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Prognostic value of spermatological parameters as predictors of in vitro fertility of frozen-thawed bull semen

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Abstract

Cryopreservation imposes irreversible damage to sperm membranes, such as swelling and disruption of plasma and acrosome membranes, changes in membrane fluidity, altered influx of calcium, and changes in enzyme activity. Morphological integrity of the sperm plasma membrane has been widely studied using different techniques, including exposure of spermatozoa to hypoosmotic solutions (provides information concerning the biochemical activity of the sperm tail membrane), supravital test using eosin stain (yields information regarding sperm head membrane integrity), and Trypan-blue Giemsa stain (TBG; reveals both sperm plasma membrane and acrosome integrity). The objective of this study was to combine these tests in order to provide information about the integrity of the whole sperm surface, as well as acrosome status, and determine if the results of these tests were associated with sperm in vitro fertilizing ability. Stepwise regression analyses yielded a model in which fertility (maintain variable) was expressed as a combination of the results of different spermatological parameters (independent variables). The results of a test combining supravital eosin staining of samples previously submitted to hypoosmotic swelling test (STHOS) accounted for the greatest proportion of variation in fertilization rates (78%). Inclusion of the results of dual staining with TBG increased the proportion of variation in fertility rate that could be accounted for to 82%. Therefore, sperm plasma membrane integrity and function, and acrosome integrity can be considered important variables for normal sperm function and STHOST and TBG could be used for the prognosis of the potential fertility of bovine semen samples used for IVF or AI.

Keywords: Frozen-thawed bovine spermatozoa; Semen assay; Membrane integrity; Fertility

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1. Introduction

Standard parameters evaluated in order to assess male fertility, i.e. sperm morphology and motility, have shown different associations with fertility. Particularly, subjective evaluation of post-thaw sperm motility, the mostly widely used single parameter to determine bovine semen quality, has been reported to be strongly or poorly correlated with fertility [1,2]. Zhang et al. [3] demonstrated significant correlations among in vitro semen tests (total sperm concentration and motile sperm concentration), zona pellucida-binding assay, and in vitro blastocyst production. The integrity of the plasma membrane reflects sperm viability and cryopreservation damages this membrane [4–8]. The damage includes swelling and disruption of plasma and acrosome membranes, changes in fluidity [9–11], altered influx of calcium [12,13], and changes in enzyme activity [8,11,14–16]. Furthermore, capacitation, acrosome reaction, and binding of spermatozoa to the oocyte surface require a biochemically active membrane [14,17,18]. Destabilization of sperm membranes following cooling resembles that of the physiological sperm capacitation [4,12,19]. Considering that capacitated and/or acrosome-reacted spermatozoa have a limited life span [20], this would result in impaired fertility.

Some assays have been developed to evaluate sperm membrane integrity, including the supravital test with eosin stain (ST) and the hypoosmotic swelling test (HOST). The ST yields information regarding the integrity of the sperm head membrane [21], while the HOST evaluates the integrity of the sperm tail membrane. The principle of the HOST is based on the observation of coiling of the sperm tail when exposed to hypoosmotic conditions [9,10,14,18,22–27]. The ability of the sperm tail to swell and coil in the presence of a hypoosmotic solution is a sign that the transport of water across the membrane occurs normally and that the membrane has normal functional activity [14]. Several studies have reported the use of HOST to evaluate spermatozoa, but the results of the test are not highly correlated to either in vivo or in vitro fertility [5,9,23,24,26,27]. By combining ST with HOST, Chan et al. [21] demonstrated that the relationship with sperm penetration assay results was improved when compared to the use of each test separately. A combination of Trypan-blue supravital stain and Giemsa stain (TBG) can be used to determine the integrity of both sperm plasma and acrosomal membranes.

The objective of the present study was to determine if specific, precise and accurate laboratory tests to evaluate sperm and acrosomal membranes could be used to predict the fertility of frozen-thawed bovine semen. In addition, we determined whether a combination of ST and HOST (STHOST) has greater potential to predict fertility than each test used separately.

2. Materials and methods

2.1. Semen samples, sperm morphology, and motility

Commercial frozen semen samples from 21 Aberdeen Angus bulls, packaged in 0.5 ml straws (approximately 40×10^6 spermatozoa/ml), were thawed in a water bath at 37 °C for 10 s and diluted (1:1) with TCM 199 (Gibco; Grand Island, NY, USA). Four straws of each

bull obtained from four different batches were used for evaluation of sperm morphology, motility, membrane integrity, and acrosome status.

Sperm morphology was evaluated by mixing equal drops of semen, Chinese ink, and formol-saline at room temperature on a slide with the edge of another slide and smeared. Stained semen smears were air-dried and evaluated with light microscopy with differential interference contrast (DIC) under 400× magnification. A total of 200 spermatozoa were examined in at least five different fields. The percentage of progressively motile spermatozoa was determined by placing a drop of semen on a warm slide and placing cover slip over it. A total of 200 spermatozoa were observed with phase contrast microscopy under 400× magnification in at least five different fields.

2.2. Evaluation of sperm plasma and acrosome membranes

Evaluation of sperm viability was performed using the supravital stain eosin-nigrosin, as recommended by the WHO [28]. Aliquots (20 μl) of semen sample were added to the same volume of staining solution and smeared on slides [29]. Eosin penetrated non-viable, dead spermatozoa with disrupted membrane, which appeared stained in red. Nigrosin offered a dark background in order to facilitate the detection of viable, unstained spermatozoa [30]. Two hundred spermatozoa were examined with light microscopy and DIC under 400× magnification.

The HOST was performed as described by Jeyendran et al. [14]. The hypoosmotic solution consisted of sodium citrate (7.35 g/l; Sigma Chemical Co., St. Louis, MO, USA) and fructose (13.51 g/l, Sigma Chemical Co.). The final osmolarity was adjusted to 150 mOsm/l with pH 7.2. Aliquots (10 ml) of semen were mixed with 500 μl of the prewarmed hypoosmotic solution in 1.5 ml tubes and incubated at 33 °C for 20 min. After incubation, an aliquot (10 ml) was pippeted on to a slide, a cover slip was placed on top of the droplet, and the preparation was observed by phase-contrast microscopy at 400×; 200 spermatozoa in at least five different fields were examined in each preparation.

The STHOST was developed in order to evaluate the integrity of the whole sperm surface, a parameter that positively correlates with the results of in vitro sperm penetration assay [31]. Whenever the STHOST test was performed, an aliquot (10 ml) of the solution was pipetted on a warm slide after the HOST incubation period and a droplet (10 ml) of Eosin (0.5% (w/v) sodium citrate 2.92%) was mixed for 10 s. A coverslip was placed on the mixture and it was evaluated with light microscopy (400× magnification). A total of 200 spermatozoa per preparation were observed in at least five different fields. Clear heads and tails and swollen tails indicated intact, biochemically active sperm membranes, while pink heads and tails and unswollen tails indicated disrupted, inactive sperm membranes [21].

The dual staining procedure with TBG was performed as described by Kovacs and Foote [32]. Briefly, the double stain procedure includes the use of the supravital stain Trypan-blue to distinguish live and dead spermatozoa and Giemsa (Sigma Chemical Co.) to evaluate the integrity of the acrosome membrane. Equal drops of Trypan-blue and semen were placed on a slide at room temperature and quickly mixed. Smears were air-dried and slides were fixed with formaldehyde-neutral red for 5 min. After rinsing with running distilled water, 7.5% Giemsa stain was applied for 4 h. After rinsing and air-drying, cover slips were mounted with Balsam of Canada (Sigma Chemical Co.). Two hundred spermatozoa were

evaluated in at least five different fields in each smear by light microscopy at 400× or 1000× magnification. Trypan-blue penetrates non-viable, dead spermatozoa with disrupted membrane, which appeared stained in blue, while live, intact spermatozoa appeared unstained. Giemsa accumulates in spermatozoa with an intact acrosome, staining the acrosome region in purple. Sperm with no blue staining and a purple acrosome were considered viable.

2.3. IVF

The technique for in vitro maturation was adapted from Revel et al. [33]. Immature oocytes were collected from ovaries of cows (in an abbatoir) for IVM and IVF. Cumulus-oocyte complexes (COCs) were recovered by aspiration from follicles 3 to 6 mm in diameter. After washing three times in TCM199, 10 intact COC, with several dense layers of cumulus cells were selected for IVM. Maturation was performed in TCM199 supplemented with 10% (v/v) fetal calf serum, 10 μg FSH/ml, 1 μg LH/ml and 1 μg estradiol/ml (Sigma Chemical Co.) for 24 h at 39 °C in a humidified atmosphere of 5% CO_2 in air. At the end of the maturation period, the COCs were inseminated using the technique of IVF described by Marquant-Le Guienne et al. [34]. Briefly, frozen-thawed spermatozoa (the remainder after morphological and motility assessment), separated by the swim-up technique and capacitated with heparin (0.02 mg/ml), were used for insemination (concentration, 0.5×10^6 spermatozoa/ml) in 50 ml drops of fertilization medium under heavy mineral oil. Eighteen hours after insemination, COCs were rinsed in HEPES-buffered TCM199 and denudated by repeat pippeting, before fixation in glacial acetic acid:95% ethanol (1:3) for 24 h and staining with 1% aceto-orcein in 45% acetic acid. The oocytes were mounted on slides and observed under the microscope to determine the presence of two pronuclei to evaluate the fertilization rate. A total of 3657 COC (13–22 COC/batch/bull) were used in three replicates, using three semen batches from each of the 21 bulls.

2.4. Statistical analysis

Forward stepwise regression analysis [35] was performed to obtain the model that best explained the variation in fertility rate using spermatological parameters as independent variables. Probabilities ≤ 0.05 were considered significant.

3. Results

Table 1 summarizes in vitro fertilization rates and spermatological parameters. There was considerable variation in fertilization rate and in sperm morphology, motility, and plasma and acrosome membranes integrity among bulls. In a stepwise regression model for in vitro fertility, STHOST results appeared as the best single predictive variable, accounting for 78.2% of the variation in fertilization rate. Including the results of TBG to the model improved the ability to explain the variation in fertilization rate to 82.4% (Table 2). Other spermatological parameters did not contribute significantly to the model. One bull produced outlying residuals with every model and was excluded from the analysis.

Table 1
In vitro fertilization rate and spermatological parameters in 21 Aberdeen Angus bulls

	Mean	Range	S.D.
In vitro fertilization rate (%)	63.6	75.6–52.3	14.5
Morphologically normal sperm (%)	9.1	12.1–6.8	1.7
Progressively motile sperm (%)	62.2	65.0–55.6	3.4
ST live sperm (%)	69.8	77.3–59.1	5.3
HOST active sperm (%)	58.8	63.1–51.3	2.6
STHOST live, active sperm (%)	51.2	58.0–39.7	5.5
TBG live, acrosome intact sperm (%)	51.2	57.1–41.3	4.5

ST: eosin–nigrosin supravital stain; HOST: hypoosmotic swelling test; STHOST: eosin–nigrosin supravital stain combined with hypoosmotic swelling test; TBG: Trypan-blue Giemsa stain.

Table 2
Stepwise regression model for bovine in vitro fertility, using results from different spermatological parameters as independent variables

Independent variable	Coefficient	Standard error	Probability
Constant	2.69	7.13	
STHOS	0.62	0.20	0.006
TBG	0.62	0.24	0.02

Total model $R^2 = 82.4$. STHOST: eosin–nigrosin supravital stain combined with hypoosmotic swelling test; TBG: Trypan-blue Giemsa stain.

4. Discussion

When evaluating semen samples, the goal is to predict fertility before they are used for in vivo or in vitro fertilization. Considering the biological and economical importance of predicting potential fertility of frozen-thawed bull semen, it is necessary to design precise and accurate laboratory tests to evaluate irreversible damage to the sperm membranes imposed by cryopreservation. Therefore, stepwise regression was performed to establish the association of in vitro fertility and different laboratory semen tests. The value of subjective motility as a predictor of the fertilizing capacity of bull spermatozoa has been disputed [13,14,17,36]. Amann [37] and Pace et al. [38] had demonstrated that low fertility had often occurred even when frozen-thawed semen contained an adequate number of progressively motile spermatozoa. Similarly, sperm morphology and motility were not good predictors of in vitro fertility in the present study. Several events during fertilization, including sperm capacitation, acrosome reaction, and fusion with the ovum, require an intact and functional sperm plasma membrane and an intact acrosome. The results of the present study demonstrated that laboratory tests to evaluate these spermatological parameters are better predictors of in vitro fertility than evaluation of sperm morphology and motility.

During the HOST, spermatozoa with a biochemically active plasma membrane exposed to the hypoosmotic solution will undergo swelling and will increased in volume due to intracellular influx of water [10,23,39]. Supravital stains like eosin and Trypan-blue are

indicators of plasma membrane physical integrity. As fertilization will not occur if the sperm plasma membrane is physically intact but biochemically inactive, the HOST could be regarded as more conclusive than supravital staining. However, perhaps spermatozoa with borderline membrane integrity will exhibit swelling, hence yielding false positive results after HOST. In the present study, combining ST with HOST reduced the number of false positive results and enhanced the ability to detect differences in sperm plasma membrane properties. The STHOST was the single best fertility predictive test, accounting for over 78% of the variation in fertility rate, while the results of ST and HOST were not good predictors when done separately. Chan et al. [21] also demonstrated that the STHOST was positively correlated to the sperm penetration assay in humans.

Using bovine frozen-thawed semen samples, Anilkumar et al. [31] described the correlation between spermatological parameters and the distance of sperm penetration in polyacrylamide gel. These authors observed that the major characteristic determining the capacity of spermatozoa to penetrate the gel was the acrosome integrity. Moreover, Ikeda et al. [40] reported that acrosome integrity rather than sperm motility had a significant effect on in vitro fertilization rates when cryopreserved epididymal spermatozoa were evaluated. In the present study, evaluation of sperm plasma membrane and acrosome integrity using the TBG dual staining was a valuable fertility predictive test. When the results of the TBG staining where include in the regression model with the results of STHOST, a greater proportion (>82%) of the variation in fertilization rate were accounted for.

In vitro fertilization assays have been performed to investigate their correlation with fertility after AI. Marquant-Le Guienne et al. [34] reported that IVF might be a valuable tool for evaluating in vivo fertility. Moreover, Zhang et al. [3] proposed that the combined analyses of swim-up separated sperm traits, zona pellucida-binding assay and IVF can be used to predict bull fertility. More recently, Rodríguez-Martínez [1] reported that the ability of post-thaw semen to fertilize in vitro has been significantly related to the observed fertility after AI. Therefore, evaluation of frozen-thawed semen in vitro fertility could also allow the elimination of sub-fertile bulls before their semen is used for AI.

In conclusion, the present study demonstrated the potential of some laboratory tests to predict bull semen fertility. The results of concomitant evaluation of sperm plasma membrane physical integrity and biochemical activity using the STHOST and concomitant evaluation of sperm plasma and acrosome membranes integrity using the TBG dual staining accounted for a high proportion in the variation of in vitro fertility. These two methods of sperm evaluation are easy and accessible and could be used as tests for predicting the potential fertility of bovine semen samples used for IVF or AI.

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