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Research Article

# Study of Sperm Evaluation Parameters to Estimate Cryopreserved Bovine Semen Fertility

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**Abstract**

In cattle production, reproductive biotechnologies such as artificial insemination with Cryopreserved semen samples show the same performance as insemination with fresh semen. The most representative method to evaluate semen fertilizing capability is through the results obtained by *in vivo* fertility. However, as it is difficult to apply this method in practice, routine laboratory tests can be used to evaluate semen quality. The aim of this work was to evaluate functional spermatic parameters in cryopreserved bovine semen samples from males presenting high and low field fertility rates. Semen samples were obtained from bulls presenting low or high fertility in a program of artificial insemination. Routine and functional parameters were determined in both groups. As regards routine tests, no significant differences were observed in progressive motility and vigor between samples of animals with high and low fertility *in vivo*, but differences were detected in vitality and acrosome integrity ($p<0.05$). Within functional tests, no differences were detected in thermo resistance and capacitation induction, but HOS test, acrosomal reaction induction and IVF showed significant differences ($p<0.05$). These results assert that routine parameters (progressive motility and vigor) have a limited value to predict field fertility. Therefore, the incorporation of some functional tests may offer a better estimation of fertilizing capability.

**Keywords:** Bull sperm; Semen fertility; Functional parameters; *In vitro* fertilization

## Abbreviations

IVF: *In Vitro* Fertilization; ICSI: Intracytoplasmic Sperm Injection; HOS test: Hypoosmotic Swelling Test; TALP: Tyrode's Albumin Lactate Pyruvate; BSA: Bovine Serum Albumin; MSOF: Modified Synthetic Oviductal Fluid; TB: Trypan Blue; ANOVA: Analysis of Variances; AI: Artificial Insemination

## Introduction

In cattle production, reproductive biotechnologies such as Artificial Insemination (AI) with cryopreserved semen samples show the same performance as insemination with fresh semen. The most representative method to evaluate semen fertilizing capability is through the results obtained by *in vivo* fertility. However, as it is difficult to apply this method in practice, routine laboratory tests (progressive motility, vigor, vitality and acrosome integrity) can be used to evaluate semen quality, although their usefulness to predict semen fertility is limited mainly due to the complexity of sperm and the fertilization process [1]. Some studies report that *In Vitro* Fertilization (IVF) developed with frozen semen from bulls of high fertility yielded higher cleavage and blastocyst formation rates [2].

The study of the sperm membrane functional status is of particular importance since an intact and functionally active membrane is required for cell metabolism, capacitation, acrosome reaction, attachment and penetration of the oocyte [3]. The elucidation of these mechanisms is of fundamental importance to resolve cases of infertility and to choose the optimal conditions for the performance of assisted reproductive techniques, such as IVF and Intracytoplasmic Sperm Injection (ICSI) [4,5]. Thus, the assessment of the sperm membrane functional status appears to be a significant marker for the fertilizing capacity of spermatozoa [6]. The Hypoosmotic Swelling (HOS) test was designed to evaluate the function of the sperm membrane [3].

The fertility of a bull has traditionally been evaluated by test inseminations in the field and while this method is considered reliable, it is expensive and time consuming [7]. Consequently it would be a benefit for the cattle industry to have an accurate, simple and efficient *in vitro* method of predicting the potential fertility of semen, where aspects such as time, cost and practicability are considered [8].

Therefore, the aim of this work was to evaluate routine and functional sperm parameters in cryopreserved bovine semen samples from males presenting high and low field fertility rates.

## Materials and Methods

Semen samples were obtained from four bulls (Aberdeen Angus, 15-20 months of age) which belonged to a bovine reproduction center and were being evaluated for their possible inclusion in a controlled program of AI. These bulls presented different performances in preliminary field tests, showing low (below 60% pregnancy rate) or high field fertility rates. The semen pellets from bulls presenting low (1 sample, 5 replicates per sample) or high (3 samples, 5 replicates per sample) were thawed in Tyrode's Albumin Lactate Pyruvate (TALP) medium without calcium or Bovine Serum Albumin (BSA) at 37°C in a 1:2 ratio. After equilibration (10 min), samples were centrifuged




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at 300 xg for 5 min to separate seminal plasma and freezing extender. The pellet was resuspended in the same medium and centrifuged as described before. Samples were then suspended in a TALP medium containing 2 mm $CaCl_2$ and 6 mg/ml BSA ($1.5x10^7$ sperm/ml) to perform the routine and functional tests.

### Evaluation of progressive motility and sperm vitality

Progressive motility and vigor were evaluated by light microscopy (x400) with a thermal stage (37°C) three times by the same observer after each treatment (45 min). The percentage of live spermatozoa was determined by the supravital eosin/nigrosin technique. At least 200 spermatozoa were counted in each sample.

### Acrosome integrity

Two hundred spermatozoa were evaluated to determine acrosome integrity in live cells with the combined technique of 0.25% trypan blue and differential interference optical contrast.

### Thermoresistance test

The capability of spermatozoa to resist incubation at 37°C for 2h was evaluated in each treatment assessing the progressive motility.

### HOS test

HOS test was performed by adding 50 µl of sperm suspension to 200 µl fructose-citrate hypoosmotic solutions (50 MSOF) and incubating for 30 minutes. Two hundred spermatozoa were evaluated at the end of incubation to determine the swelling patterns.

### Capacitation induction

Sperm suspensions were incubated for 45 min at 38°C under 5% $CO_2$ in humidified air in the presence of heparin (capacitation inducer). Determination of capacitation was made by the chlortetracycline epifluorescence assay using an epifluorescence microscope (Jenamed 2, Carl Zeiss, Jena, Germany) at x400. Two hundred sperm were examined and designated as one of the following: F-pattern (intact sperm), where the fluorescence was detected over the whole region of the sperm head; B-pattern (capacitated sperm), where the fluorescence was detected in the sperm head, except in the post-acrosomal region; and AR-pattern (acrosome reacted sperm), with no head fluorescence. The percentages of the B and AR patterns were obtained by subtracting from the values obtained in the control and treated samples, the ones obtained at zero time, in order to rule out cells destabilized during the freezing-thawing process.

### Acrosome reaction induction and evaluation

The ability of the capacitated sperm to undergo acrosome reaction was assessed as follows: samples were incubated with bovine follicular fluid (30% (v/v) for 15 min in the same capacitating conditions). The acrosome reaction was evaluated by a combined technique of differential-interferential contrast microscopy and a supravital stain Trypan Blue (TB). Sperm (200/sample) were assessed at x1000 magnification (Carl Zeiss Jenamed 2 microscope, Jena, Germany). The percentage of live acrosome reacted sperm was evaluated by counting the percentage of live acrosome reacted sperm in each treatment from which the one obtained at zero time was subtracted, in order to rule out cells destabilized during the freezing-thawing process.

### *In vitro* fertilization

IVF was carried out using cryopreserved semen samples from bulls of low and high field fertility rates. Semen was thawed at 37°C in Modified Synthetic Oviductal Fluid (MSOF) [9] with 10 mmol/l theophylline, centrifuged at 500 xg twice for 5 min and then resuspended in fertilization medium to a final concentration of 2 $x10^6$ motile spermatozoa/ml. Co-incubation of COCs and spermatozoa was performed in IVF–MSOF medium, consisting of MSOF supplemented with 10 IU/ml heparin and 5 mg/ml BSA, under mineral oil at 39°C, 5% $CO_2$ in humidified air during 24 h. IVF in semen samples was evaluated by cleavage rates.

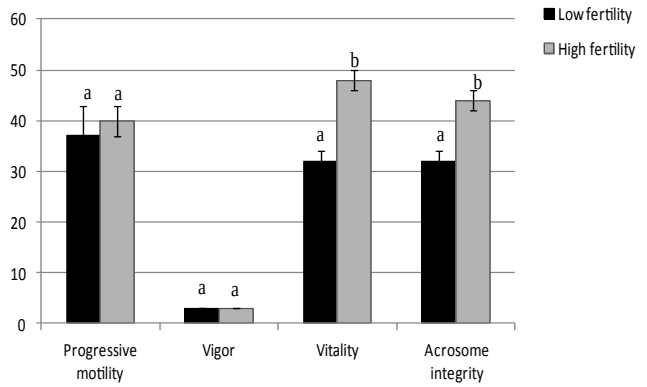


**Figure 1:** Routine sperm parameters in cryopreserved bovine semen from bulls of low (n =1) and high (n=3) field fertility rates (5 replicates per sample). Data were expressed as mean ± SEM. [a,b] Values with different superscripts present significant differences ($p<0.05$).

### Statistical analysis

Data were expressed as mean ± SEM. Values were compared using Analysis of Variances (ANOVA). A p-value < 0.05 was considered significant.

## Results

As regards routine tests, no significant differences were observed in progressive motility and vigor between samples of animals with high and low fertility *in vivo*, but differences were detected in vitality and acrosomal integrity ($p<0.05$) (Figure 1). Within functional tests, no differences were detected in thermoresistance (data not shown) and capacitation induction, but HOS test, acrosomal reaction induction and IVF showed significant differences ($p<0.05$) (Figure 2).

## Discussion

Conventional *in vitro* evaluation of semen quality following the freeze - thaw process, such as the assessment of sperm concentration, motility and morphology are of limited value in assessing field fertility [10]. Various fluorescent staining techniques have also been used to evaluate sperm viability [11], capacitation status [12], membrane integrity [13], chromatin integrity [14], acrosome status [15] and mitochondrial activity [16], proving to be useful for *in vitro* assessment of sperm, but having a limited ability to predict field fertility. [17-19] pointed out that the combination of tests which make possible to evaluate sperm function is the most reliable approach to increase the accuracy of estimating the potential fertility of semen samples.

The thermoresistance test is another routine analysis commonly used in bovine reproduction centers, although the usefulness of this

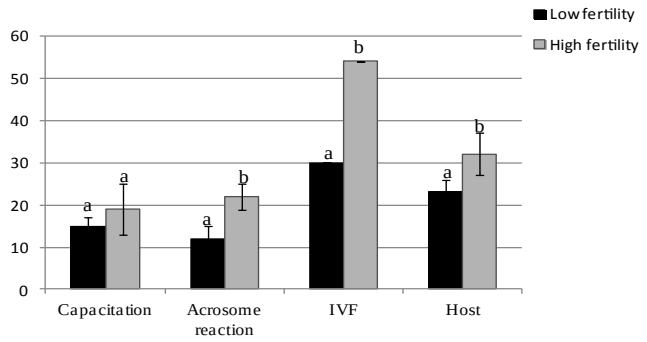


**Figure 2:** Functional sperm parameters in cryopreserved bovine semen from bulls of low (n =1) and high (n=3) field fertility rates (5 replicates per sample). Data were expressed as mean ± SEM. [a,b] Values with different superscripts present significant differences ($p<0.05$).

test has not been demonstrated [20]. In our study, no significant differences were observed between high or low field fertility semen samples.

Studying the capacitation status could also be important to determine fertilizing capacity. If capacitation diminishes the ability of spermatozoa to bind to the oviductal epithelium, the change in status described for cryopreserved spermatozoa may interfere with the establishment of the isthmic sperm reservoir. Failure to establish the normal sperm–epithelial interactions may contribute to the reduced fertility and shorter lifespan of the frozen–thawed spermatozoa [8]. Poor fertility associated with a compromised ability to survive within the female tract and to establish sperm reservoirs can be overcome by carefully timing insemination with frozen–thawed semen to coincide closely with ovulation. However, in our study the determination of the capacitation state by the CTC stain was not enough to demonstrate significant differences between semen samples, being the induction of acrosome reaction and the evaluation of the percentage of live reacted sperm a more accurate estimator of field fertility.

Our results coincide with previous studies, in which sperm characteristics, measured on a percentage basis, were higher for high than for low fertility groups. The results obtained in those works also showed that the total number of motile, morphologically normal spermatozoa and spermatozoa with intact acrosome were similar but tended to be higher in high fertility bulls [15]. These observations correspond with the protocol employed by the AI industry, in which the dose per straw is calculated on the basis of the number of motile and morphologically normal spermatozoa [21]. In contrast, differences between high and low fertility bulls were more noticeable for the total number of swollen spermatozoa. The evaluation of sperm swelling patterns also seemed to be of clinical and practical significance in evaluating bulls of high *vs*. low field fertility rates, as shown by the results obtained in the HOS test.

Considering the relative or low significance of the mentioned tests, the use of IVF could be the most adequate technique to estimate field fertility. However, previous studies showed controversial results. Some authors have reported that IVF can be used as a tool to predict the field fertility of a bull or to discriminate among bulls of different field fertility in terms of both cleavage and blastocyst formation rates [2,22,23]. Others have reported a correlation between field fertility and cleavage rates alone [24,25] or blastocyst formation rates alone [26].In contrast, other authors reported that IVF is not a useful predictor of field fertility [27,28]. According to our results, the rates of IVF were significantly higher in samples of semen corresponding to high field fertility rates.

These results assert that routine parameters (progressive motility and vigor) have a limited value to predict field fertility in the evaluated samples. According to our results, the combination of morphological (vitality and acrosome integrity evaluation) and functional tests (IVF and HOS test) may offer a better estimation of fertilizing capability of cryopreserved bovine semen. It is necessary to increase the number of animals in future studies to maximize the impact of these findings on the AI practice in bovine production.

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