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Utilisation of sperm-binding assay combined with computer-assisted sperm analysis to evaluate frozen-thawed bull semen

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Summary

Due to homologies between the chicken egg perivitelline membrane with mammalian zona pellucida proteins, spermatozoa of several species are able to bind to this membrane. However, adequate standardisation is required to attest possible applications of this technique for semen evaluation of a given species. Therefore, we thawed and divided cryopreserved semen samples into two aliquotes, one kept in water bath at 37 °C (thawed) and the other submitted to snap-freezing to damage sperm cells (dead spermatozoa). Aliquotes were mixed into different ratios of thawed:dead cells and analysed for motility, membrane and acrosomal integrity, and mitochondrial activity. In parallel, chicken egg perivitelline membranes were inseminated with these ratios, and the number of spermatozoa bound per mm^2 of membrane was assessed by conventional microscopy (CM) and computer-assisted sperm analysis (CASA). Linear regression showed high correlation between thawed:dead sperm ratio and number of spermatozoa bound to the membrane (CM: $r^2 = 0.91$ and CASA: $r^2 = 0.92$ respectively). Additionally, positive correlations were found between the number of spermatozoa bound to the membrane and acrosomal integrity, membrane integrity, mitochondrial activity and motility. These findings indicate that sperm-egg-binding assay associated with CASA is a reliable, practical and inexpensive method for examining the fertilising capacity of cryopreserved bull semen.

Introduction

Assisted reproduction techniques such as artificial insemination (AI), *in vitro* fertilisation (IVF) and intracytoplasmic sperm injection (ICSI) are essential for programmes aiming to preserve endangered species, to propagate genetic material of high value animals and to treat infertility in human medicine (Lasley *et al.*, 1994; Pellicer *et al.*, 1995; Galli & Lazzari, 1996). However, their outcome is highly dependent on sperm quality, which can be impaired due to semen cryopreservation, a key process for the application of these techniques (Hammerstedt *et al.*, 1990; Aitken, 2006).

Conventional semen analysis (e.g. sperm motility, vigour, concentration and morphology) has been routinely used to predict the probability of generating pregnancy with acceptable results (Bonde *et al.*, 1998). However, limitations of this analysis are also known, especially in cases of male idiopathic infertility, due to its inaptitude to detect some functional abnormalities responsible for impaired sperm capacity (Saleh *et al.*, 2003; Aitken, 2006). In this regard, sperm functional assays are extremely useful because they are capable of evaluating the functionality of distinct cellular structures including plasma membrane (Jeyendran *et al.*, 1984), acrosome (Pope *et al.*, 1991), mitochondria (Gravance *et al.*, 2000) and DNA (Sakkas & Alvarez, 2010). These techniques are extremely useful to detect probable causes of unexplained infertility (Aitken, 2006). A prominent example of such approach is the interaction between spermatozoa and zona pellucida, which appears to predict with considerable accuracy their capacity to fertilise oocytes (Bhattacharyya & Kanjilal, 2003).

Notwithstanding, tests using spermatozoa–zona pellucida interaction can be strongly influenced by factors as *in vitro* conditions or oocyte variability (Graczykowski *et al.*, 1998). Therefore, alternative methods aiming to solely evaluate the sperm binding, not considering further embryo development, are very appealing for the routine of reproductive laboratories. Sperm binding to homologous hemizona represents one of these optional diagnostic tools (Burkman *et al.*, 1988). Nevertheless, depending on the species, homologous zona pellucida assays exhibit logistical difficulties concerning oocyte availability and excessive labour (Criscuolo *et al.*, 2010). Another option is the zone-free hamster egg penetration test, in which the zona pellucida of hamster oocytes is removed enabling spermatozoa of various species to penetrate the oocyte. Although this technique allows us to assess the penetration capacity of the spermatozoa, the collection of hamster oocytes also is extremely laborious and raises ethical questions around euthanasia (Yanagimachi, 1984; Kamiguchi & Mikamo, 1986).

The chicken egg perivitelline membrane (EPM) displays homology to the mammalian ZP3, which enables the binding of spermatozoa from several mammalian species (Waclawek *et al.*, 1998; Bausek *et al.*, 2000). Consequently, sperm-egg-binding tests using EPM have been used for poultry, mice, horses, bulls, rams, pigs and humans (Robertson *et al.*, 1997; Barbato *et al.*, 1998; Waclawek *et al.*, 1998; Amann *et al.*, 1999; Corcini *et al.*, 2012). This technique is relatively simple and requires inexpensive equipment (Criscuolo *et al.*, 2010) and if it is combined with a computer-assisted analysis and DNA-specific dye (Hoechst bisbenzimide) would provide a more reliable and fast analysis, requiring only a few seconds to evaluate a given sample without the possible interference of detritus from the membrane itself. Thus, the objective of the present study was to standardise the sperm-egg-binding assay combined with computer-assisted sperm analysis (CASA) using chicken egg perivitelline membrane on bovine cryopreserved semen as an practical alternative to functionally assess indirectly bovine sperm fecundity ability.

Material and methods

The sperm-binding assay in perivitelline membrane of chicken eggs was performed on six batches of bull cryopreserved semen according to the technique used by Criscuolo *et al.* (2010) in human semen. However, due to semen peculiarities of each species, standardisation was required to test efficacy of this technique using bull semen. Unless otherwise stated, all chemicals were purchased from Sigma Chemical® (St. Louis, MO, USA).

Thawing and sperm processing

Cryopreserved semen samples (egg yolk–tris–glycerol extender) were thawed at 37 °C in a water bath, and sperm concentration was measured in a Neubauer chamber. Samples were then diluted to a final concentration of 5×10^6 spermatozoa per ml using a medium for *in vitro* fertilisation at 37 °C (Modified TALP medium; 100 mM NaCl, 3.1 mM KCl, 25 mM $NaHCO_3$, 0.3 mM NaH_2PO_4, 21.6 mM lactate, 2 mM $CaCl_2$, 0.4 mM $MgCl_2$, 10 mM HEPES, 1 mM pyruvate, 6 mg ml^{-1} bovine serum albumin and 50 μg ml^{-1} gentamycin; (Parrish *et al.*, 1988). Immediately after dilution, samples were divided into two aliquots: one kept in a water bath (37 °C; *'thawed'*) and another submitted to snap-freezing to induce cell injury (immersed in liquid nitrogen and thawed in a water bath at 37 °C for three times: *'dead cells'*). Afterwards, these aliquots (thawed and dead spermatozoa respectively) were mixed in proportions of 0%, 25%, 50%, 75% and 100% (thawed/dead sperm ratio). From these proportions, the sperm-binding assay, computerised analysis of motility and function tests were performed.

Egg perivitelline membrane (EPM) processing and sperm-egg-binding assay

Egg perivitelline membrane was isolated from the yolks of freshly laid, unfertilised hen's eggs and washed with PBS until the membrane and PBS became translucent. Segments of the EPM (0.5 cm^2 each) were then transferred to a plate containing wells and incubated with 25 000 spermatozoa diluted in 250 μl of modified TALP medium. This incubation was performed at 37 °C for 1 h (Amann *et al.*, 1999). After incubation, the membranes were washed in macrocentrifuge tubes containing 10 ml of PBS to remove unbound spermatozoa. Membranes were then placed on a glass slide, overlaid with a cover slip and observed under CASA or phase contrast microscope (400× magnification).

To perform the evaluation of the number of spermatozoa bound to the EPM using the CASA, perivitelline membranes were stretched on microscope slides and submitted to the fluorescent probe Ident® (Hamilton Thorne Biosciences, Beverly, MA, USA) according to the manufacturer's instructions. In brief, the stain was diluted in 500 μl of BWW (Biggers–Whitten–Whittingham) medium and mixed vigourously using a vortex. Fifty microlitre of the solution was added to the membrane and incubated for 7 min. This staining was used to avoid the influence of the membrane on sperm counting. After this period, a cover slip was placed over the membrane and this slide was evaluated using the CASA (Hamilton-Thorne, Ivos 12.3, USA) according to manufacturer's rec-

ommended setup for the Ident® stain (number of frames – 30; frames per second – 60 Hz; minimum contrast – 20; minimum cell size – 3 pix; static intensity gates 0.40–2.00; static size gates 0.25–5.00; static elongation gates 20–100). Spermatozoa bound to the EPM were counted in 3–5 fields, and the results were expressed in spermatozoa bound per mm^2 of membrane. The same procedure was performed in the conventional microscope (CM), where the number of spermatozoa bound to the membrane was evaluated in a 400× magnification (usually in three fields) using a phase contrast microscope (Nikon® E200, Nikon, Tokyo, Japan). Results were also expressed in number of spermatozoa bound per mm^2 of membrane.

Computer-assisted sperm analysis

Ratios of thawed and dead spermatozoa were submitted to motility analysis by computer-assisted sperm analysis (CASA, Hamilton-Thorne, Ivos 12.3, USA). The following variables were examined: motile, progressive motile, VAP (average path velocity, μm s^{-1}), VSL (straight-line velocity, μm s^{-1}), VCL (curvilinear velocity, μm s^{-1}) ALH (amplitude of lateral head displacement, μm), BCF (beat cross-frequency, Hz) STR (straightness, %), LIN (linearity,%) and the percentage of spermatozoa rapid (%), medium (%), slow (%) and static (%) (Goovaerts *et al.*, 2006).

Functional tests

Subsequently to CASA analysis, ratios of thawed/dead spermatozoa were evaluated for mitochondrial activity and integrity of the plasma and acrosome membranes. Integrity of the plasma membrane was investigated by the eosin–nigrosin staining (Barth & Oko, 1989). To perform the technique, 5 μl of eosin–nigrosin stain was mixed with 5 μl of semen on a microscope slide and smeared using another slide. Two hundred cells were counted in a CM (1000×) and classified as intact and damaged.

Acrosomal integrity was assessed using fast-green/bengal-rose staining (Pope *et al.*, 1991), which was performed by mixing 5 μl of stain with 5 μl of semen on a microscope slide. After 60 s, this mixture was smeared on the glass slide, and two hundred cells were counted in a CM (1000×) and classified as intact or injured acrosomes. The mitochondrial activity was analysed using 3.3′ diaminobenzidine (DAB) staining (Hrudka, 1987). An aliquot of each sample was incubated with DAB in an amber microcentrifuge (1:1) for 1 h at 37 °C. After incubation, the mixture was smeared in microscopy slides and fixed in 10% formalin for 10 min. Slides were then examined under phase contrast microscope (1000×), where two hundred cells were counted and classified in four different classes: *Class I* with 100% of the mid-piece stained indicating full mitochondrial activity; *Class II* with more than 50% of the mid-piece stained indicating medium activity; *Class III* with less than 50% of the mid-piece stained indicating low activity; and *Class IV* with the absence of staining in the mid-piece indicating no mitochondrial activity.

Statistical analysis

All data were evaluated using SAS System for Windows (SAS Institute Inc., Cary, NC, USA). The effect of proportion of thawed/dead cells (0%, 25%, 50%, 75% and 100%) was determined using parametric (general linear model procedure (PROC GLM)) and nonparametric (Wilcoxon) tests, according to the residue normality (Gaussian distribution) and variance homogeneity of each variable. A probability value of $P < 0.05$ was considered statistically significant. Results are reported as untransformed means ± SEM. Pearson correlation was used to calculate the relationship between variables studied in each temperature group.

Results

Increases in the number of spermatozoa bound to the EPM followed rises in the ratio of thawed/dead spermatozoa in both CASA (0%: 1.43 ± 0.35; 25%: 3.53 ± 0.67; 50%: 7.50 ± 1.14; 75%: 14.50 ± 1.50; and 100%: 21.25 ± 2.25) and CM analysis (0%: 1.07 ± 1.0; 25%: 6.79 ± 3.02; 50%: 21.13 ± 5.97; 75%: 64.49 ± 1.96; 100%: 100.14 ± 6.12; Table 1). In fact, linear regression coefficients showed a consistent relationship between these two parameters in both techniques (Fig. 1).

Similarly, membrane and acrosome integrities increased according to raises in the proportion of thawed/dead spermatozoa (Table 1), circumstance also observed in the percentage of spermatozoa showing full mitochondrial potential. On the other hand, percentages of spermatozoa showing medium, low and absence of mitochondrial activity (DAB II, DAB III and DAB IV respectively) decreased with the increment in thawed/dead spermatozoa (Table 1). As expected, percentages of spermatozoa showing total and progressive motilities increased whenever ratios of thawed/dead spermatozoa were increased, and the opposite occurred with the percentage of static spermatozoa.

Positive correlation was found regarding CASA and CM analysis of the number of spermatozoa bound to EPM (0.962, $P < 0.0001$). Moreover, both techniques were positively correlated with the plasma membrane integrity (CASA – 0.845, $P < 0.0001$ and CM 0.846, $P < 0.0001$, Table 2), acrosome integrity (CASA 0.846,

Table 1 Relationship among thawed/dead sperm ratios and the following variables: number of spermatozoa bound to EPM evaluated by CASA (Spermatozoa per mm^2 – CASA) and CM (Sperm per mm^2 – CM), plasma membrane integrity (IM), integrity acrosomal (IA), mitochondrial activity (DAB I, II, III and IV), total motility (motile), progressive motility (progressive) and statics spermatozoa (Static)

	Ratio (thawed/dead spermatozoa)				
	0%	25%	50%	75%	100%
Spermatozoa per mm^2 (CASA)	1.43 ± 0.35^{d}	3.53 ± 0.67^{d}	7.50 ± 1.14^{c}	14.50 ± 1.50^{b}	21.25 ± 2.25^{a}
Spermatozoa per mm^2 (CM)	1.07 ± 1.01^{d}	6.79 ± 3.02^{d}	21.13 ± 5.97^{c}	64.49 ± 1.96^{b}	100.14 ± 6.12^{a}
Intact membrane (%; IM)	0.17 ± 0.17^{d}	9.00 ± 1.32^{d}	23.83 ± 3.18^{c}	34.33 ± 3.57^{b}	46.00 ± 4.78^{a}
Intact acrosome (%; IM)	27.50 ± 4.37^{e}	44.33 ± 4.73^{d}	59.17 ± 3.54^{c}	72.67 ± 2.69^{b}	85.67 ± 2.25^{a}
DABI (%)	9.33 ± 5.11^{c}	19.50 ± 5.12^{c}	36.50 ± 5.31^{b}	46.67 ± 6.58^{b}	66.50 ± 6.00^{a}
DABII (%)	40.67 ± 2.60^{a}	38.67 ± 2.65^{a}	42.00 ± 3.62^{a}	34.17 ± 3.79^{ab}	25.83 ± 4.06^{b}
DABIII (%)	33.50 ± 4.86^{a}	27.50 ± 4.10^{a}	15.00 ± 3.99^{b}	14.00 ± 2.18^{b}	4.83 ± 1.19^{b}
DABIV (%)	16.17 ± 2.77^{a}	14.50 ± 3.19^{a}	6.50 ± 1.38^{b}	5.17 ± 1.14^{b}	2.83 ± 1.01^{b}
Motile (%)	0.00 ± 0.00^{c}	1.50 ± 0.43^{c}	3.00 ± 0.26^{bc}	6.00 ± 0.89^{b}	16.00 ± 2.18^{a}
Progressive (%)	0.00 ± 0.00^{c}	0.17 ± 0.17^{c}	1.20 ± 0.49^{bc}	4.20 ± 1.11^{b}	8.83 ± 2.10^{a}
Static (%)	99.33 ± 0.67^{a}	94.67 ± 1.98^{ab}	83.80 ± 5.56^{ab}	79.40 ± 3.88^{b}	60.00 ± 10.29^{c}

Different superscripts in the same line indicate significant differences between the thawed/dead sperm ratios ($P < 0.05$).

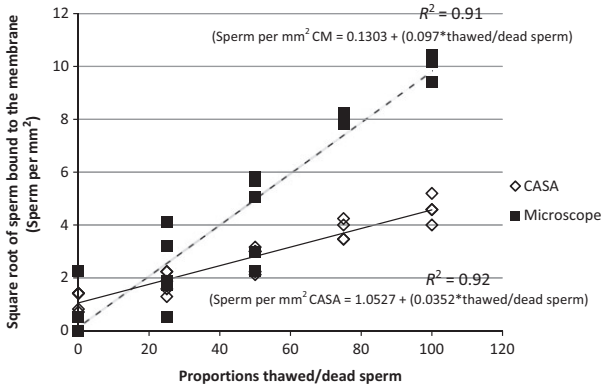

Fig. 1 Linear regression between thawed/dead sperm ratios with CASA and microscope analysis of the number of spermatozoa bound per mm^2 of EPM.

$P < 0.0001$; CM 0.788, $P < 0.0001$, Table 2) and DAB I (CASA: 0.759, $P < 0.0001$; microscope: 0.768, $P < 0.001$, Table 2). Additionally, we demonstrated robust correlations between the number of spermatozoa bound to EPM and variables provided by the CASA, exceptions made for VAP, VSL, VCL and BCF (data not shown). Correlations were positive for ALH, STR, LIN, Mot, Prog, Rapid, Medium and Slow and negative for Static (Table 3).

Discussion

Assays to predict fertilising capacity of semen samples are extremely valuable in the routine of a laboratory, especially when expensive-assisted reproductive techniques are required or rare and high value genetic materials are used (Aitken, 2006). In this regard, although many functional assays exhibit some precision, it seems that assessment of the spermatozoa–zona pellucida binding indicates with higher accuracy, the fertilising capacity of a given sample (Barbato *et al.*, 1998; Amann *et al.*, 1999). Several studies indicate that proteic components of the EPM are highly conserved between species, with possible inter-species differences in N- and O-linked oligosaccharides of the carbohydrate components of glycoproteins (Ohtsuki *et al.*, 2004; Rodler, 2011). These EPM characteristics led Stewart *et al.* (2004) to suggest that the high incidence of hybrids in birds is probably due to the ability of heterologous perivitelline membrane to induce rooster sperm binding and acrosome reaction. Hence, considering the homology of EPM glycoproteins to some mammalian ZPs, especially ZP1 and ZP3, sperm-egg-binding assays figure as an inexpensive and practical method to complement conventional and functional semen analysis (Barbato *et al.*, 1998; Bausek *et al.*, 2000; Mann, 2008).

The sperm capacity to bind to the EPM have been previously reported by Barbato *et al.* (1998). These authors verified a relationship between fertility and the number of rooster spermatozoa bound to the EPM. Notwithstanding, during the standardisation of this technique in other species (such as horses, rat, human and bovine), they only showed that an increase in the number of spermatozoa applied on the EPM rendered a rise in the number of cells bounded to it. Herein, we kept sperm concentrations constant while amounts of viable cells were changed; circumstance that confirmed that the number of spermatozoa bound to the EPM is highly correlated by the ratio of thawed/dead spermatozoa. Likewise, positive correlation was found between CASA and CM, indicating that both techniques can be used; the faster and nonsubjective evaluation performed by CASA or the more inexpensive analysis achieved by CM.

It has been speculated that the binding of avian spermatozoa to homologous EPM is mediated by ZP1 with a

Table 2 Correlations among analysis of the number of spermatozoa bound per mm^2 (CASA and CM) and values of plasma membrane integrity (IM), acrosomal integrity (IA) and mitochondrial activity (DAB I, II, III and IV)

	Spermatozoa per mm^2 (CASA)	Spermatozoa per mm^2 (CM)	IM	IA	DAB I	DAB II	DAB III	DAB IV
Spermatozoa per mm^2 (CASA)	1.0000	0.962 (<0.0001)	0.845 (<0.0001)	0.846 (<0.0001)	0.759 (<0.0001)	−0.490 (<0.017)	−0.624 (<0.0015)	−0.624 (<0.001)
Spermatozoa per mm^2 (CM)		1.0000	0.846 (<0.0001)	0.788 (<0.0001)	0.768 (<0.001)	−0.489 (<0.0242)	−0.633 (<0.0021)	−0.705 (<0.0003)

Table 3 Correlations (respective significance) among analysis of the number of spermatozoa bound per mm^2 (CASA and CM) and other sperm parameters assessed by CASA

	ALH (μm)	STR	LIN	Mot	Prog	Rapid	Medium	Slow	Static
Spermatozoa per mm^2 (CASA)	0.738 (<0.0001)	0.583 (<0.0043)	0.628 (<0.0017)	0.890 (<0.0001)	0.861 (<0.0001)	0.867 (0.0001)	0.445 (<0.0379)	0.435 (<0.0425)	−0.686 (<0.0004)
Spermatozoa per mm^2 (CM)	0.650 (<0.0014)	0.540 (<0.0115)	0.642 (<0.0017)	0.806 (<0.0001)	0.919 (0.0001)	0.932 (<0.0001)	0.484 (<0.0260)	0.557 (<0.0086)	−0.711 (<0.0003)

possible involvement of the ZP3. Bausek *et al.*(2004) proved that the inhibition of ZP1 by the P95 antiserum induces a drastic decrease on EPM hydrolysis by rooster sperm-associated proteases. These findings led the authors to conclude that ZP3 (ZPC) may be associated with the initial binding, which in turn allow the hydrolysis of the ZP1. Therefore, it is feasible that ZP3 is the primary receptor for both sperm–oocyte-binding and induction of acrosome reaction (Hinsch & Hinsch, 1999). Another hypothesis raised by Ohtsuki *et al.* (2004) is that the ZP3 binds specifically to ZP1 forming insoluble fibres, which may be involved in the sperm activation and binding to the EPM. In porcines, such interaction between spermatozoa and oocyte is also attributed to ZP1–ZP3 heterocomplexes (Yurewicz *et al.*, 1998).

Other key point to be considered when using EPM for semen analysis is the presence of the egg yolk in semen extenders, because both egg yolk and EPM are derived from chicken. Ricker *et al.* (2006) demonstrated that lipids in egg-yolk-based extenders may associate with the spermatozoa membrane, producing lipid aggregates on their surface. Besides, the low-density lipoprotein fraction in egg yolk has been shown to bind to seminal plasma proteins preventing the deleterious influence of these proteins in bull spermatozoa (Manjunath *et al.*, 2002). Although we do not expect any influence of the egg-yolk-based extender on the binding ability of cryopreserved spermatozoa to EPM, further studies will be necessary to evaluate this interference on the sperm-egg-binding assay.

The importance of mitochondria for sperm functionality has been demonstrated widely; mitochondria is the main source of energy necessary for motility and cellular homeostasis (Travis *et al.*, 1998; St. John, 2002). Despite several studies indicating the role of glycolysis on sperm motility rather than mitochondrial oxidative phosphorylation (Mukai & Okuno, 2004; Ford, 2006; Nascimento *et al.*, 2008), mitochondria appear to be a key organelle for both physiological and pathological conditions. As a source of reactive oxygen species (ROS) (Koppers *et al.*, 2008), mitochondria may influence sperm hyperactivation (de Lamirande & Cagnon, 1993), capacitation (Aitken *et al.*, 2004), acrosome reaction (Griveau *et al.*, 1995; de Lamirande *et al.*, 1998), binding to the oocyte and zona pellucida (Aitken *et al.*, 1995), processes that are dependent of limited concentrations of ROS. The oxidative stress is known to cause damages to the sperm acrosome, membrane and DNA (Griveau & Lannou, 1997; Halliwell, 1999), leading to impaired fertilising capacity. This could be observed in our results, in which a high correlation was found between the number of spermatozoa bound to the perivitelline membrane and mitochondrial activity; positive for the full mitochondrial activity (DAB I) and negative for all classes showing impaired activity (DAB II, III and IV).

The use of the computer-assisted sperm analysis combined with the sperm-binding assay allowed a reliable and faster analysis, which could be an important point when using such technique as a tool for routine semen analysis. Furthermore, results found in the present study provided consistent data regarding the motility-related variables obtained with the CASA and the ability to bind to the EPM. Several studies have been performed aiming to correlate the variables obtained using the computer-assisted sperm analysis with fertility (Hirano *et al.*, 2001; Shibahara *et al.*, 2004; Oliveira *et al.*, 2013). Similar to our study, correlations were found between total motility and

progressive motility with conception rates in cows (Oliveira *et al.*, 2013). Also, the amplitude of lateral head displacement has been demonstrated to present a strong correlation with both *in vitro* and *in vivo* fertilities (Hirano *et al.*, 2001; Verstegen *et al.*, 2002; Oliveira *et al.*, 2013). Despite the absence of correlations between VSL, VCL, VAP and BCF with the sperm ability to bind to the perivitelline membrane observed in the present study, Verstegen *et al.* (2002) previously verified that semen samples that generate more than 50% of fertilised oocytes showed higher values in these variables, when compared to samples generating less than 50% of zygotes. Also, several studies have demonstrated a correlation between these variables and fertilisation rates (Barratt *et al.*, 1993; Irvine *et al.*, 1994; Cox *et al.*, 2006; Oliveira *et al.*, 2013). Therefore, the importance of these variables for fertility assessment should not be ignored. However, for all subpopulations of motile spermatozoa (i.e. rapid, medium and slow), positive correlations were found with the number of spermatozoa bound to the membrane. These results may indicate that regardless of velocity, even slow spermatozoa are able to fertilise. Further studies are necessary to assess the real contribution of each motility trait for fertility; however, the sperm-binding test may be an important tool to establish standard parameters of motility of fertile samples.

In conclusion, our results indicate that the sperm-egg-binding assay using may be a valuable parameter to determine sperm viability in cryopreserved bull semen. However, further *in vivo* studies are necessary to establish the validity of this assay under field conditions.

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