter values of 3 groups of bulls with ERCR classification. VAP = average path velocity; VSL = straight line velocity; STR = straightness of track; ALH = amplitude of the lateral head displacement; VCL = curvilinear velocity; ERCR (estimated relative conceptions rates): 1 – high; 2 – medium; 3 – low.

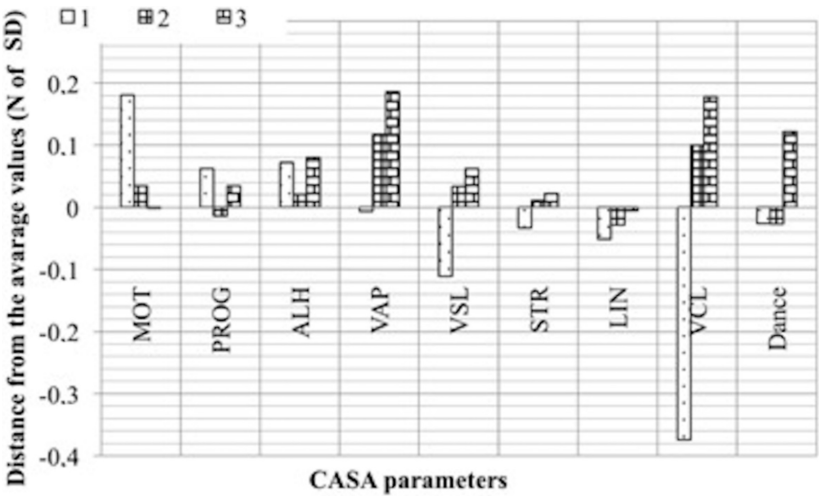

Figure 3. Distance from the average values (number of standard deviations) of the CASA parameters divided for the three classes of ERCR. MOT = motility; PROG = progressive motility; ALH = amplitude of the lateral head displacement; VAP = average path velocity; VSL = straight line velocity; STR = straightness of track; LIN = linearity of track; VCL = curvilinear velocity. ERCR (estimated relative conceptions rates): 1 – high; 2 – medium; 3 – low.

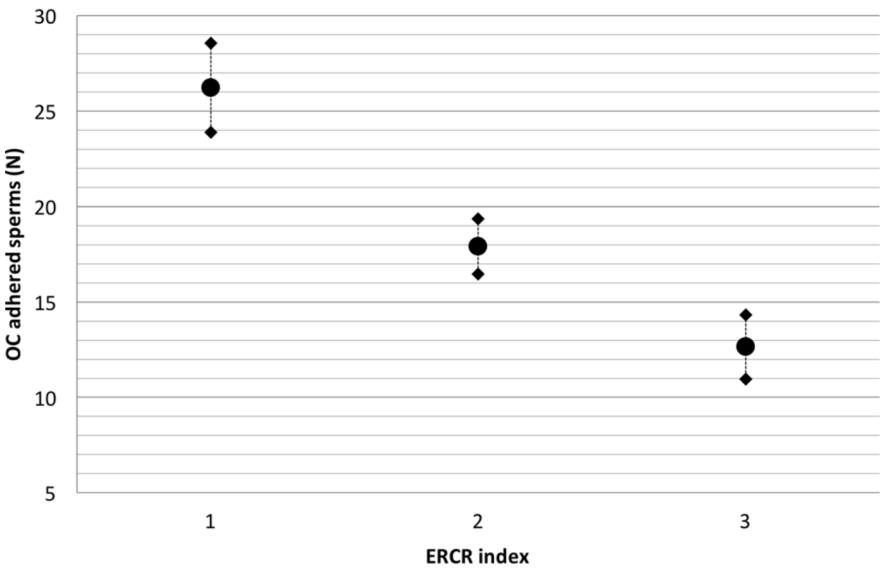

Figure 4. Values (mean ± SD) of adhered sperms to oviductal cells explant in three different classes of ERCR index. OC: oviductal cell; ERCR (estimated relative conceptions rates): 1 – high; 2 – medium; 3 – low.

much as possible to mirror the conditions that arise in vivo. It was also demonstrated that there is a higher density of binding of the sperm using the explant rather than the cell monolayer [19]. Other researchers indicated that the binding of sperm isthmic epithelium is fundamental to maintain motility and for sperm to reach the ampulla [18]. Secretions from the oviductal cells vary in type and quantity in different sections as well as in different estrous periods [17]. Analyzing the adhesion of the sperm at this level could give the possibility to assess the fertilizing capacity of semen more realistically. If the hyperactivated sperm population is high, these cells would presumably be hypoviable due to the loss in energy reserves. Therefore, it is interesting to correlate the ERCR with parameters such as hyperactivity. Estrogen and progesterone regulation processes have a major role in the adhesion and release

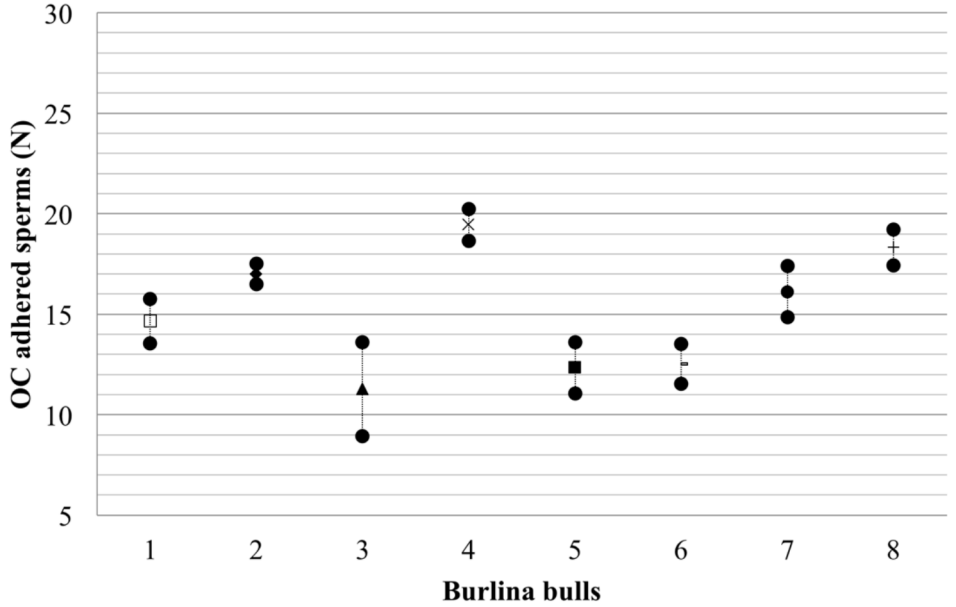

Figure 5. Values (mean ± SD) of adhered sperms in 8 endangered Burlina breed bulls. OC: oviductal cell.

of sperm at the oviductal level as well as capacitation and hyperactivation [22]. We tried to standardize the tests using a single medium instead of different media for sperm and oviduct as carried out in previous studies [15]. During the processing of the protocol for our test, the use of PBS or h-TALP did not interfere with the results. De Pauw et al. [19] corroborated the use of h-TALP compared to other mediums (such as IVF-TALP and TCM-199) because it allows obtaining a greater conservation of sperm viability over time. The use of PBS both for sperm and oviduct has been reported in different studies and they concluded that PBS does not cause variations in the results compared to TALP [23,24]. Based on the literature and also our test results it was decided to consider only the use of PBS, which is much simpler to prepare, more easily storable, and cost-effective. Studies by Petrunkina [7] and Apichela et al. [15] showed that smaller explant surface oviductal taken into consideration (< 10,000 μm^2) can give a better vision of the sperm's ability to adhere to the epithelium and decrease the risk of errors during the evaluation. In this work, it was decided to withdraw about 0.1 × 1 mm (0.1 mm^2) of mucosa through the use of a stereomicroscope. Fundamental to standardize observations eligible for light microscopy is rather defining the explant extension or to better determine a standardized area unit. In this study, we choose the microscopic field as a measure, respecting the objective of our work that is to create a practical adhesion test easily applicable without the use of expensive equipment, capable of measure the explant. The literature regarding the counting of the adherent spermatozoa shows the need to define the area of explant [15] and to calculate the adhesion by using a fluorescence intravital staining [19]. De Pauw et al. [19] showed that 30 min of incubation of the coculture is sufficient to evaluate the adhesion. In our study, we confirmed that 20 and 30 min give similar results in terms of adhesion. The CASA parameters in our study did not differ from those reported in the literature [23,25] for the bovine species. The CASA system represents a good tool to compare the quality of fresh and frozen semen [26] but its efficiency in predicting the capacity to fertilize the field is still unclear [25,27]. We found a lack of variation between the CASA data depending on the ERCR index. The hypothesis to justify this point is the inability of CASA to assess hyperactivity (HA) with standards derived from the instrument parameters. There are some developed formulas for the fertility estimation of the HA values with CASA [21,28]. To date, the literature reports that hyperactivation is distinguishable through increased VCL and ALH parameters with a corresponding decrease of LIN. There is also a derived value from basic parameters defined as DANCE (= VCL × ALH) that seems to be more linked to the HA level [21,29]. Analyzing the data in our work and specifically those related to the DANCE derived parameter, it is possible to assume that there is a higher degree of HA in bulls with low fertility, which would justify the absence of significant difference among ERCR index classes in terms of CASA parameters.

The adhesion test represents a functional test because it excludes hyperactive sperms unable to adhere to the oviductal cells. The adhesion test differed among the ERCR classes and individuals. The RSDs of the three classes were acceptable enough for the number of samples considered and future assessments could increase the level of repeatability. Puglisi et al. [27] have stated that membrane

integrity was found to be the most closely related parameter to fertility than conventional analysis made on the semen. The advantage of this test is the discrimination of infertile spermatozoa that without intact acrosome cannot adhere to AOC as a new selection criterion [13,30]. The sperm that bind to oviductal explants are characterized by a noncapacitated, intact acrosome, good morphology, and normal chromatin structure. It has also been shown that the proportion of noncapacitated sperm present in the frozen semen is positively correlated with fertility [19]. During the third phase of the study, the test was applied to Burlina breed bulls, for which no data regarding fertility are available due to the small breed population. According to the results, eight bulls have an average of similar adhesion between them and when applying the test to the three doses of semen for each bull there is considerable repetition. Comparing these data with those relating to Frisona breed bulls, it is evident that none of them reach the values of adhered sperms similar to those of the highest ERCR class and many have values similar to those of a lower fertility class. The reason for the different behavior of the Burlina breed semen can only be assumed and should be correlated with field data for evaluation of the actual bulls' fertility. Among the hypotheses that can be considered are different responses of sperm to the specific breed adhesion test on oviductal cell explants, reduced fertility of the breed, and lower resistance to the manipulation/preservation of the semen. The developed protocol has proven its worth and repeatability, even at this stage. The benefits of the protocol are ease of execution, low cost, and fewer consumables and basic equipment (not requiring the use of either fluorescent microscope or dyes) than other adhesion test protocols. Our study has confirmed that the number of sperm bound to oviductal explants is a sensitive and reproducible parameter, like others [7].

Although establishing a relationship between fertility and kinetic parameters is needed, it is important to associate other tools to increase the successful evaluation of semen quality. The adhesion test in its conceptual simplicity and implementation is found to be closely related to the field of fertility, showing more significant data than some CASA parameters and highly repeatable parameters. Although further studies should be conducted to ascertain the link between this test and the fertility of the semen, an emphasis should be placed on hyperactivity as it is the key for comparison between the methods. Sperm adhesion to oviductal cells could be included in the BSE protocols as a collateral and functional test.

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References

1. Holt WV, Van Look KJW. Concepts in sperm heterogeneity, sperm selection and sperm competition as biological foundations for laboratory test of semen quality. Reproduction 2004;127(5): 527-535. doi: 10.1530/rep.1.00134
2. Varner DD. Odyssey of the spermatozoon. Asian Journal of Andrology 2015; 17 (4): 522-528. doi: 10.4103/1008-682X.153544
3. Druart X. Sperm interaction with the female reproductive tract. Reproduction in Domestic Animals 2012;47 :348-352. doi: 10.1111/j.1439-0531.2012.02097.x.
4. Juyena NS, Stelletta C. Seminal plasma: An essential attribute to spermatozoa. Journal of Andrology 2012;33(4): 536-551. doi: 10.2164/jandrol.110.012583
5. Gwathmey TM, Ignotz GG, Mueller JL, Manjunath P, Suarez SS. Bovine seminal plasma proteins PDC-109, BSP-A3, and BSP-30-kDa share functional roles in storing sperm in the Oviduct1. Biology of Reproduction 2006;75(4): 501-507. doi: 10.1095/biolreprod.106.053306
6. Yanagimachi R. Fertility of mammalian spermatozoa: Its development and relativity. Zygote 1994; 2(4): 371-372. doi: 10.1017/S0967199400002240
7. Petrunkina AM, Gehlhaar R, Drommer W, Waberski D, Töpfer-Petersen E. Selective sperm binding to pig oviductal epithelium in vitro. Reproduction 2001; 121(6): 889-896. doi: 10.1530/rep.0.1210889
8. Coy P, García-Vázquez FA, Visconti PE, Avilés M. Roles of the oviduct in mammalian fertilization. Reproduction 2012; 144(6): 649-660. doi: 10.1530/REP-12-0279
9. Liu M. Capacitation-associated glycocomponents of mammalian sperm. Reproductive Sciences 2016; 23(5): 572-594. doi: 10.1177/1933719115602760
10. Yanagimachi R. The movement of golden hamster spermatozoa before and after capacitation. Reproduction, Fertility and Development 1970; 23(1): 193-196. doi: 10.1530/jrf.0.0230193
11. Suarez SS, Pacey AA. Sperm transport in the female reproductive tract. Human Reproduction Update 2006; 12(1): 23-37.
12. Suarez SS. Control of hyperactivation in sperm. Human Reproduction Update 2008; 14(6): 647-657. doi: 10.1093/humupd/dmn029
13. Apichela SA, Stelletta C. Prove in vitro di adesione degli spermatozoi e il loro valore per stimare la fertilità negli animali da reddito. Large Animal Review 2012; 18(1): 7-11.

14. Bosch P, Wright RW. The oviductal sperm reservoir in domestic mammals. Archivos de Medicina Veterinaria 2005; 37(2): 95-104. doi: 10.4067/S0301-732X2005000200002

15. Apichela S, Jiménez-Díaz MA, Roldan-Olarte M, Valz-Gianinet JN, Miceli DC. In vivo and in vitro sperm interaction with oviductal epithelial cells of llama. Reproductionin Domestic Animals 2009; 44(6): 943-951. doi: 10.1111/j.1439-0531.2008.01125.x

16. Lefebvre R, Suarez SS. Effect of capacitation on bull sperm binding to homologous oviductal epithelium1. Biology of Reproduction 1996; 54(3): 575-582. doi: 10.1095/biolreprod54.3.575

17. Gualtieri R, Talevi R. In vitro-cultured bovine oviductal cells bind acrosome-intact sperm and retain this ability upon sperm release. Biology of Reproduction 2000; 62(6): 1754-1762. doi: 10.1095/biolreprod62.6.1754

18. Abe H, Hoshi H. Bovine oviductal epithelial cells: Their cell culture and applications in studies for reproductive biology. Cytotechnology 1997; 23(1-3): 171-183. doi: 10.1023/A:1007929826186

19. De Pauw IMC, Van Soom A, Laevens H, Verberckmoes S, de Kruif A. Sperm binding to epithelial oviduct explants in bulls with different nonreturn rates investigated with a new in vitro model. Biology of Reproduction 2002; 67(4): 1073-1079. doi: 10.1095/biolreprod67.4.1073

20. Gaviraghi A, Deriu F, Soggiu A, Galli A, Bonacina C, Bonizzi L et al. Proteomics to investigate fertility in bulls. Veterinary Research Communications 2010;34 (1), 33-36.doi: 10.1007/s11259-010-9387-0

21. Mortimer ST. A critical review of the physiological importance and analysis of sperm movement in mammals. Human Reproduction Update 1997; 3(5): 403-439. doi: 10.1093/humupd/3.5.403

22. Cerny KL, Garrett E, Walton AJ, Anderson LH, Bridges PJ. A transcriptomal analysis of bovine oviductal epithelial cells collected during the follicular phase versus the luteal phase of the estrous cycle. Reproductive Biology and Endocrinology 2015; 13(1): 84. doi: 10.1186/s12958-015-0077-1

23. Farrell P, Presicce G, Brockett C, Foote RH. Quantification of bull sperm characteristics measured by computer-assisted sperm analysis (CASA) and the relationship to fertility. Theriogenology 1998; 49(4): 871-879. doi: 10.1016/s0093-691x(98)00036-3

24. Lefebvre R, Chenoweth PJ, Drost M, LeClear CT, MacCubbin Met al. Characterization of the oviductal sperm reservoir in cattle. Biology of Reproduction 1995; 53(5): 1066-1074. doi: 10.1095/biolreprod53.5.1066

25. Kathiravan P, Kalatharan J, Karthikeya G, Rengarajan K, Kadirvel G. Objective sperm motion analysis to assess dairy bull fertility using computer-aided system - a review. Reproduction in Domestic Animals 2011; 46(1): 165-172. doi: 10.1111/j.1439-0531.2010.01603.x

26. Defoin L, Granados A, Donnay I. Analysing motility parameters on fresh bull semen could help to predict resistance to freezing: A preliminary study. Reproduction in Domestic Animals 2008; 43(5): 606-611. doi: 10.1111/j.1439-0531.2007.00964.x

27. Puglisi R, Pozzi A, Foglio L, Spanò M, Eleuteri Pet al. The usefulness of combining traditional sperm assessments with in vitro heterospermic insemination to identify bulls of low fertility as estimated in vivo. Animal Reproduction Science 2012; 132(1-2): 17-28. doi: 10.1016/j.anireprosci.2012.04.006

28. Mortimer ST, Van Der Horst G, Mortimer D. The future of computer-aided sperm analysis. Asian Journal of Andrology 2015; 17(4): 545-553. doi: 10.4103/1008-682X.154312

29. Holt WV, O'Brien J, Abaigar T. Applications and interpretation of computer-assisted sperm analyses and sperm sorting methods in assisted breeding and comparative research. Reproduction, Fertility and Development. 2007; 19(6): 709-718. doi: 10.1071/RD07037

30. Talevi R, Gualtieri R. Sulfated glycoconjugates are powerful modulators of bovine sperm adhesion and release from the oviductal epithelium in vitro. Biology of Reproduction 2001; 64(2): 491-498. doi: 10.1095/biolreprod64.2.491