In vitro evaluation of sperm quality related to *in vivo* function and fertility

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The potential fertility of a sire can not be evaluated in the field simply by assessment of mating ability and physical examination, although these procedures can expose his limitations as a breeder. Finding a laboratory test that accurately estimates the potential fertility of a semen sample or a sire is also distant, as shown by the modest correlations that present tests have with fertility. Due to the complex nature of male fertility any sought for laboratory method must include testing of most sperm attributes relevant for both fertilisation and embryo development, not only in individual spermatozoa, but within a large, heterogeneous sperm population. Although such a task has proven difficult, it is both challenging and attractive for ruminants, where methods with good estimative power are available to evaluate the many attributes required for fertilisation. Among these methods are the isolation of highly viable spermatozoa by swim-up followed by their ability to respond to capacitation or acrosome reaction challenges and their capacity to penetrate homologous or heterologous zona pellucidae (ZP). Identification of fertility markers in, for instance, seminal plasma would further aid in identifying low-fertility sires. Future efforts should concentrate on finding how many spermatozoa in the semen sample are competent for fertilisation, perhaps by screening sperm linear motion, membrane integrity and membrane stability by multi-parametric methods, linked to the ability of males to provide a stable population of spermatozoa in a repeatable manner.

Introduction

The fertility of males is best evaluated after breeding an adequate number of females either by natural mating or artificial insemination (AI). However, this is time consuming, involves major costs, good identification of bred animals and proper recording of the outcome. The potential fertility of bulls or rams can also be evaluated in the field by a clinical andrological examination, including a thorough evaluation of the ejaculate and assessment of libido and serving ability. Such screening, often referred to as a breeding soundness evaluation, has diagnostic value for assessing testicular and epididymal function, and/or the normality of the genital tract of the male (Barth 2000). Although it has proven useful to eliminate clear-cut cases of sub-

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fertility, breeding soundness evaluation appears insufficient to estimate the level of fertility that the males would actually achieve, whether used in natural service or for AI.

Determination of the potential level of fertility of a sire, or a particular ejaculate, by using the results of clinical and/or laboratory examinations has been the goal of veterinary andrologists for decades. However, due to the complex character of male fertility, estimating the expected level of fertility remains a challenge, particularly for sires in natural service (Parkinson 2004). Scrotal circumference (SC) is directly related to testicular size and thus indirectly to potential sperm production, but its relationship to fertility is confounded by several factors including semen quality and breeding pressure. On the other hand, SC is easily measured, it is highly heritable and relates to the age of puberty of male and female progeny; thus it is advantageous for sire selection despite its low predictive value for potential fertility. Sires can also be evaluated for their responsiveness to hormonal challenges (for example, GnRH-induced luteinising hormone [LH] or testosterone surges); however, a relationship to fertility has yet to be proven (Parkinson 2004).

Evaluation of a semen sample can determine its degree of normality before it is processed for AI or *in vitro* fertilisation (IVF). Usually, this evaluation includes recordings of volume, sperm concentration and motility and less often sperm morphology and the presence of foreign cells. In recent years, more detailed screenings of sperm function have been incorporated (reviewed by Silva & Gadella 2006), other than sperm motility which is by far the most widely used test in any species of domestic animals. Sperm numbers, sperm motility and sperm morphology are related to potential *in vivo* fertility, but only to a modest extent. Other measures of sperm function may have stronger correlations to fertility, but still provide only estimates of the outcome even when used in a battery of tests. Therefore, they are unrealistic practical alternatives and keep distant our ultimate goal to find a single *in vitro* test to reliably predict the level of fertility (Rodriguez-Martinez 2003).

Many reviews of the relationship between semen assessment and fertility in ruminants are available (Rodriguez-Martinez *et al.* 1997b; Saacke *et al.* 1998; 2000; Larsson & Rodriguez-Martinez 2000; Rodriguez-Martinez 2000; 2003). These reviews confirm that some laboratory methods can often correctly estimate the potential fertilising capacity of a semen sample and in some cases of the male, provided they are designed to test sperm attributes relevant for fertilisation and embryo development. However, such estimations depend on the type of test used to study a very heterogeneous suspension of terminal cells, the spermatozoa, whose intrinsic lability make assessments of attributes needed for fertilisation rather difficult (Holt & Van Look 2004).

The handling of semen during processes such as cooling, cryopreservation or high pressure flow cytometric sex-sorting, damages spermatozoa to various extents and further increases the complexity of fertility analyses. Moreover, these processes blur the relationship between male fertility and our (in)capacity to preserve spermatozoa. Nevertheless, the challenge of estimating level of fertility is closer to being met in ruminants, where methods are already available to evaluate an important quantity of the attributes of fertilisation in semen and relate them to the fertility outcome (most often by AI), compared to the situation in other domestic animals, where numbers of females are insufficient or proper records are not available (Amann 2005).

The present review describes the state of the art regarding *in vitro* methods available for sperm analysis as estimators of fertility, particularly those measuring sperm function and competence for physiological events such as sperm transport, storage and interaction with the female genital tract and the oocyte. It attempts to critically appraise the value and constraints of these laboratory methods as predictors of fertility when the male is used in natural mating situations and when semen is used by AI. It also considers the ability of *in vitro* methods to assess the effects of procedures such as semen extension, cool storage, cryopreservation and sperm sexing. The review hopes to avoid reiteration of the large amount of relevant descrip-

tions of available methods and includes results obtained in the laboratories of the authors on work done on ruminant livestock; in particular, cattle and sheep.

In vitro assessment of ruminant semen: state of the art

Bull, ram or buck semen can be collected during ejaculation with an artificial vagina or by electro-ejaculation, a procedure that, albeit contested due to animal welfare issues, is routinely practiced worldwide. In bulls, spermatozoa can also be collected, primarily for diagnostic purposes, by trans-rectal massage of the ampullae (Palmer *et al.* 2005). Ejaculated semen, from either source, is immediately evaluated for volume, sperm concentration and sperm motility, usually using ocular, photometric and light microscopic aids. Sperm concentration can be evaluated manually (counting chambers) or automatically (photometers, Coulter instruments, flow cytometry). Computer assisted motility analysis (CASA) instruments are now less costly and therefore more widespread. Most of these instruments digitise microscope images of sperm trajectories providing information on proportions of motile spermatozoa, motility patterns and other kinematic variables, while other computerized sperm analysers determine the number of particles (spermatozoa) crossing fields of view, yielding a regression algorithm of sperm numbers and translation classes. While the first named can describe patterns of movement of comparatively few spermatozoa per sample, followed in most cases for a few seconds, the second one analyses thousands of spermatozoa per sample but can only provide numerical classes. In other words, these instruments are more and more sophisticated, yielding much data with a degree of objectivity. However, subjective motility evaluations are still practical for immediate assessment of sperm viability, due to easiness and low cost.

Sperm morphology is sometimes screened, but far too seldom, to determine the proportion of spermatozoa with deviating morphology or showing specific defects (Barth & Oko 1989). Morphological abnormalities may be studied in unstained wet preparations of formalin-fixed spermatozoa and/or stained spermatozoa with light microscopy. Accurate detection of some abnormalities, head defects in particular (Chacón 2001), make necessary both staining and the counting of a large number of spermatozoa in order to obtain repeatability (Kuster *et al.* 2004). Proportions of defective spermatozoa are often grouped as primary vs. secondary, or major vs. minor according to their relative importance, while in other cases, morphological deviations are grouped by origin in order to determine underlying testicular or epididymal pathology, or as artefacts caused by mishandling of the semen. Abnormalities may also be grouped as compensable or un-compensable (Saacke *et al.* 2000). Sub-fertility caused by abnormal spermatozoa which are not transported to the oviduct, or which fail to penetrate the ZP could be compensated for by increasing the dose of spermatozoa at AI in order to obtain adequate numbers of normal spermatozoa at the site of fertilisation. Sub-fertility due to spermatozoa that fertilise, but fail to participate in the development of a normal embryo, can not be compensated for by simply increasing sperm numbers in the AI-dose. For this reason, it is not only important to know the number of morphologically normal spermatozoa in a sample but also the number of un-compensable abnormalities. Ideally, as we shall see later, it is the number of spermatozoa holding most intact attributes essential for fertility within a semen sample that matters. Presence and relative amount of foreign cells other than spermatozoa are easy to account for provided discontinuous smears are prepared to form dense ridges before drying and staining. These provide valuable information in cases of inflammation, testicular degeneration or other pathologies (Rodriguez-Martinez *et al.* 1997b).

Manual assessment of sperm morphology is sometimes considered highly variable within and between technicians. This variability may be related to technician training and the assess-

ment of too few spermatozoa per sample. In any case, automated, computer-assisted sperm head morphometry analysis instruments (ASMA, Gravance *et al.* 1998) have been designed to analyse sperm head dimensions objectively. These instruments have been found to give repeatable results when used with bull (Gravance *et al.* 1999), ram (Gravance *et al.* 1998) or goat spermatozoa (Marco-Jimenez *et al.* 2006). When combined with multivariate statistical analyses, ASMA allowed identification of sub-populations in the ejaculate (Peña *et al.* 2005a). However, ASMA is restricted to measurement of the sperm head surface area and lacks the ability to discern other morphological abnormalities.

While sperm numbers, motility and morphological features are important, sperm attributes required for interaction with the female genitalia, activation (capacitation), fertilisation and early embryo development have also been investigated. Among these attributes are the presence of intact and competent membranes, organelles (such as, the acrosome, mitochondria, proximal centriole and flagellum) and a haploid genome (sperm nucleus). While membrane abnormalities and abnormalities of many organelles would be compensable by dose, abnormalities of the proximal centriole (for example, accessory tails) and genome abnormalities (for example, pyriform sperm head shape or presence of nuclear vacuoles) would be uncompensable by dose. Several methods to assess semen (sperm) quality can be applied to ejaculated spermatozoa: including, the ability to swim through certain media (cervical mucus, artificial media); the reactivity shown by the plasma membrane to various stimuli (glycosaminoglycans or calcium-ionophores); the outcome of IVF; or the presence of fertility-related substances in seminal plasma (Rodriguez-Martinez 2000).

Ejaculated spermatozoa are handled for use in assisted reproductive technologies (ARTs); such as, AI with chilled, frozen thawed or sexed-semen, IVF *et cetera*. Spermatozoa used for ARTs may be exposed to semen extension, fluorophore loading, ultraviolet and laser illumination, high speed sorting, cooling, cryopreservation *et cetera* and each of these procedures will impose different degrees of changes in sperm function following introduced damage to sperm membranes, organelles or the DNA. Therefore, several assays have been recently developed to monitor these sperm parameters *in vitro*.

Plasma membrane integrity and stability of lipid architecture

A functionally intact plasma membrane is a pre-requisite for sperm life and function. The plasmalemma not only acts as a boundary for the sperm cytoplasm; by its semi-permeable features it maintains a chemical gradient of ions and solutes and also holds specific structural proteins that act as transporters for water, energy source substrates and signalling receptors: all relevant for sperm metabolism and the ability to interact with the surroundings. Loss of this functional integrity threatens sperm function and life to various extents, from decreased fertilizing capacity to cell death. If the plasmalemma is intact but functionally unstable, the spermatozoon is unable to interact with its environment and unable to fertilise. Plasma membrane integrity is usually assessed with membrane impermeable dyes, using the rationale that spermatozoa that can exclude these dyes are alive. Examples of impermeable dyes include eosin, many DNA-binding fluorescent probes such as Hoechst 33252, ethidium homodimer and propidium iodide (PI) (Rodriguez-Martinez *et al.* 1997b). A similar rationale lies behind the hypo-osmotic test (HOST) (Jeyendran *et al.* 1984; Correa & Zavos 1994; Zou & Yang 2000) in which sperm membranes able to react to a hypo-osmotic environment have a functional membrane. Combinations of fluorophores are now more often used. Membrane impermeable dyes are combined with penetrating acylated (AM) dyes which, taking advantage of their amphiphatic nature, can penetrate intact and functional membranes, are de-acylated in the cytoplasm, become impermeant

and are retained in the living cells. Examples of these AM-probes are fluorescein diacetate (FDA) and its carboxy(methyl) derivatives, such C-FDA (Haugland 2004). There are also acylated fluorescent dyes (for example, SYBR-14) that can bind to DNA and may be combined with other non-penetrating fluorescent DNA-probes such as PI. Together, these would stain the nuclei of sperm with and without deteriorated membranes and simultaneously inform us of the percentages of membrane-intact sperm (Garner *et al.* 1994; Garner & Johnson 1995). This allows examination of ruminant spermatozoa without worrying about the medium in which they were suspended (for example, milk or egg yolk containing extenders) that usually confound measurements with other fluorophores (Nagy *et al.* 2003; 2004). The latter fluorophores can be used with fluorescent microscopy or flow cytometry, allowing us to evaluate large numbers of spermatozoa.

The plasma membrane of the spermatozoon is a highly dynamic structure with a tendency to become unstable if the glycocalix is freed from adsorbed proteins, usually of epididymal and/or seminal plasma origin (reviewed by Flesch & Gadella 2000). Such instability starts with a loss of the order of the phospholipids present in the plasma membrane, a disorder that can be temporary (Harrison *et al.* 1996) or lead to membrane disorganisation (Harrison 1996). This membrane lipid scrambling is associated with cholesterol efflux and is considered as one of the initial steps in sperm capacitation (Harrison & Gadella 2005). Similar phenomena occur during cooling of spermatozoa and early changes in the architecture of the plasma membrane, well ahead of visible changes, can be monitored by loading spermatozoa with the lipid dye Merocyanine-540 whose significant increase in fluorescence can be determined with flow cytometry (Sostaric *et al.* 2005; Bergqvist *et al.* 2006). Recently, Annexin-V staining has been used as an alternative method to detect subtle membrane changes that involve redistribution of the phospholipid phosphatidylserine (PS) from its normal inner leaflet position to the outer leaflet of the sperm plasmalemma. (Januskauskas *et al.* 2003). Changes similar to those detected during the later part of sperm capacitation and the acrosome reaction (AR) (Tienthai *et al.* 2004) can also be indirectly visualized by the incubation of viable spermatozoa with the fluorescent antibiotic chlortetracycline (CTC) which monitors the displacement of $Ca2+$ in the sperm head plasmalemma (Fraser *et al.* 1995; Januskauskas *et al.* 2000a; Hallap *et al.* 2006).

Assessment of organelle integrity

Flagellar intactness can be easily determined by light microsopy on fixed wet preparations and during motility assessment. Specific abnormalities can be explored further using electron microscopy (Andersson *et al.* 2000). Mitochondria and their ATP production have classically been related to flagellar function, a concept that is today challenged (Silva & Gadella 2006) under the major argument that flagellar movement is related to the local ability to produce ATP anaerobically by glycolysis (Miki *et al.* 2004) while aerobically produced mitochondrial ATP is used for housekeeping metabolism at the mid-piece and head domains. This hypothesis, albeit interesting, still requires more experimental evidence. Mitochondrial integrity and functionality can be measured using specific fluorophores such as Rhodamine 123 and the family of Mitotracker dyes (Gravance *et al.* 2001) including JC-1 which differentiates whether mitochondria are functional or quiescent by detecting the changing re-polarisation of the inner mitochondrial membrane (Martinez-Pastor *et al.* 2004; Hallap *et al.* 2005). Disruption of the acrosomal outer membrane can be readily observed by phase contrast microscopy but intactness can not be assured in all cases. Acrosome integrity can be examined by microscopy or flow cytometry after using fluorescent conjugated lectins that bind to specific carbohydrate moieties of acrosomal glycoproteins. The most commonly used lectins are derived from peanuts (PNA) for assess-

ment of the outer acrosomal membrane or from green peas (PSA) for labelling of acrosomal matrix glycoproteins. Using both PNA and PSA conjugated to fluoresceins with different wavelengths it is possible to identify intact and disrupted acrosomes (Guillan *et al.* 2005). Alternative methods include the use of antibodies against acrosomal membrane proteins or fluorophores such as the Lysotracker family, or Deep Red TM, that label cell lysosomes (reviewed by Guillan *et al.* 2005).

Simultaneous assessments of plasma membrane and organelle intactness

Several sperm traits can be simultaneously assessed using flow cytometric multi-parametric analyses after staining spermatozoa with 4 dyes to identify: (a) membrane integrity using SYBR-14/PI; (b) acrosome integrity using phycoerythrin-conjugated peanut agglutinin (PE-PNA); and (c) functional status of the mitochondria using Mitotracker deep red (Nagy *et al.* 2003). Such analyses can be done during several hours of sperm incubation using multi-colour flow cytometry to disclose changes occurring in spermatozoa over time (Nagy *et al.* 2004).

Integrity of chromatin structure and DNA integrity

The spermatozoa of ruminants are characterized by having a highly condensed chromatin where protamines tightly pack and protect the haploid DNA. Optimal sperm DNA packing seems essential for full expression of male fertility potential (Spano *et al.* 2000) and handling of spermatozoa, in particular cryopreservation, has been related to a deterioration of DNA integrity (Peris *et al.* 2004). Over the past 25 years, several methods have been designed to determine DNA damage including the Comet assay, TUNEL, the acridine orange test (AOT), the tritium-labelled 3H-actinomycin D (3H-AMD) incorporation assay, the *insitu* nick translation (ISNT) assay, DNA breakage detection-fluorescence *in situ* hybridisation (DBD-FISH) and the sperm chromatin structure assay (SCSA) (reviewed by Fraser 2004). While most assays are basically microscopy methods, the TUNEL and the SCSA assays can advantageously use flow cytometry (Evenson & Wixon 2006). Although all methods indicate DNA strand breaks, the SCSA is used most commonly. The SCSA characterizes sperm nuclear chromatin stability based on the increased susceptibility of altered DNA to *in situ* de-naturation when exposed to very low pH. De-naturation is detected by staining with the metachromatic dye acridine orange which results in green fluorescence for native DNA and red fluorescence for denatured DNA. The degree of de-naturation within each sperm nucleus is quantified by flow cytometry (Evenson *et al.* 1980). The SCSA provides data of spermatozoa with or without DNA fragmentation, the extent of DNA fragmentation and the proportion of immature spermatozoa (Evenson & Wixon 2006).

In vitro assessment of sperm quality and its relationship to fertility

A multitude of laboratory semen evaluation methods have been designed during the last 3 decades to estimate male fertility, aiming to avoid the costs and the time otherwise needed to measure fertility through the AI of hundreds or thousands of females (Rodriguez-Martinez 2003). Among the large variety of tests, fertility appears to be more closely related to membrane integrity than to overall sperm motility, a fact which is especially clear when a large number of spermatozoa are evaluated with fluorescence-activated cell sorting (FACS) or fluorometry (Januskauskas *et al.* 2001).

However, most of these statistically significant relationships between semen tests and fertility varied greatly among studies; for example, correlations between *sperm motility* and fertility ranged between 0.15 and 0.83 (Kjaestad *et al.* 1993; Stålhammar *et al.* 1994; Bailey *et al.* 1994; Amann *et al.* 2000; Januskauskas *et al.* 2003). Even analyses of semen samples from AI-bulls with CASA instruments have shown variable correlations between patterns of post-thaw motility and *in vivo* fertility (r^2 = 0.45-0.63: Zhang *et al.* 1998; Januskauskas *et al.* 2001). Statistical analyses of combinations of motility patterns yielded stronger correlations (r = 0.68-0.98: Farrell *et al.* 1998) and predictive values could be presented (r^2 = 0.83) when the outcome of motility assessments were combined with other parameters of sperm function (Januskauskas *et al.* 2001) in AI-bulls. *Mitochondrial integrity* and *functionality*, quantified with specific fluorophores, still present both variable and low relationships with AI-fertility in rams and bulls (Martinez-Pastor *et al.* 2004; Hallap *et al.* 2005).

Relationships between *sperm morphology* and fertility have also been shown to exist, but vary widely (r = 0.06-0.86: Graham *et al.* 1980), depending on the type of abnormality (Barth 1989; Barth *et al.* 1992; Ostermeier *et al.* 2001), the quality of the examined semen and the methodology standards used (Kuster *et al.* 2004). Such variability has led to frustration among scientists and clinicians alike when individual bulls often perform much better or much worse than predicted by the breeding soundness evaluation (Higdon *et al.* 2000) and has led to continuous requests for thresholds of abnormalities. Such thresholds for the tolerable levels of abnormal spermatozoa date as far back as the 1920s and 1930s (Williams & Utica 1920; Williams & Savage 1925; Lagerlöf 1934), have been confirmed by numerous studies since then (Barth & Waldner 2002) and so it is now widely accepted that morphology is a main criterion in semen quality in cattle (Fordyce *et al.* 2006). A major point, often disregarded, is the need for large numbers of confirmed pregnancies or, even better calves, before attempting to establish relationships with fertility (Amann 2005). In well-controlled field trials with beef bulls, the relationship between the proportion of morphologically normal spermatozoa and calf outcome (Fitzpatrick *et al.* 2002) or non-return rates (Phillips *et al.* 2004) has proven important. However, when the semen examined is of good quality, such relationships tend to disappear (Rodriguez-Martinez *et al.* 1997b; Saacke 1999).

Membrane integrity, when assessed in large numbers of spermatozoa by fluorometry (Alm *et al.* 2001; Januskauskas *et al.* 2001) or flow cytometry (Guillan *et al.* 2005) is related to fertility after AI but correlations are low. More subtle, early membrane changes (lipid scrambling) indicated by Merocyanine-540 (Hallap *et al.* 2006) or the outer localisation of phosphatidylserine (PS) when examined by Annexin-V staining in frozen-thawed bull spermatozoa (Januskauskas *et al.* 2003), showed low (r = -0.22 to -0.27) negative correlations to fertility.

DNA integrity evaluated by the SCSA has shown a relationship to fertility (Evenson *et al.* 1994; Evenson & Jost 2000). The proportion of sperm nuclei outside the general population [COMPat], nowadays called the DNA fragmentation index (DFI), has been related to high, moderate or very low fertility in humans, when the COMPat (DFI) was 0-15%, 16-29% and >30%, respectively (Evenson & Jost 2000). Similarly, DFI thresholds for sub-fertility in boars and bulls have been proposed as 18% for boars and 20% for bulls (Rybar *et al.* 2004). Unfortunately, this latter report used semen from young, unproven Simmental bulls with low fertility (40-60% of 90d-NRR) and from boars without fertility data, thus leaving these thresholds of sub-fertility with little or no inherent value. However, in several well-controlled studies of AI-sires with proven and varied fertility it has been clear that proven bulls do not reach these high DFI values, even when there is a statistical relationship between SCSA and fertility. For instance, Januskausakas *et al.* (2001; 2003) found the COMP aat (DFI) of AI bulls with different fertility ranged from 1.2 to 8.0%; figures similar to those found in other AI-bulls in other countries

(Hallap *et al.* 2005). Therefore; significant; but largely variable relationships (r = 0.33-0.94) between this parameter and fertility have been reported for bull frozen-thawed semen used for conventional AI (Januskauskas *et al.* 2001; 2003) or heterospermic AI followed by paternity screening of fetuses using genetic markers (Ballachey *et al.* 1988).

In summary, it seems clear that only when studying handled spermatozoa, such as frozen-thawed semen for AI, are significant relationships found between modern rather than conventional semen analyses methods and the fertility achieved in the field. However, such relationships are modest and are disturbingly variable between laboratories.

How should we analyze a heterogeneous semen sample?

The ejaculate of a bull, ram or buck (or any other domestic mammal for that matter) is heterogeneous in the sense that none of the spermatozoa are equal to each other in terms of haploid genomic information and in attributes for fertilisation. Andrologists have been trying for decades to solve the question of which spermatozoa are involved in fertilization or how fertile a particular semen sample is. Sperm transport through the female genital tract imposes a series of sperm selection steps which eliminate a proportion of spermatozoa at each step, so that eventually only a small number gain access to the oocyte. Fertilisation seems, therefore, a matter of probability, based on sperm numbers and on proportions of spermatozoa with attributes that favour survival during the journey through the female genitalia and the effective interaction with the female epithelia and the oocyte. This concept suggests the presence of sub-populations of competent spermatozoa within the ejaculate, as previously discussed by Rodriguez-Martinez (2000; 2003) and Holt & Van Look (2004). Whether there is a relationship between the proportion of these competent (that is, potentially fertile) spermatozoa and the fertility of the semen sample (or the male) remains to be proven.

Considering fertilisation as a multi-factorial process, the combined assessment of a large number of parameters would lead to a higher accuracy of the test employed. To reach this goal, semen samples have been subjected to functional *in vitro* tests which are able to discern the ability of spermatozoa to undergo specific steps in the process of fertilisation and the triggering of the development of the early embryo. Among these methods we found the selection of spermatozoa via swim-up or double-phase partition in aqueous systems, the ability to bind to genital epithelia, the ability to undergo sperm capacitation or AR by exogenous stimuli, the ability to bind and penetrate the ZP and to fertilise *in vitro* (IVF). To determine whether these tests have biological value and would be able to determine the level of potential fertility of the semen before its application in the field, the results obtained have been related statistically with the semen or sire fertility (reviewed by Larsson & Rodriguez-Martinez 2000; Rodriguez-Martinez 2003).

Linearity of sperm movement - swim-up tests

Most ejaculated spermatozoa show a typical progressive and linear motility, and many display an innate ability to traverse fluids of a certain viscosity, which led to the so-called "swim-up tests" where spermatozoa are assessed for their capacity to traverse fluid barriers simulating *in vivo* conditions such as traversing the cervical barrier (Rodriguez-Martinez *et al.* 1997a). Experiments with frozen-thawed bull semen, using a simple swim-up across a column of culture medium have indicated the number of viable spermatozoa with linear motility post-swim-up reflects the innate fertilising capacity of a seminal sample (Zhang *et al.* 1998). Swim-up is,

moreover, a suitable method to separate a sub-population of spermatozoa with most attributes for further testing, specially those with stable plasmalemmae (Januskauskas *et al.* 2000a; 2000b; Hallap *et al.* 2004; 2005). Another method to isolate spermatozoa with linear motion is by centrifugal counter-current distribution analysis (CCCD). This is an aqueous two-phase partition system that has proven valuable for revealing sperm heterogeneity in semen samples and results have been correlated to fertility, albeit correlations were low ($r = 0.44$: Pérez-Pé *et al.* 2002).

Sperm binding to oviductal epithelium

Binding of spermatozoa occurs in the sperm reservoir of the oviduct, one of the presumed barriers to sperm progression to the site of fertilisation, prolonging sperm viability. Binding to oviductal epithelial cells also prolongs sperm life *in vitro*, presumably because the binding occurs only with non-capacitated spermatozoa (Levfebre & Suarez 1996). Consequently, sperm co-culture with oviductal epithelial explants has been used to determine the capacity of a semen sample to colonise the tubal reservoir and was reported to be correlated with bull fertility, but only when high quality sperm samples were tested (De Pauw *et al.* 2002).

Measurement of sperm capacitation-like phenomena in spermatozoa

Sperm calcium levels and CTC patterns relate to *in vivo* fertility of frozen-thawed bull spermatozoa (Collin *et al.* 2000). In Swedish dairy AI-bulls of known fertility, 30-40% of frozen-thawed spermatozoa showed capacitation-like changes when screened by the CTC-assay (Thundathil *et al.* 1999). The percentage of "non-capacitated" spermatozoa showed a significant relation to fertility ($r = 0.50$: Thundathil *et al.* 1999; Gil *et al.* 2000), indicating that the method could be used for prognostic purposes. For instance, the proportion of CTC-assessed "uncapacitated" bull spermatozoa recovered after swim-up of frozen-thawed AI-samples correlated positively ($r = 0.48$, $P < 0.05$) with their fertility in the field (Januskauskas et al.; 2000a; 2000b). When semen from AI-boars and bulls has been examined fresh and/or following cryopreservation, increases in "capacitation-like changes" (for example, destabilisation of the plasma membrane) measured with Merocyanine-540 (Peña *et al.* 2004; Hallap *et al.* 2006) or Annexin-V (Peña *et al.* 2003; Januskauskas *et al.* 2003) were detected, and even correlated to fertility (Januskauskas *et al* 2003; Hallap *et al.* 2006). However, in the boar these capacitation-like changes appear not to be similar to *in vivo* capacitation (Guthrie & Welch 2005) and are probably a consequence, reversible or not, of the process of cryopreservation.

Capacity for in vitro *acrosome reaction*

The AR can be induced in vitro by exposure to homologous, solubilised ZPs (Gil *et al.* 2000). It can also be induced by exposure to particular glycosaminoglycans (GAGs) known to be present in the oviductal fluid, where spermatozoa bathe before and during fertilisation. Heparin (Parrish *et al.* 1985; Whitfield & Parkinson 1992; Januskauskas *et al.* 2000a), heparan sulphate (Bergqvist *et al.* 2006) and chondroitin sulphate (Ax & Lenz 1987; Lenz *et al.* 1988) were able to induce the AR in vitro, and the degree of AR was significantly related to fertility. The AR can also be induced by treatment with calcium ionophores such as Hoechst A23187 which promotes a massive influx of Ca^{2+} into spermatozoa similar to that occurring during binding to the ZP. Significant correlations ($r = 0.60$) have been reported between the degree of AR-responsiveness and fertility (Whitfield & Parkinson 1995; Januskauskas *et al.* 2000b).

Zona pellucida binding tests

Binding of spermatozoa to the ZP is a critical step in the process of fertilisation. Capacitation is a pre-requisite to binding and it is followed by the AR induced by the ZP. The combination of these progressive steps is the rationale for *in vitro* sperm-ZP binding tests using whole, or hemi-ZPs. Using ZP-binding tests, significant correlations ($r = 0.50$) have been obtained with AI-fertility in bulls (Zhang *et al.* 1998).

Accessory sperm counts and in vitro penetration tests

Spermatozoa that did not penetrate the ZP entirely during fertilisation due to the zona reaction (an effective block to polyspermia) are trapped in the ZP of oocytes or early embryos and named accessory spermatozoa. These spermatozoa have demonstrated the attributes needed to penetrate the ZP and should therefore be considered as potentially fertile (Saacke *et al.* 1998). Rates of in vivo fertilisation and the number of accessory spermatozoa are statistically correlated (Saacke *et al.* 2000). Fertility can be also evaluated in vitro using oocyte penetration tests (Henault & Killian 1995; Brahmkshtri *et al.* 1999), where the presence of spermatozoa or pronuclei in the ooplasm determines the success of the test. Relationships between in vitro penetration and the fertility of the sires have been reported in bulls (Henault & Killian 1996) where spermatozoa from several sires were tested simultaneously. The spermatozoa from each sire were marked with fluorophores that render different fluorescence and thus their relative penetrability was measured, substantially diminishing variation.

In vitro fertilisation (IVF)

In vitro fertilisation closely mimics gamete-to-gamete relationships occurring during fertilisation *in vivo*, allowing measurement of different end-points in the stages of early embryo development. Thus IVF has been used to determine the relative fertility of semen samples in cattle: statistically significant relations between IVF fertility and fertility *in vivo* were sometimes found, mostly in retrospective studies (Larsson & Rodriguez-Martinez 2000) but also in prospective ones (Zhang *et al.* 1999). However, IVF is largely dependent on laboratory stability, thus making comparisons among studies and controls of variation difficult (Papadopoulus *et al.* 2005). Furthermore, as with many other tests of oocyte-spermatozoa interaction, the sperm:oocyte ratio *in vitro* is largely that *in vivo* thus reducing its diagnostic value (Hunter & Rodriguez-Martinez 2002).

Indicators of male fertility in seminal plasma

Analyses of seminal plasma have shown a series of proteins and other seminal factors with indirect relationships to fertility, particularly in bulls. Some of these substances are related to sperm function; for example, heparin binding proteins (HBPs)(Bellin *et al.* 1994; Moura *et al.* 2006), platelet activating factor (PAF) (Brackett *et al.* 2004), bovine seminal plasma protein A3 (BSP-A3) (Roncoletta *et al.* 2006), acid seminal fluid protein (aSFP) (Roncoletta *et al.* 2006) or fertility associated antigen (FAA) (Bellin *et al.* 1998; Sprott *et al.* 2000). Other substances are related to oviductal attachment, such as osteopontin (Cancel *et al.* 1997; Moura 2005; Moura *et al.* 2006) and sperm membrane antigen P25b (Parent *et al.* 1999), or to prostaglandin synthesis (for example, lipocalin-type prostaglandin D2 synthase: Gerena *et al.* 1998; Moura *et al.* 2006). Although correlations with fertility have been reported, this does not mean that these factors are general predictors of fertility, but

rather they indicate the complexity of factors involved in fertility. However, they may be useful to pre-screen individuals and separate extreme populations from further use.

Can the fertilizing capacity of a given semen sample be estimated *in vitro*?

A combination of results from a series of *in vitro* tests analysed by multiple regression has often been used to show significant relationships between laboratory assays and fertility in bulls (Linford *et al.* 1976; Januskauskas *et al.* 1996; Zhang *et al.* 1997; Januskauskas *et al.* 1999; 2000a; 2000b; 2001; Tanghe *et al.* 2002). These studies had a retrospective nature using bulls with wide ranges of fertility; that is, they were not predictive *per se* (Rodriguez-Martinez 2003). However, it has been argued that *in vitro* data could be predictive of *in vivo* fertility if the results of various *in vitro* methods of frozen semen evaluation could be combined to calculate the *expected fertility* of, for instance, young bulls. This approach was used with frozen-thawed semen from unproven bulls (11-13 months of age) whose potential fertility was estimated in the laboratory before their real fertility in the field was assessed by AI (Zhang *et al.* 1999). With this approach, a strong relationship ($r = 0.90$) was found between predicted and real fertility. This *in vitro* testing enabled identification of sub-fertile bulls, whose expected and real fertility was below the limit considered for sub-fertility (62% non-return rate), while the other young bulls predicted to have satisfactory fertility had non return rates of $\geq 65\%$. However, a large variation was evident among ejaculates within a young sire, both *in vitro* and *in vivo*, even when ejaculates were collected within a relatively short period (Zhang *et al.* 1998). This implies that it is necessary to analyse a large proportion of ejaculates from a large bull population in order to lower this variation to a minimum (Zhang *et al.* 1997). Moreover, semen parameters might change with age (Hallap *et al.* 2005), impairing our ability to make reliable predictions over time.

Conclusions

Male sub-fertility is a problem of complex etiology, requiring a complete andrological work-up for proper diagnosis and prognosis. At present, veterinary andrologists have access to a large battery of diagnostics aids but still lack simple comprehensive tests to estimate levels of fertility in male ruminants before they are used for natural mating or their semen is processed for breeding via AI or other ARTs. A major point derived from the present review is the lack of strategies to determine the presence of sperm subpopulations with the ability to surpass sperm selection steps present *in vivo*. Some clear examples appear in the value of swim-up procedures, in the screening of handicapping morphology, in the ability of spermatozoa to respond to pre-fertilisation stimuli (for example, bicarbonate-mediated triggering of membrane scrambling and the associated changes in metabolism), and at the same time measure their lifespan after these stimuli, and their resilience to acrosome exocytosis. Obviously, defining sperm subpopulations is difficult when handling billions of spermatozoa in an ejaculate. Common sense calls for the preparation of AI-doses with low sperm numbers that can be tested for fertility both *in vitro* and *in vivo* before the sire can be allocated into a certain level of potential fertility. This must be accompanied by a complete andrological examination before breeding and proper strategies (management as well as nutritional) to ensure good development of the male from calfhood. Measurement of LH surge and of insulin growth factor-I (IGF-I) following challenge with GnRH in bull calves are simple procedures that might monitor potential testicular development and, consequently, estimate potential fertility. Post-pubertal screening of fertility-associated factors for pre-allocation of sires is a promising strategy.

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