FERTILITY PROGNOSIS BY SEMEN ANALYSIS

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Abstract

The ejaculates of AI bulls are routinely assessed for sperm concentration and sperm motility and, whenever needed, also for sperm morphology (including presence of foreign cells). This scrutiny assures proper monitoring of the bull normal semen production and the screening of semen for processing. The processed (extended, cooled and frozen) semen is solely evaluated for presence of acceptable (usually 50%) post-thaw motility. Major efforts have been made to establish a predictive value for the in vitro scrutiny of these single semen parameters, since they are considered relevant for the fertility of the bull and his processed semen AI-doses. Unfortunately, none of these single sperm traits have, so far, shown predictive value for the fertility of a single semen sample or even for the sire. This presentation reviews in vitro methods that evaluate multiple functions of frozen-thawed AI-bull semen and their relation to field fertility, paying particular attention to their predictive value and seeking to identify the least fertile (or sub-fertile) bulls. The methods included the computer-assisted evaluation of patterns of sperm motility, analyses of chromatin stability, sperm separation by swim-up, sperm morphology, the intactness of the sperm membranes, the degree of sperm capacitation, the ability to respond to inducers of the acrosome reaction, the capacity to bind to homologous zona pellucidae (ZP-binding) and to undergo *in vitro* fertilisation and early embryo development (IVF), thus enabling the assessment of many sperm attributes of importance for fertility. Multivariate analyses incorporating sperm linear motility, total concentration and concentration of motile spermatozoa after swim-up, frequency of uncapacitated spermatozoa, readiness to acrosome react to ionophore-exposure, ZP-binding rates, as well as cleavage and blastocyst yields allowed the calculation of predictive values of fertility that were significantly related to the observed fertility after AI in young AI-bulls and clearly identified those bulls considered sub-fertile in the population.

Introduction

Artificial insemination (AI) has been the reproductive biotechnology behind the successful development of national and international breeding improvement programs in cattle. Massive use of AI allows the dissemination of desired characters in a cattle population as well as it enables the testing of bulls aiming to the selection of genetically superior sires. Although the AI-enterprises are actually selling genetics, there is a basic need to select those andrologically sound sires yielding the highest possible fertility after AI with their processed (mostly frozen-thawed) semen. The fertility of the sires is therefore constrained by the handling of the semen, the use of low numbers of spermatozoa present in the AI-doses and further, by the application of proper AI-procedures on the female population, including heat detection and time of AI. The higher the number of AI-doses produced the better profits are to be made,

maximising the number of AI´s that can be performed with a single ejaculate, provided these doses have a good fertility. To achieve this goal there is a need for using the best possible cryopreservation methodology, together with the most accurate diagnosis of the viability and fertilising capacity of the processed semen. Handling of bull semen, even using the best possible currently applied methodology often kills and affects a large proportion of the spermatozoa present in the ejaculate. It particularly affects (and can destroy) the membranes (plasmalemma, acrosome, mitochondria) as well as the axoneme, thus impairing viability and leading to cell death. Cryopreservation can also affect bull spermatozoa in a subliminal way, inducing membrane changes that resemble capacitation (Cormier et al., 1997), which although it does not necessarily cause cell death, certainly shorten the fertilising life span of the spermatozoa. Under current conditions of semen processing, it has been demonstrated that the semen of each individual AI-bull has a threshold number of viable (assessed as motile) spermatozoa per AI-dose, in direct relation to its fertility, either as absolute numbers (Shannon and Vishwanath, 1995; den Daas et al, 1998) or as numbers of viable spermatozoa (assessed for motility or membrane integrity; Januskauskas et al., 1996).

The fertility assessment of the processed semen (and thus of the sires entering the breeding programmes) is done by insemination of a certain number of females (usually a couple of thousands). Although this procedure provides an accurate value for the fertility level of the bull, it is time-consuming and costly, especially if sub-fertile bulls are present. To be able to detect these least fertile males as soon as possible, and if possible using less expensive in vitro methods, is thus of major importance.

A review of current methods for sperm analysis of frozen-thawed bull AI-semen constitutes, therefore, the core of this presentation, focusing on their eventual prognostic value.

Sperm analysis of frozen-thawed semen: what are we looking for ?

Bull spermatozoa are terminal cells, highly specialised for delivering a genomic package contained in their nuclei, as well as a centriole, components the oocyte counterpart requires for fertilisation to succeed with the development of a zygote that can develop to an early embryo. For this deliverance to succeed, the bull ejaculate contains billions of spermatozoa, for the sake of increasing the chances of probability for sperm-oocyte encounter. Spermatozoa are able to disclose a forward motility that can pass fluid barriers (cervical mucus), a motility that changes pattern in relation to capacitation (hyperactivated motility) allowing penetration of the oocyte coverings (cumulus cells and the zona pellucida, ZP). Capacitation also prepares spermatozoa for sperm-ZP recognition, binding and the occurrence of the acrosome reaction (exocytosis of the acrosome). All these events seem to occur in a random fashion, that is, not all spermatozoa are necessarily in the same level of events (at least from what is gathered in vivo and mostly extrapolated from other species than the bull) but require the morphological and biochemical integrity of the cell, enabling spermatozoa to act metabolically (mostly catalitically) and pursue the expression of these particular attributes, all of them conveying to the occurrence of fertilisation (rev. by Rodriguez-Martinez and Larsson, 1998). Considering fertilisation to depend to a major extent on the presence of several (often interrelated) sperm characteristics, the question arises as to whether the sperm analysis we usually perform is enough to measure the fertilising capacity of a semen sample, particularly when the sample has been extended, cooled, frozen and thawed, as it is the case with AI-bull semen doses

(Amann, 1989; Amann and Hammerstedt, 1993).

Analyses of single sperm attributes; are they related to fertility ?

Sperm motility is the most widely parameter assessed to measure sperm viability and it is practised subjectively in most AI-stations in order to determine the quality of an ejaculate before processing and also of the processed semen, post-thaw. In most cases, a motility threshold of 50% is applied to refuse use of the processed semen. Significant correlations (r= 0.55) have been described for a group of 18 bulls with fertility (as non-return rates, NRRs) above 65% (Kjaestad et al., 1993). Unfortunately, in studies including a much larger bull population (117 AI-bulls), subjectively assessed motility was a poor predictor (b= 0.13-0.24) for field fertility, when motility values were within ranges around or above 50% (Stålhammar et al., 1994). Computer-assisted motility assessment devices (CASA) have been used lately. Even when these devices are still not devoid of programming or operator-related bias (Rodriguez-Martinez et al., 1997b), the analyses of standardised sperm samples have shown that certain patterns of sperm motility, such as linearity, correlate significantly (r^2= 0.45-0.63) with field fertility (Zhang et al., 1998; Januskauskas et al., 2000c). Stronger correlation can be found when several patterns of sperm motility are statistically combined (within motility; r^2= 0.68-0.98, Farrell et al., 1998) or with other parameters of sperm function (r^2= 0.83, Januskauskas et al., 2000c).

Evaluation of sperm morphology is a major component of the clinical andrological evaluation, as part of the spermiogramme. Sperm morphology indicates deviations in spermatogenesis and epididymal maturation processes and its outcome is used to eliminate bulls with semen of poor quality for AI in cases where morphological deviations indicate major pathologies (Söderquist, 1991; Rodriguez-Martinez et al., 1997b). Unfortunately, when morphological parameters are within acceptable limits, this parameter does not provide enough information about the expected level of fertility of the semen after AI, either when done subjectively (Zhang et al., 1998) or using a computer-assisted assay (ASMA, Gravance et al., 1999). Evaluation of the morphology of frozen-thawed bull spermatozoa can, however, provide some clues regarding sperm ability to survive freezing-thawing (Gravance et al., 1998). Furthermore, a relation between variation in sperm morphometry of bull spermatozoa with abnormal chromatin structure (the latter having a relation to fertility) has been reported (Sailer et al., 1996).

Evaluation of the status of the sperm chromatin, done via the analysis of the degree of DNA denaturation suing flow cytometry (the so-called SCSA method, Evenson et al., 1980; Evenson, 1999) may be a valuable complement for the routinely practiced microscopic evaluation of sperm morphology of semen from bulls in regular production schedules, since some of the parameters tested relate to bull fertility (Ballachey et al., 1988; Januskauskas et al., 2000c).

The assessment of the integrity of the sperm plasma membrane has been used to explore the frequency of sperm viability (live-dead) and the degree of membrane damage after cryopreservation (Rodriguez-Martinez et al., 1997a). The simplest method is the exposure of spermatozoa to hypo-osmotic solutions (Correa and Zavos, 1997; Gil et al., 2000), although their outcome do not always correlate with the fertility of the samples investigated. A major breakthrough to functionally assess frozen-thawed bull spermatozoa has been the development of fluorescent probes for DNA, intracytoplasmic enzymes, lectins or membrane potential (Rodriguez-Martinez et al., 1997a). Combinations of these fluorophores can nowadays be used to determine the integrity of sub-cellular sperm compartments (mitochondrial function

[Evenson et al., 1982], plasmalemma [Garner and Johnson 1995; Garner et al., 1997] and acrosome integrity [Januskauskas et al., 2000a]). Fluorophores have most often been used in connection with operator-screened fluorescent microscopy to determine the number of spermatozoa with an intact membrane present in a sample. Unfortunately, the data did not reveal a consistent significant relation between membrane integrity levels and levels of fertility achieved after AI, when AI-bulls were studied (Januskauskas et al., 1996; 1999). Fluorescent microscopy is a methodology which, albeit inexpensive, only screens a few hundred spermatozoa per sample, making this technique less accurate than flow cytometry (FACS Analysers), where thousands of spermatozoa can nowadays be analysed per minute. Assessment of sperm viability of bull frozen-thawed spermatozoa using SYBR-14/PI and FACS showed a significant relation to fertility (as 56d-NRR) of r= 0.45 (Januskauskas et al., 2000a, b). Unfortunately, FACS are costly instruments (in purchase and operational costs) hindering its use for routine evaluation of semen. An alternative instrumentation is the use of computerised fluorometry analysers (Halangk and Bohnensack, 1982). In a recent study, fluorometry was used to read fluorescence in samples containing large sperm numbers, being sufficiently accurate and rather quickly to be applied for routine evaluation of semen quality (Januskauskas et al., 2000c). Moreover, the assessment of membrane integrity in these large sperm populations was significantly related to fertility, both at batch (r=0.39) and bull (r=0.57) levels.

Acrosome status (integrity) has been retrospectively and significantly related to the fertility of frozen-thawed spermatozoa (Saacke et al., 1980) and it is used as an indicator of the degree of sperm damage inflicted by the cryopreservation procedures.

Determination of fertility potential using protein analysis

The ejaculated spermatozoa bathe in fluids of the epididymis and the accessory sexual glands that built up the seminal plasma. Seminal plasma influences the physiology of spermatozoa appearing as inhibitor of the destabilisation changes that characterise capacitation. Accessory sex gland fluid (Henault et al., 1995) and seminal plasma (Henault and Killian, 1996) appear to influence the fertility of bull spermatozoa, and promising research has linked some particular “high-fertility seminal plasma proteins” (Cancel et al., 1997; Gerena et al., 1998) to the fertility of AI-bulls, indicating these fertility-associated proteins can be valuable predictors of fertility. However, accessory gland fluid is detrimental for sperm survival (Way et al., 2000) and rapid removal of spermatozoa from seminal plasma (by centrifugation or extension) is critical for ensuring maximal sperm viability.

A 30-kDa heparin-binding protein (named fertility-associated antigen, FAA) has been identified in sperm membranes of beef bulls having higher fertility than bulls lacking this FAA (Bellin et al., 1998). Data concerning this FAA in a larger, better defined bull population is still not available.

Assessment of sperm fertilizing ability by functional in vitro tests; do they relate to field fertility ?

As seen above, sperm fertility seems to be more closely related to membrane integrity than to the estimated motility, especially when analyses of large sperm populations are used (using FACS or Fluorometry, see above). However, with these

exceptions, the analysis of a single sperm viability parameter (defined as membrane and acrosome intact, motile and with a normal chromatin) in a sample of frozen-thawed bull semen of acceptable quality does not always allow the prediction of its fertility after AI. A major reason for this is that male fertility is a complex trait and several sperm characteristics must remain unaffected by the cryopreservation in order for the thawed semen to show an acceptable fertility (Wood et al., 1986; Amann, 1989). For this reason, spermatozoa have been tested for their ability to reveal more complicated traits that combine single attributes such as plasmalemmal composition, intactness and fluidity, motility behaviour, decondensation ability of the nucleus, chromatin integrity, centriolar intactness, etc. Among these in vitro tests we find the swim-up tests, the assessment of capacitation status, the ability to bind to homologous ZP and to undergo acrosome reaction upon exogenous stimuli. As well, the capacity to fertilise homologous oocytes in vitro (IVF) and to be able to induce embryo development in vitro. The outcome of each of these laboratory tests has, obviously, been related to the field fertility of the tested sample, looking for a single, ultimate test for bull spermatozoa.

Swim-up tests

The population of spermatozoa present in a normal semen sample shows certain heterogeneity of morphology and motility. Most spermatozoa show a typical forward, rather rectilinear direction and are denominated linear motile spermatozoa. Linearity (see above) has been related to fertility and therefore tests based upon the innate ability of the spermatozoa to traverse fluids of a certain viscosity, the so-called swim-up tests have been developed (see Rodriguez-Martinez et al., 1997a), and used to predict fertility (Lathrop and Foote, 1986). More important is to combine the number of linearly motile spermatozoa after a swim-up procedure (about 90% of spermatozoa depict this motility pattern), since it is the combined amount of spermatozoa with this motility pattern that would offer a population of spermatozoa with potential fertility, with this procedure mimicking conditions prevailing in vivo (sperm selection for good morphology and motility during transport through the female genital tract). Using a simple swim-up through a column of culture medium, Zhang et al. (1998) was able to establish that the concentration of linear motile spermatozoa after swim-up was significantly related (r= 0.43 and 0.63 for batch and bull, respectively) to fertility (as 56d-NRR). Similar results have been obtained by Januskauskas et al. (2000b), which indicates the number of viable spermatozoa after swim-up might reflect the innate fertilising capacity of a semen sample.

Assessment of capacitation-like status and the ability to acrosome-react to stimuli

Sperm capacitation-like changes can be monitored by the incubatiion of bull spermatozoa with the antibiotic chlortetracycline (CTC, Tsien, 1989). The antibiotic fluoresces while monitoring Ca^{++} displacement in the sperm head membrane and therefore, since calcium displacement relates to the presence of capacitation-like membrane changes and the occurrence of the acrosome reaction, the method shows different status of the sperm membrane fluidity, at the head domains. When frozen-thawed bull spermatozoa with fully known AI-fertility were assessed with CTC, capacitation-like changes were present in up to 30-40 % of the spermatozoa, thus confirming previous data relating cryopreservation with induction of capacitation-like changes (Cormier et al., 1997). Furthermore, these studies revealed the percentage of un-capacitated (un-reacted) spermatozoa in an AI-semen batch appeared significantly

related to field fertility (Thundathil et al., 1999; Gil et al., 2000), implying the method could be used for prognostic tests (r= 0.48) of fertility (Januskauskas et al., 2000a).

The acrosome reaction (AR) can be induced in vitro by exposure to exogenous calcium ionophores (as the A23187), which promote a massive Ca^{2+} influx, bypassing intracellular regulatory mechanisms. A significant correlation (r= 0.60) between the degree of induced AR and the 56d-NRRs of the bulls used has been found in a retrospective study (Januskauskas et al., 2000a). Also using a prospective design, Whitfield and Parkinson (1995) found a significant correlation between the induced AR and the 90d-NRR of the bulls after AI. Acrosome-reactions can also be induced by exposure to glycosaminoglycans (GAGs) such as heparin (Parrish et al., 1985). The rate of AR after heparin treatment has been shown to be highly significant correlated with both in vivo and in vitro fertility (Henkel et al., 1993). However, these results have not been repeated when spermatozoa were exposed to heparin after being retrieved from a swim-up procedure (Januskauskas et al., 2000b). Whether the procedure diminishes the number of heparin-binding sites on the spermatozoa or any other methodological difference is causing these differences remains to be tested.

Sperm binding to the ZP

The effective binding of spermatozoa to the ZP is an early and critical step in the process of fertilisation (Gould et al., 1983) which increases after capacitation and relates to the AR by the ZP (Topper et al., 1999). This cascade of events, necessary for fertilisation to occur, has led to the design of in vitro ZP-sperm binding assays (ZBA), with the ultimate aim of predicting the fertility of the tested sample (Fazeli et al., 1993). In the bull, two main types of ZP-binding assays have been used, incubating non-capacitated frozen-thawed spermatozoa with intact homologous oocytes (Zhang et al., 1995; 1998) or with bisected empty hemizonae (hemizona binding assay, HZA, Fazeli et al., 1997). The first mentioned method is very simple, provided that a large number of oocytes and a suitable number of replicates are used (Zhang et al., 1995), to diminish the inherent variation among offal oocytes. Thus, the rate of sperm binding to the ZP has been able to show significant correlations (r= 0.49 and 0.50 for batches and bulls, respectively) with field fertility (56d-NRR)(Zhang et al., 1998). The other variant, the HZA, requires the micro-bisection of the oocytes into two matching hemizonas, where one is incubated with spermatozoa from a control bull and the other with test semen. HZA-results have correlated significantly with fertility (r= 046), even though a low number of bulls and semen batches were used (Fazeli et al., 1997). In any case, these correlations (for both intact ZBA and HZA) were present when the semen tested had large differences in field fertility and allowed to identify which bulls (or batches) were sub-fertile. When the intact ZBA was used to test a population of bulls with narrower high fertility, these correlations were not present (Zhang et al., 1999).

In vitro fertilisation and its relationship with AI-fertility

The outcome of IVF is known to vary with the sperm batch used (Shamsuddin and Larsson, 1993; Lonergan, 1994). Being IVF probably the in vitro test method that most closely imitates conditions during in vivo fertilisation, this led to a large series of studies looking for a single test for frozen-bull semen. However, this bull-related variation in vitro is not always accompanied by a significant relation with field fertility (Ohgoda et al., 1988; Schneider et al., 1999) although most studies have shown, in retrospectively designed trials, such a relation (Hillery et al., 1990; Marquant Le Guienne et al., 1990; 1992; Shamsuddin and Larsson, 1993), even with levels of r=

0.59 for cleavage rates (Zhang et al., 1997), when bull semen with large differences in field fertility was tested. Cleavage yield, measured 48 h after IVF, reflects the potential of the fertilised oocyte to undergo mitosis to the 2-8 cell stage. The IVF examinations of frozen-thawed semen measure not only this end-point but also blastocyst yield, which reflects the ability of the obtained 2-8 cell embryo to develop up to blastocyst (usually 7-9 days post-insemination). Blastocyst yield has, whenever related, a lower correlation (r= 0.35, Zhang et al., 1997) to field fertility. This lower relation has been explained by the higher dependence of the early bovine embryo on the culture conditions to reach the blastocyst stage. Noteworthy, these correlations were not present when semen from bulls with a high but narrower field fertility was tested (Zhang et al., 1999).

Is there any prognostic value in the outcome of in vitro semen analyses ?

As seen in the previous descriptions, few single sperm attributes, when analysed in vitro appear related to in vivo fertility. Some of these relations also arise when preparatory procedures are performed, such as a selection of highly motile spermatozoa by swim-up of culture with oviductal explants (Schneider et al., 1999). However, these analyses were done in a retrospective design, e.g. the tested semen was having a known, usually large range of fertility after AI. When a prospective design was used (e.g. the fertility of the semen tested was unknown), no single sperm attribute correlated significantly with field fertility, which appeared to be high and rather narrowly distributed (Zhang et al., 1999).

When the outcome of several tests, of sperm attributes considered relevant for the definition of sperm fertilising ability, was combined in multiple regression analyses some of these sperm parameter in vitro assays showed a clear (albeit retrospective), relation with field fertility (Hiaro, 1975; Amann, 1989; Zhang et al., 1997, 1998; Januskauskas et al., 1999, 2000a, b, c).

However, correlation analyses are retrospective and not forward, that is they are not predictive per se, unless the data gathered in vitro lead to the calculation of predictive fertility (as predictive NRR-values for instance) which is to be confronted to the actual field fertility of the tested semen. Such an approach was used to calculate a prognostic value for the expected fertility of frozen-thawed semen from young AI bulls, where seven different sperm parameters tested at the bull level (i.e. post-thawed sperm motility, linear motility, concentration and concentration of linear motile spermatozoa after swim-up, sperm ZP-binding as well as cleavage and blastocyst rates, based on the analyses of three semen batches), were combined to calculate predictive NRRs before the actual field fertility was known (Zhang et al., 1999). The predicted bull fertility (as NRR) varied from 61.8 to 67.5% and correlated with observed NRR (r= 0.90). The results indicate it is possible to use a combination of laboratory tests to determine semen quality in vitro and to do a prognosis over the potential fertility of the bull. Especially important, the analysis of the data allowed to identify the least fertile bull from a population with a narrow fertility range.

Some words of caution must, however, be given. Firstly, there is an important variation in fertility among ejaculates from one and the same young bull, as it was found both in vitro and in vivo, even when the ejaculates were collected within a relatively short period (Zhang et al., 1998), thus calling for the analysis of a large bull population and a large number of processed semen batches. Such a variation among ejaculates can for instance impair the prediction of fertility for a bull when his progeny testing has been completed and the bull could be selected as an elite sire (about 1-

2% of the young bulls in Sweden). Longitudinal studies where changes of in vitro and in vivo fertility of young bulls are monitored from fertility through progeny testing periods are therefore being carried out at present in our laboratory. Secondly, there is a tendency for seeking the identification of the males with highest fertility, rather than those least fertile which are, actually, more interesting to exclude as soon as possible. The procedures described in this review might, nevertheless, be effective to screen the semen from young AI-bulls with the purpose of excluding those with lower fertility from further progeny testing in the AI-programme, thus allowing the restricted fertility and progeny testing space available for young bulls to be used more efficiently, with obvious economic savings.

Conclusions

The ultimate goal of finding a laboratory procedure that would allow the prognosis of fertility for a given semen sample or an AI-bull is still out of range. With male fertility being a complex trait, the analysis must include most sperm attributes of importance for fertility in a large sperm population, and not on individual spermatozoa. The development of such analyses are, hopefully, to be expected in the near future.

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