Anim. Reprod., v.10, n.3, p.268-276, Jul./Sept. 2013

Sperm biotechnologies in domestic species: state of the art

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Abstract

Modern livestock breeding is basically dependent on the proper use of semen for artificial insemination (AI) of females and of other reproductive biotechnologies such as the production of embryos in vitro for embryo transfer (IVP). Both these techniques have made possible not only the wide dissemination of genetic material onto breeding populations but also enhanced the selection of best sires, owing to the development of better diagnostic techniques for sperm function and of preservation of seminal material over time. Although use of liquid semen cooled to room temperature, to intermediate temperatures (+16-20°C) or chilled (+5°C) dominates in different species, cryopreservation is preferred in bovine AI and it is advancing in other species by the design of new containers, freezing methods and the use of better insemination strategies. Techniques to separate the aliquot of most robust spermatozoa from an ejaculate have shown a renascent particularly for sires with low sperm quality, and technological advances in separating spermatozoa for chromosomal sex make the technique suitable for commercial use, following application of novel findings in sperm and seminal plasma (SP) diagnostics and function. Alongside, knowledge of the epigenome and signalling capabilities of the semen (sperm and SP) calls for further studies regarding transgene production via ICSI for IVP or AI.

Keywords: AI, cryobiology, IVF, selection, semen, sexing.

Introduction

Generations of reproductive biotechnologies have been developed as routine applications to safely propagate genetic material among breeding populations. Moreover, they intend to shorten generational intervals and to focused selection of specific traits and production of specific genders. In some of these technologies such as the predominant AI and in IVP for embryo transfer, focus is on spermatozoa and recently on SP. Furthermore, new challenges in cryobiology, sperm desiccation, sperm selection and sperm survival to manipulations have arose, particularly in species where selection for sperm quality has not been primarily addressed. Semen is still the "cheapest" component of artificial breeding, something that explains the dominancy of AI over any other reproductive biotechnology. AI is, alongside its sanitary advantage, the best technique to issue the large dissemination of desirable genetic characters on a female population, propagating the genetic material of selected stud sires, which are continuously replacing the best ones we presently use (Rodriguez-Martinez, 2012a). Successful freezing of semen of all livestock is a long lasting priority, tied not only to acceptable cryosurvival and lifespan after thawing but also to the devise of rational techniques that can provide largest possible numbers of doses for AI at the lowest possible cost. Last but not least, the deposition of these doses is to be easy and yield acceptable fertility, i.e. close to use of cooled semen or even natural mating. Unfortunately, a better knowledge of the moment of spontaneous ovulation is mandatory, demanding a holistic approach to the technology of semen processing and use.

The present review summarizes the state-of-the-art in some prevailing semen biotechnologies such as sperm (cryo)preservation for use in AI or IVF, focusing on low-sperm number AI-doses, and spermatozoa subjected to sperm selection techniques including sex-sorting via flow cytometry or other techniques. Particular attention is taken on the feasibility of these techniques for commercial application in view of the high susceptibility of spermatozoa to be modified or succumb alongside the treatments. Renascent manipulations such as alternative preservation methods or their use as DNA/RNA adsorbed vectors for transgene production is also included.

Sperm preservation

Semen preservation has historical roots that document back to the 18th century, with a boom experienced during the first half of the 20th century in relation to the development of AI with liquid semen. From the 1950s, the application of cryoprotectants contributed to the wider use of semen freezing, particularly for the application of intrauterine AI in dairy cattle. During the past 40 years, the development and use of AI with preserved semen have grown exponentially and on a global scale, particularly in the breeding of dairy cattle (>200 million of the first AIs in

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Received: May 6, 2013
Accepted: June 13, 2013

the world use frozen semen) and pigs (>160 million used cooled liquid semen doses). In Europe, the Americas and South-east Asia, sows are basically bred via AI with liquid (non-frozen) semen, mimicking the situation already reached in dairy cattle. Cattle semen is cryopreserved using standardized methods for extension, cooling, freezing and thawing basically all over the world, with only subtle differences between *B. Taurus*, *B. indicus*, *Bubalus bubalis* or *Bos javanicus* (Rodriguez-Martinez, 2007a; Rodriguez-Martinez and Barth, 2007).

However, the current available methods to preserve semen as a genetic resource and its successful dissemination via AI and other assisted reproductive technologies (ARTs) are still sub-optimal (Rodriguez-Martinez, 2012a, c). This is a fact even in dairy cattle, visible when sperm numbers per dose are lowered after sex sorting, and survival of potentially fertile spermatozoa reaches a threshold and fertility does not reach values higher than 60-70% of the fertility of the conventional (non-sexed) semen (Rodriguez-Martinez, 2012a, c). Other species, such as porcine, equine, canine and ovine, seem condemned to using extended chilled liquid semen despite its limited shelf-life, its decline in fertility over transit time, and risks of damage due to temperature, pressure or handling changes. While use of liquid-chilled semen has grown exponentially over the past decades, use of frozen semen is restricted for these species to 1-3% of total AIs worldwide (Rota *et al*., 1997; Gil *et al*., 2003; Riesenbeck, 2011). Semen is still being "best" cryopreserved using slow-freezing protocols originally devised in the mid 1960-1970´s with empirical modifications introduced over time (Katkov, 2012; Morris *et al*., 2012; Rodriguez-Martinez, 2012b). In general, extension is done using egg yolk or milk-containing media in most livestock following the extension or removal of most of the SP. The freezing media most often includes glycerol as cryoprotectant (CPA) and in some species a surfactant (often laurylsulphate, Orvus es Paste-OEP) is also included. Spermatozoa are cooled beyond the eutectic temperature at 30 to 50°C/min and thawing is done at 1,000-1,800°C/min (see Katkov, 2012). The entire freezing procedure takes up to 8-9 h from semen collection to storage of the frozen doses in LN_2, and for some species yields few AI-doses per ejaculate (Rodriguez-Martinez, 2012b). Variation is still present between ejaculates and among males for their capacity to survive cryopreservation. Often, cryopreservation protocols have to be modified to accommodate males with suboptimal sperm freezability (so-called bad freezers), particularly regarding glycerol concentration and warming rates (Knox, 2011). Those changes usually allow for minimum acceptable cryosurvival (i.e. between 30 and 40%), confirming its suboptimality. Moreover, the weakening of the surviving cells (lifespan, deteriorated attributes, fertilizing capacity, genome damage), leads to lowered fertility (Rodriguez-Martinez, 2012a), which implies we are far from reaching the goals set up by the industry for frozen-thawed semen: >80% conception rates and, for pigs, a litter size of 11 piglets (Knox, 2011). This scenario limits most frozen semen to research, genetic banking or the export of semen for selected nuclei lines, impairing its wider use. Publications in species with clearly suboptimal freezing capacity increased from 18 in 1980 to 2,103 in 2012 (Pubmed http://www.ncbi.nlm.nih.gov/pubmed/. Access on April 12, 2013), indicating the solution is yet to come. Obviously new cryopreservation methods ought to be developed.

Advances in sperm cryobiology?

Over the past decade, the cryobiology of semen has diminished its empiric approach towards (i) the determination of *in vivo* features (particularly regarding SP-composition), (ii) the action of specific additives and different CPA, (iii) the use of automated freezers and of directional gradient freezing and, (iv) the freezing of lower-sperm numbers/dose owing to intrauterine AI or use of sexed semen (Rodriguez-Martinez, 2012c). Yet, there is an intrinsic male-to-male variation that needs clarification. Methods for cryopreservation have not changed much. Spermatozoa are frozen slowly, with extracellular ice formation, dehydration, a toxic hyper-concentration of intracellular solutes which does not resolve during thawing, and jeopardizing cell survival or affecting vital cell functions post-thaw (Rota *et al*., 1997; Gil *et al*., 2003; Saragusty *et al*., 2009; Morillo-Rodriguez *et al*., 2011; Rodriguez-Martinez, 2012b). Solute-caused damage can be minimized by CPAs (such as glycerol, dimethyl sulphoxide [DMSO], ethylenglycol [EG], propyleneglycol [PG]) all highly soluble, permeating compounds of low-to-medium toxicity at low concentrations. They increase the total concentration of all solutes in the system, thus reducing the amount of ice formed at any given sub-zero temperature; *slow equilibrium freezing* (Pegg, 2007; Saragusty and Arav, 2011). With sufficient CPA introduced, high cooling rates (i.e. dipping into LN2) would eventually solidify the sperm suspension into a metastable glassy, vitreous state with no ice formed; rapid, non-equilibrium *vitrification* (Pegg, 2007; Saragusty and Arav, 2011). Ultra-high rates void the need of toxic penetrating CPA and opens for non-penetrating CPA (such as sucrose or trehalose). Use of these CPAs has made possible the vitrification of dog, human and rabbit spermatozoa, yet with low survival (Sánchez *et al*., 2011; Isachenko *et al*., 2012; Rosato and Iaffaldano, 2013). Ultra-high cooling rates (10,000 ^{o}C/min; Rodriguez-Martinez, 2012b) are seen a major prerequisite for improvement (Arav and Natan, 2009; Saragusty and Arav, 2011).

Basic research opens new possibilities

In species with fractionated ejaculation (canine, porcine, equine, human) spermatozoa are mainly

ejaculated in SP with specific SP-proteins (Rodriguez-Martinez *et al.,* 2011) and anti-oxidant enzymes, such as paraxonase-1 (PON1, Verit *et al*., 2009), which effectively sustain sperm survival. Although ejaculated spermatozoa are an heterogenous population, they certainly share basic concepts: (i) a sperm head with substantially less free water than the neck and tail segments (Morris *et al*., 2012), (ii) consistent use of anaerobic glycolysis alongside aerobic respiration (Silva and Gadella, 2006), (iii) a membrane with water channels including glycerol-transporting aquaglyceroporins (Ekwall, 2007), (iv) plasmalemmal propensity to lipoperoxidation (LPO) by exposure to reactive oxygen species (ROS; Ortega Ferrusola *et al*., 2009), which (v) facilitates for oxidative DNA damage under sperm storage (Aitken and Koppers, 2011). Stem cells (Darzynkiewicz and Balazs, 2012), appear often in niches surrounded by low O_2 levels (pO_2 1-6%, against 2-9% in most tissues or 21% in air). Such "hypoxia" levels prevent cell differentiation (Eliasson and Jönsson, 2010). Hyaluronan (HA, the *in vivo* existing polyanionic form of hyaluronic acid) an abundant, ubiquitous, non-antigenic glycosaminoglycan, well-conserved over taxa (Volpi *et al*., 2009) is also present in these niches. By comparison, the low pH, low bicarbonate and low O_2 conditions of the epididymal caudae preserves sperm survival and fertilizing capacity (Rodriguez-Martinez *et al*., 1990; Rodriguez-Martinez, 1991), as when lowering extender pH (Lafluf *et al*., 1990; Rodriguez *et al*., 1994). The same conditions seem to be present in the oviductal sperm reservoir (Rodriguez-Martinez, 2007b; Preston and Sherman, 2011) with HA being conspicuous in pigs and cows (Rodriguez-Martinez, 2001) preventing sperm capacitation and acrosome exocytosis (Tienthai *et al*., 2004; Bergqvist *et al*., 2005). HA has been reported as protecting cells from ROS-induced DNA damage by chelating Fe^{2+} and Cu^{2+} ions that contribute to formation of OH-radicals (Balogh *et al*., 2003). Spermatozoa express HA receptors on the cell surface, apparently with specific roles for maturation, motility and fertilization (Tienthai *et al*., 2003). HA favors preservation of stemness during long-term culture of embryonic stem cells (Ramirez *et al*., 2011) and embryo development in several species (Gardner *et al*., 1999; Suzuki *et al*., 2002; Palasz *et al*., 2006). Considering the above and the fact that HA has proven a good CPA for somatic cells (Ujihira *et al*., 2010) and beneficial for boar sperm cryosurvival (Peña *et al*., 2004), a combination of relative low O_2 levels (hypoxia), HA and specific SP-components ought to be tested while preserving spermatozoa via vitrification.

MicroRNAs, not to be forgotten…

Semen contains spermatozoa with a nuclear genome that has to be intact to participate in embryo development, so DNA is explored for intactness when sperm is manipulated and cryopreserved (see above). Semen also delivers a series of small regulatory non-coding RNAs (ncRNA, 19-22 nucleotides; Bartel, 2009), microRNAs (miRNAs) shed both in the SP (Belleannee *et al*., 2012; Wu *et al*., 2012) and also present within each spermatozoon (Hamatani, 2012; McIver *et al*., 2012). These miRNAs are key post-transcriptional modifiers of gene expression, e.g acting epigenetically, and play an important role in the acquisition and maintenance of male fertility (Dadoune, 2009). Numerous in bull sperm, they show differential expression in relation to fertility levels of the sires (Govindaraju *et al*., 2012). Delivered to the oocyte at fertilization, they modulate first cleavage divisions (Liu *et al*., 2012). In freeze-resistant insects but also vertebrates, they are responsive to freezing, and may act to rapidly regulate metabolic responses to survive freezing stress (Biggar *et al*., 2009).

But, does cryopreservation cause epigenetic alterations in chromatin structure?

Reports are controversial. Suboptimal cryopreservation leads to alterations in chromatin structure, often in relation to cell viability restrictions in spermatozoa of several species (Rodriguez-Martinez, 2012b), in human oocytes (Monzo, 2012) or in murine blastocysts (Larman *et al*., 2011). Significant down regulation of house-keeping and function related genes have been seen (Karimi-Busheri *et al*., 2013), as well as changes in transcriptomes in embryonic stem cells (Wahg *et al*., 2011), while opposite reports are also available regarding mesenchymal stem cells (Angelo *et al*., 2012). Hence, there is a large need to resolve how different methods of cryopreservation impact the epigenetic fingerprint of spermatozoa.

Low-sperm numbers in AI doses: the prevailing trend

Despite the above exposure, preservation of semen for AI is routine providing semen of good quality for AI on commercial basis, despite using excessive sperm numbers per AI-dose. The reason behind is the low cryosurvival we experience (most often less than 50% of the spermatozoa survive) and, despite we still struggle in trying to understand how spermatozoa lose their capacity to remain fertile upon freezing and thawing, many of the survivors have changed attributes, among which a shortened life-span is predominant. There is an overall tendency among AI-semen cattle producers to reduce sperm numbers per AI-dose. Several reasons are behind this trend; (i) to increase revenues, (ii) to select sires by their innate optimal fertility level and, (iv) to accommodate the increasing use of sex-sorted semen. While spermatozoa from most sires survive cryopreservation using current protocols, some others do not, for reasons not yet fully understood. More crucial is the freezing of flow-cytometrically sex-sorted

spermatozoa, whose membrane is clearly affected by the processing and thus require of particular modifications in the freezing protocols (Parrilla *et al*., 2012; Balao da Silva *et al*., 2013) including the restoration of SP (de Graaf *et al*., 2008) or of specific SP-proteins (Caballero *et al*., 2012). The AI of low-sperm doses, including those containing sex-sorted semen by flow cytometry (Garner and Seidel, 2008), is usually carried out more or less deep into the uterine horn with acceptable results in bovine (Ballester *et al*., 2007; Seidel and Schenk, 2008; Schenk *et al*., 2009) or porcine using the deep-intrauterine AI procedure (Roca *et al*., 2011) specially when using frozen-thawed boar semen or sex-sorted semen effective by properly trained deep AI (Wongtawan *et al*., 2006; Roca *et al*., 2011).

Sperm selection

Sperm selection is a wide term, comprising methods for separation of spermatozoa for *in vitro fertilization (IVF),* for sperm size, for membrane and DNA intactness, for motility, or for the enrichment of the population of spermatozoa bearing either an X- or a Y-sexual chromosome (sex-sorting). For IVF, washing by extension and centrifugation, filtration/gradient separation or self-motility (the so-called swim-up or -down) techniques are most common (Rodriguez-Martinez *et al*., 1997). The grade of enrichment of the most robust spermatozoa from the ejaculate (i.e. the effectiveness of the method) depends, however, on the sperm numbers present and whether the sample contains a high proportion of abnormal spermatozoa. Neither case is common among livestock ejaculates. Some techniques are, however, used for enrichment after thawing, where the amount of surviving spermatozoa is low and there is an interest to remove dead, moribund and abnormal spermatozoa. Most farm animal spermatozoa in a normal semen sample show a typical progressive, linear motility. Spermatozoa use this innate linearity to convey natural fluid barriers such as the cervix or the uterine-oviductal junction and thus it has been related to fertility in farm animals, thus leading to methods that mimic *in vivo* situations, as swim-up. By overlaying a semen sample with an appropriate volume of a suitable fluid (often culture medium but also with more complex preparations of varying viscosity) followed by incubation, during which time the motile spermatozoa migrate actively into the overlay medium, this simple procedure selects for sperm motility and membrane integrity, essential parameters for fertilization. Sperm numbers and their speed when traversing the column might define the most fertile sample. The method separates a sperm sub-population with most intact attributes for further testing, such as membrane, acrosome, mitochondrial, and DNA integrity as well as a higher penetrability of the zona pellucida (ZP), and higher ability to produce blastocysts after IVF (Zhang *et al*., 1998). Additives for the swim-up media have proven beneficial; for instance, recovery and cleansing of high quality bulls spermatozoa are facilitated by adding homologous or heterologous cervical mucus or hyaluronan (a component of the oviductal fluid) to the swim-up medium (Shamsuddin and Rodriguez-Martinez, 1994). Another method to separate by linearity is the centrifugal counter-current distribution analysis (CCCD), an aqueous two-phase partition system, which has proven valuable for revealing sperm heterogeneity in semen samples and, indirectly, shown correlations to fertility (revised by Rodriguez-Martinez, 2007c). Novel methods have recently been developed using alternative multiple microfluidic flow streams for sperm self-migration which allow for the sorting of motile spermatozoa (Smith *et al*., 2011; Wang *et al*., 2011). In sum, self-migration procedures select spermatozoa as *in vivo*, but they can not isolate large sperm numbers, becoming mostly suitable for IVF.

Other methods have, therefore, put forward as substitutes where a higher output of an intact population is selected, a prerequisite when dealing with farm animals having large ejaculates. Examples of these methods are the centrifugation through columns of adherent particles (Sephadex or glass-wool, Januskauskas *et al*, 2005) or the differential centrifugation through discontinuous density gradients of silane-coated silica spheres (Rodriguez-Martinez *et al*., 1997). Centrifugation through a single column of species specific formulations of colloid (based on silate-coated spheres, the SLC method) has proven successful to harvest the most robust spermatozoa from any (raw or serially processed) semen suspension, in most species tested so far (Morrell and Rodriguez-Martinez, 2009, 2010; Morrell *et al*., 2010). The selective power of the latter method is clearly related to species differences in osmolarity and density of the colloid (Morrell *et al*., 2011), being more advantageous in terms of less damage and better recovery rate for spermatozoa with intact fertilizing attributes.

Sperm sex-sorting

Gender selection is, in livestock production, highly desirable; heifers for dairy cattle, males for beef cattle. In pig production, it would also allow the production of either male or female crossbred lines, or ameliorate the incoming problem of the banning of male piglet castration in Europe. Using the Beltsville Sperm Sexing Technology, based on high speed flow cytometry sorting of DNA-stained spermatozoa with difference in size (and thus emitted fluorescence to a laser beam exposure) between the sex chromosomes (revised by Johnson *et al*., 2005) the enrichment of AI-doses for either X- or Y-chromosome-bearing spermatozoa is now a commercial reality. At commercial level, doses, enriched to >90% level have resulted in the birth of >50,000 documented calves of the desired sex, with

>95% or success, and figures are increasing (Garner, 2006; Schenk and Seidel, 2008; Garner and Seidel, 2008; Seidel, 2009). Although the numbers of sorted spermatozoa per hour reach at present larger figures than a decade ago (100-200 million compared to 350000), these numbers yet imply few sperm doses produced, impairing their application for conventional AI. However the technology is facing today a strong wave of increasing commercialization in cattle (Garner and Seidel, 2008; Seidel, 2009) as well as it is becoming promising for other species (porcine, equine), including the provision opportunities for sex selection of IVP-embryos, surpassing the need for sex diagnosis of embryos (Blondin *et al*., 2009; Carvalho *et al*., 2010). Although the only fully validated technology for pre-selecting offspring for sex available at present is the Beltsville method, new applications are coming into the market such as use of antibodies against sex-specific proteins, which can -by immobilizing spermatozoa of a certain sex- allow for handling of the spermatozoa bearing the other (Cattle Logic Ltd, UK). Sex-sorting is however, still too costly (a flow sorter costs above US$ 300000), slow, and still yields spermatozoa with reduced lifespan (Lonergan, 2007; Gosalvez *et al*., 2011). Nevertheless, the products (male- or female-sorted spermatozoa) are available and becoming more competitive by the day (Hayakawa *et al*., 2009; Underwood *et al*., 2009, 2010).

A series of problems yet shadows the commercial application of sex-sorted boar or stallion semen for AI; firstly, the well-known low survival of the sorted spermatozoa, a matter that affects spermatozoa from all species; related to the high pressure and to the extreme sperm extension applied during the process (Vazquez *et al*., 2009), conveying detrimental effects of the absence of SP-components (Caballero *et al*., 2012). Secondly, sperm sexing is slow, thus inappropriate for standard pig AI, requiring deep-intrauterine AI (see above). The above mentioned drawbacks of the current technology have been compensated by the use of additives to the sperm-media (mostly as SP) and the growing application of deep intrauterine AI (Vazquez *et al*., 2009). Freezing of sex-sorted boar spermatozoa has been tested and proven usable for IVF; the embryos obtained are capable of establishing pregnancies to term (but not carry to term) after nonsurgical ET (Bathgate *et al*., 2007). Obviously, owing to the enormous impact that sperm-mediated gender selection would have in pig production, a further development of the flow cytometry sex-sorting technique or alternative methods are hoped for, demonstrating how far they might be from commercial use.

Spermatozoa as vectors for transgene production

Sperm-Mediated Gene Transfer (SMGT) has been shown to be a practical method to produce transgenic animals, without requiring embryo handling or expensive equipment (Lavitrano *et al*., 1989). Although the mechanism governing foreign DNA integration is not well understood and yet controversial (Eghbalsaied *et al*., 2013), SMGT has proven to be highly efficient in integrating the transgene into the genome of the pig embryo (57-80%, based on ~200 generated pigs, compared to microinjection (Lavitrano *et al*., 2006). Transgenic pig lines produced by SMGT have been reported since 1997 (Lavitrano *et al*., 1997, 2002, 2003) and the methodology is well established (Lavitrano *et al*, 2013). SMGT efficiency has been shown to be increased by ICSI of spermatozoa coated with DNA (Kurome *et al*., 2006) and proven to work in other domestic animals, such as equine (Zaniboni *et al*., 2012) or bovine (Simões *et al*., 2012).

Future trends

Development of better (cryo)preservation methods for pigs are a priority for our research group. AI is a low-cost technology for commercial pig production and therefore making it interesting to maintain and select specific stud boars, similarly to what is practice in cattle. For instance, if the aim is to diminish undesirable alleles in a general population, a logical way is to start genotyping stud boars genetic markers related to these alleles and amortize such investment (and that of semen freezing) by selling the semen doses of those boars found free. The other interested party is the producer with an elite breeding herd, who is the most interested to buy semen from the genotyped boars with two good alleles for a particular gene, since they can, using AI, reduce the frequency of an undesirable allele in their population by 50% on each generation. Once this elite breeding herd is free, the original selection has paid off, when semen from these elite stud boars is used in multiplier and commercial herds. Associated with gender selection, freezing of marker-assisted selected stud sire herds appears extremely synergistic.

Cryopreservation of semen is, however, suboptimal since variation between species, individuals, and even between sperm subpopulations is so clearly marked. Selection pressure put on freezability is not always possible. It has worked for dairy bulls (one basic freezing method is used to discriminate among young sires) but will probably not be used with the same impetus when fewer bulls are to be recruited by genome selection of calves, as it is applied at present. We would therefore see situations similar to other species, where semen cryopreservation methods are “adapted” for a particular sire in order to “save” the genetic value it represents. Preselection of the most robust spermatozoa is considered for instance to increase the use of otherwise “weak” sires (as in equine), but will not be effective in other species unless related to sex-sorting, of functional genomic selection. Despite suboptimality,

conventional slow equilibrium freezing methods are thus to continue being the major option. Alternative methods such as vitrification (which avoid the risk of ice damage and solute toxicity) are less likely to be applied simply because of the size of the sperm sample that needs to be frozen, unless a breakthrough is reached. The same applies for freeze-desiccation since the number of sperm needed for the only commercially competitive method of AI is too large. On the other hand, both vitrification and sperm-drying of small sperm suspension volumes are realistic options for IVF and production of embryos *in vitro* for ET, in any livestock species and most realistic in equine, where ICSI is routinely practiced.

Diagnostic tools for semen assessment are going to continue developing, involving both spermatozoa and the surrounding SP. Whether this will require use for sophisticated instrumentation (as flow cytometers still are) or if the level of simplification in this sector with advance more rapidly that what has been the case leading to more accessible bench- or field instruments is yet to be awaited. Most likely we shall see cheaper, operator-friendly instruments in a few years. Ultimately, development in terms of semen biotechnology will depend on the costs it implies for the designers and the enterprises. After all, semen is still the "cheapest" component of livestock breeding.

Acknowledgments

The studies of the author have been made possible by grants from The Swedish Research Council FORMAS, and the Swedish Research Council (VR), Stockholm, Sweden.

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