Semen technologies in domestic animal species

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Implications

• Artificial insemination (AI) has increased the dissemination of genetic material and enhanced the selection of best sires and dams.
• Use of cooled liquid semen for AI still dominates in most species, while cryopreservation is the rule in dairy cattle. However, advances in the development of new containers, freezing methods, and AI strategies are likely to increase the use of frozen semen in other species.
• Gradient separation methods are reascent to harvest the most robust spermatozoa from semen from sires, even those with low sperm quality or when sperm with a specific chromosomal sex are needed.
• Sperm sexing is commercially available today in dairy cattle and, following application of novel findings in sperm- and seminal plasma diagnostics, will likely lead to its commercialization in other species.
• Current research in the epigenetic capabilities of sperm should lead to improved diagnostic tests for male fertility, which could lead to new procedures for producing transgenic animals via AI.

Key words: artificial insemination, chromosomal sex-sorting, cryobiology

Introduction

Reproductive biotechnologies have been developed for routine, microbiologically-safe propagation of genetic material among breeding populations. Moreover, they also shorten generation intervals and allow for selection of specific traits in a given population. Semen still is the “cheapest” component of artificial breeding. Therefore, AI is the most prevalent reproductive biotechnology used on a global scale and, depending on the species, requires the preparation of liquid, chilled, or frozen-thawed insemination doses (Rodríguez-Martínez, 2012a). Consequently, research efforts still focus on improving the quality of sperm exposed to manipulations required during their preparation for AI, especially the ones associated with sperm cryobiology. Successful freezing of semen from all livestock is a high priority due to its numerous advantages including biosecurity and disease monitoring, conservation of specific genetics, and global commercialization of superior genotypes. Cryopreservation success is linked not only to acceptable survival and lifespan post-thaw, but also to acceptable fertility after AI.

If fertility levels with frozen semen are ever going to be similar to those with fresh semen, then a holistic approach for improving semen processing technologies must be used. This requires a critical assessment of semen and sperm function. Semen is heterogeneous population of sperm bathed in seminal plasma of heterogeneous composition (Rodríguez-Martínez et al., 2011). The spermatozoa in semen have the following unique characteristics that need to be considered during their manipulation: 1) their head contains substantially less free water than their neck and tail segments (Morris et al., 2012); 2) they use both anaerobic glycolysis and aerobic respiration for energy production (Silva and Gadella, 2006); 3) their plasma membrane contains water channels including glycerol-transporting aquaglyceroporins (Ekwall, 2007); and
4) they are prone to lipoperoxidation (LPO) by exposure to reactive oxygen species (ROS; Ortega-Ferrusola et al., 2009a), which contributes to oxidative DNA damage when they are stored (Ortega-Ferrusola et al., 2009b).

The primary focus of this review is to summarize the current state of the art in cryopreservation and storage of liquid semen for use in AI. Emphasis is placed on which technologies, at present, present the best opportunity for commercialization. A secondary objective is to discuss the production of sexed insemination doses and the potential spermatozoa have for use as DNA/RNA vectors for transgene production.

**Sperm Preservation**

The earliest documented efforts in semen preservation occurred in the 18th century. However, the most active period of research occurred during the first half of the 20th century. Since the 1950s, inclusion of cryoprotectants facilitated the wider use of semen freezing, particularly when used in conjunction with intrauterine AI. During the past 40 years, the global development and use of AI with preserved semen has grown exponentially, particularly in dairy cattle (>200 million frozen semen doses) and pigs (>160 million cooled liquid semen doses). Sows and dairy cattle in Europe, the Americas, and Southeast Asia are almost exclusively bred via AI. Bovine semen is cryopreserved using standardized methods for extension, cooling, freezing, and thawing all over the world with only subtle differences between *Bos taurus*, *Bos indicus*, *Bubalus bubalis*, or *Bos javanicus* (Rodríguez-Martínez and Barth, 2007; Rodríguez-Martínez, 2007a, 2012a). However, even for cattle, the current methods for semen freezing are suboptimal. Survival of potentially fertile spermatozoa is low and their subsequent fertility often is under 50% (Rodríguez-Martínez, 2012b). Porcine, equine, canine, or ovine species seem condemned to the use of extended chilled liquid semen for AI since development of techniques for their cryopreservation lag behind those of cattle. This is in spite of its limited shelf life, decline in fertility during storage, and damage due to temperature, pressure, or handling changes. In these species, use of frozen semen is restricted to 1 to 3% of total use of AI worldwide (Rota et al., 1997; Gil et al., 2003; Rodríguez-Martínez, 2007b; Riesenbeck, 2011).

Semen is “best” cryopreserved using slow-freezing protocols originally devised in the mid-1960s to 1970s with empirical modifications introduced since then (Katkov, 2012; Morris et al., 2012; Rodríguez-Martínez, 2012c). Generally, semen is extended in egg-yolk or milk-based media followed, in many species, by the removal of seminal plasma and inclusion of surfactants. Freezing media most often include glycerol as a cryoprotectant so that spermatozoa can be cooled beyond their eutectic temperature at 30 to 50°C/min. Inclusion of cryoprotectants also allows for increased thawing rates between 1,000 and 1,800°C/min (Katkov, 2012). The entire freezing procedure can last, depending on the species, between 2 and 9 hours from semen collection to storage of the frozen doses in liquid N₂. For some species, particularly pigs, cryopreservation yields few AI doses per ejaculate (Rodriguez-Martinez, 2012c). These aspects make the process unattractive for broad commercialization.
The ability to withstand cryopreservation varies between ejaculates and, particularly, among males. To attain minimum acceptable sperm viability, processing protocols are often modified to accommodate sires with suboptimal freezability. These are often referred to as “bad freezers.” Even with these adjustments, surviving spermatozoa have a shortened lifespan, genome damage, and reduced fertilizing capacity (Rodriguez-Martinez, 2012a). Although research in species with poor semen freezing capacity has gained more attention as seen by an increase from 18 publications in 1980 to 2,103 in 2012 (according to PubMed, 4 Dec, 2013), the goal of routinely producing litters of 11 live piglets from 80% of the sows bred, which is needed by the industry for adoption of the technology, remains elusive (Knox, 2011). To close the gap between where we currently are and where we need to be, significant investments in research and technological developments are needed.

**Developments in sperm cryobiology: towards vitrification?**

Most methods for cryopreservation were developed 30 to 40 years ago and involve the use of cryoprotectants (Rath et al., 2009). Cryoprotectants are necessary due to changes that occur in all cells during freezing, including spermatozoa. When sperm are frozen, ice forms extracellularly and cells dehydrate (Pegg, 2007; Saragusty and Arav, 2011). This leads to toxic concentrations of intracellular solutes, which do not return to basal concentrations during thawing, thus jeopardizing cell survival and handicapping vital cell functions post-thaw (Rota et al., 1997; Gil et al., 2003; Saragusty et al., 2009; Morillo-Rodriguez et al., 2011; Macias-Garcia et al., 2012a,b; Rodriguez-Martinez, 2012c). Solute-induced damage can be minimized by selective use of cryoprotectants such as glycerol, dimethyl sulfoxide, ethylene glycol, and propylene glycol. All are highly soluble, permeating compounds of low-to-medium toxicity at low concentrations. With sufficient concentrations of cryoprotectants, high cooling speeds eventually solidify the sperm suspension into a metastable glassy, vitreous state with no ice formed. This is referred to as vitrification (Pegg, 2007; Saragusty and Arav, 2011). Use of ultra-high-speed cooling, in theory, should eliminate the need for toxic penetrating counterparts such as sucrose or trehalose. Use of these non-penetrating cryoprotectants has recently made possible the vitrification of dog, human, and rabbit spermatozoa, albeit the survival rate is still low (Sanchez et al., 2011; Isachenko et al., 2012; Rosato and Iaffaldano, 2013). Ultra-high cooling rates of ~10,000°C/min are seen as a major prerequisite for further improvement in cryopreservation of spermatozoa (Saragusty and Arav, 2011; Rodriguez-Martinez, 2012c).

**Basic research behind new possibilities**

As mentioned previously, most of the efforts recently have been on the action of cryoprotectants and cooling speeds. Interestingly, species with obvious suboptimal freezing results are often those with fractionated ejaculation such as dogs, pigs, and horses where spermatozoa are mainly ejaculated in seminal plasma, which is composed of specific proteins (Rodriguez-Martinez et al., 2011) and anti-oxidant enzymes like paraaxonase-1 (Verit et al., 2009; Rodriguez-Martinez et al., unpublished). All of these components act to effectively sustain sperm survival. In the cauda epididymi, the prevailing low pH, low bicarbonate, and low O2 preserve sperm survival and fertilizing capacity (Rodriguez-Martinez et al., 1990; Rodriguez-Martinez, 1991). Thus, reducing the extender pH has yielded better cryosurvival results (Lafluf et al., 1990; Rodriguez et al., 1994). Stem cells (Darzynkiewicz and Balazs, 2012) often appear in niches surrounded by low O2 concentrations and hyaluronan, which is the in vivo existing polymeric form of hyaluronic acid (Volpi et al., 2009). Similar conditions are present in the oviductal sperm reservoir (Rodriguez-Martinez, 2007c) where hyaluronan is present in the fluid and epithelia of pigs and cows (Rodriguez-Martinez, 2001) and acts to prevent sperm capacitation and acrosome exocytosis (Tienthai et al., 2004). Hyaluronan provides further protection from ROS-induced DNA damage by chelating the Fe2+ and Cu2+ ions that lead to OH radicals (Balogh et al., 2003). Spermatozoa have hyaluronan receptors on their cell surface and hyaluronan and, when activated, assist with their maturation, motility, and fertilization (Tienthai et al., 2003). Hyaluronan also stimulates embryo development in several species (Gardner et al., 1999; Suzuki et al., 2002; Palasz et al., 2006). Finally, hyaluronan has been proven to be a good cryoprotectant for somatic cells (Ujihira et al., 2010) and boar sperm (Peña et al., 2004). Based on all of this information, it seems physiologically reasonable to determine whether a combination of low O2 levels, hyaluronan, and other specific components isolated from seminal plasma can increase the effectiveness of preserving spermatozoa via vitrification.

**Spermatozoa, not only DNA…**

The sperm genome has to be intact to participate in embryo development. It is, however, susceptible to oxidative DNA damage, so it is important to determine whether it remains intact when semen is manipulated and cryopreserved. In addition, semen contains a series of small regulatory non-coding RNA (ncRNA) that contain 19 to 22 nucleotides (Bartel, 2009). These microRNA (miRNA) are found in both the seminal plasma (Belleannée et al., 2012; Wu et al., 2012) and spermatozoa (Hamatani, 2012; McIver et al., 2012). The miRNA are key post-transcriptional modifiers of gene expression. They act epigenetically and play an important role in the acquisition and maintenance of male fertility (Dodame, 2009). They are abundant in bull sperm and show differential expression in relation to fertility levels of different sires (Govindaraju et al., 2012). They are delivered to the oocyte at fertilization and modulate the first cleavage divisions (Liu et al., 2012). In insects and some vertebrates that are resistant to the effects of freezing, they are believed to rapidly regulate metabolic responses that are critical for reducing cellular damage caused by cold stress (Biggar et al., 2009). Suboptimal cryopreservation leads to alterations in chromatin structure (Rodriguez-Martinez, 2012c). In embryonic stem cells, this is accompanied by a significant downregulation of house-keeping and function-related genes (Wagh et al., 2011). Hence, there is a great need to resolve how different methods of cryopreservation impact the sperm epigenetic fingerprint through changes in concentrations of miRNA.

**AI Doses with Low or High Sperm Numbers**

There is an overall tendency to reduce sperm numbers per AI-dose in the cattle industry. Several reasons are behind this trend: i) to increase revenues; ii) to determine the innate optimal fertility level achieved by the individual sires; and iii) to accommodate the increasing use of sex-sorted semen. While the ejaculates from many sires can be produced to produce frozen AI doses with low sperm numbers, those from others cannot.
Sperm selection is a term with many interpretations. However, it generally is used to describe methods for separation of spermatozoa for in-vitro-assisted reproduction and includes identification of sperm with a specific size, quality characteristic, or sex (those containing the X or Y chromosome). The most common techniques to select sperm include washing by extension and centrifugation, filtration/gradient separation, or self-motility, which is commonly referred to as the “swim-up” or “swim-down” test (Rodríguez-Martinez et al., 1997). The success of these techniques in terms of producing a population of the most robust or select sperm depends on the total number in the ejaculate and the proportion that are abnormal. For the most part, ejaculates from livestock species typically contain high numbers of normal sperm. However, increased numbers of abnormal sperm are common in horses and dogs. Some of these techniques can be used for enrichment after thawing, where the amount of surviving spermatozoa is low and there is a need to remove dead, moribund, and abnormal spermatozoa.

Most spermatozoa from farm animals show a typical progressive and linear motility. Spermatozoa use this innate pattern of movement to transverse natural barriers such as the cervix or the uterine-oviductal junction in the female reproductive tract and, thus, this pattern of motion has been related to their fertility. Consequently, the swim-up test mimics these in vivo situations and is believed to produce an enriched population of fertile sperm. This is done simply by placing a semen sample underneath an appropriate volume of suitable media and collecting the spermatozoa that actively migrate the furthest into the media. Both the numbers of sperm and their speed when traversing the column are used to select the most fertile sample. This method separates a sub-population of sperm that can be tested further or used for making AI doses (Zhang et al., 1998). The recovery and enrichment of high quality bull spermatozoa increased when cervical mucus or hyaluronan was used as the swim-up medium (Shamsuddin and Rodríguez-Martinez, 1994). Moreover, 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 summary, self-migration procedures select spermatozoa as is done in vivo during AI.

**Figure 1.** Confocal laser microscopy of FC-sorted stallion spermatozoa after staining with SytoxGreen (dead spermatozoa, green fluorescence, short arrows) and Merocyanine-540 (B, red fluorescence) exemplifies increased lipid disorder in the plasmalemma of some processed spermatozoa (long arrow, high fluorescence intensity) but not in others (low fluorescence intensity). C: Nomarski differential interference contrast, D: merged images A-C.

**Figure 2.** A MiniFlatPack containing a highly concentrated boar sperm suspension (A), which is depicted in the frozen state in a Cryo-SEM micrograph (B), showing morphologically well preserved boar spermatozoa from the sperm-peak portion of the ejaculate (photo courtesy of Dr Hans Ekwall).
a natural mating but cannot isolate large sperm numbers. Consequently, their use for AI, at the present, is limited.

Other methods have attempted to address this limitation. Examples of these methods are the centrifugation through columns of adherent particles such as Sephadex or glass-wool (Januskauskas et al., 2005) or differential centrifugation through discontinuous density gradients of silane-coated silica spheres (Rodríguez-Martinez et al., 1997). Centrifugation through a single column containing species-specific formulations of these colloids has proven successful in harvesting the most robust spermatozoa from any semen suspension in most species tested so far (Morrell and Rodríguez-Martinez, 2009, 2010; Morrell et al., 2010). The discriminating ability of this method is affected by species differences in osmolarity and the density of the colloid (Morrell et al., 2011).

Sex Sorting

Gender selection in livestock is highly desirable. Using the Beltsville Sperm Sexing Technology, which is based on high-speed flow cytometrical sorting of DNA-stained spermatozoa (Garner, 2006), AI-doses with >95% of either X- or Y-chromosome-bearing spermatozoa have resulted in the birth of >50,000 documented calves of the desired sex (Seidel, 2009). Because the numbers of sorted spermatozoa produced per hour are greater now compared with those achieved a decade ago (100 to 200 million compared with 350,000, respectively), the technology today is facing a strong wave of commercialization within the cattle industries (Seidel, 2009) and appears promising for use in other species such as pigs and horses (Blondin et al., 2009; Carvalho et al., 2010). In horses, a combination of sex-sorting and embryo transfer technologies will likely increase its adaptation by breeding farms provided that techniques for superovulation in the mare become commercially available (Samper et al., 2012). New applications for pre-selecting offspring for sex are coming into the market. The most promising of these are the use of antibodies against sex-specific proteins, which by immobilizing spermatozoa with either the X- or Y-chromosome, allow for enrichment of spermatozoa containing the other (Cattle Logic Ltd, UK). Sex sorting, however, still produces “weak” spermatozoa with reduced lifespans (Gosalvez et al., 2011). This phenomenon affects spermatozoa from all species and is related to the high pressure, extreme dilution rates, and removal of specific seminal plasma components associated with this process (Vazquez et al., 2009; Caballero et al., 2012; Balao da Silva et al., 2013). For porcine and equine species, sperm sexing is slow and, thus, inappropriate for standard AI.

The challenges mentioned previously have been compensated for by the use of additives such as seminal plasma proteins to the sperm media and the growing application of deep intra-uterine AI in pigs (Vazquez et al., 2009) and horses (Samper et al., 2012). Freezing of sex-sorted boar spermatozoa has been tested and proven usable for in vitro techniques (Bathgate et al., 2007). Obviously, sperm-mediated gender selection would have a tremendous impact on pig production worldwide. However, further developments of the current sex-sorting techniques or invention of alternative methods are needed before routine commercialization is possible. Similar challenges face the equine industry. However, most of these can be compensated for by the greater value of the offspring. Consequently, the cost of their continued development and eventual use has a better chance of moving forward.
Spermatozoa as Vectors for Transgene Production

Sperm-mediated gene transfer (SMGT; Lavitrano et al., 1989) is a procedure whereby DNA from one species is introduced into another via co-incubation with sperm followed by insemination. The basic premise is that the foreign DNA is absorbed by the sperm and then delivered to and incorporated in the genome of the future animal during fertilization and early development. The mechanisms governing integration of foreign DNA by this method are not well understood and somewhat controversial (Eghbalsaied et al., 2013). However, it is viewed as a practical method of producing transgenic animals without the need for surgery, embryo handling, or expensive equipment. Sperm-mediated gene transfer (SMGT) has proven highly efficient in integrating transgenes into the genome of the pig embryo with success rates of 57 to 80%, based on ~200 generated pigs, compared with microinjection (Lavitrano et al., 2006). Transgenic pig lines have been produced by SMGT since 1997, and the methodology is well established (Lavitrano et al., 2013). SMGT also has been used successfully in horses (Zaniboni et al., 2013) and cattle (Simões et al., 2012). However, more applied and basic studies are needed to fully develop this technology for commercialization.

Conclusions and Future Trends

Development of better cryopreservation methods are on top of the research agenda, especially for horses and pigs where alternative methods for freezing of specific ejaculate portions, new containers with acceptable cryosurvival, and fertility have been developed. Despite its limitations, conventional slow equilibrium freezing is likely to be used until a breakthrough is reached for alternative methods such as vitrification. Of equal importance is the further delineation of male-to-male variation in freezability. Selection pressure on this trait is not always possible. However, it has worked well initially for dairy bulls. However, this approach probably will not be used with the same impetus when fewer bulls are recruited for genome selection. It is likely that, in the future, semen cryopreservation methods will be “adapted” for a particular sire to “save” the genetic value it represents instead of simply selecting for “good freezers” and ignoring other traits.

Pre-selection of the most robust spermatozoa is necessary to increase the use of otherwise “weak” sires, which are prevalent in horses, but will not be effective in other species unless used in conjunction with other semen technologies such as sex-sorting.

Diagnostic tools for semen assessment are going to continue to be developed but will involve both spermatozoa and the accompanying seminal plasma. Whether this will require use of sophisticated instrumentation that can be adopted to field conditions is yet to be seen. Hopefully, economical and operator-friendly instruments will be developed in the near future. One major advantage of flow-cytometry and other sorting or sperm enrichment technologies is the possibility that several tests conducted independently now can be combined and measured simultaneously. This “multi-test” approach will likely provide better estimates of fertility. Ultimately, development and commercialization of semen biotechnologies will depend on the economics associated with their design and implementation. After all, semen is still the “cheapest” component of livestock breeding.

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