Optimal characteristics of spermatozoa for semen technologies in pigs

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Despite the great potential of sperm technologies such as sperm cryopreservation and sperm sex sorting for the improvement of different aspects of swine production, artificial insemination with fresh or stored semen is currently the only sperm technology used at a commercial scale in the pig industry. The lower reproductive performance associated with the use of these sperm technologies is the reason for such limited use. Since optimal characteristics are required for successful application of frozen-thawed and sex-sorted boar spermatozoa, the present paper summarises the value of the current available methods for their functional assessment as well as the effects of these technologies on boar sperm functionality. In addition, strategies developed to reduce sperm damage and improve the yields of both sperm technologies in swine production are also reviewed with particular attention to the contributions of the authors.

Introduction

Over the past decade, considerable interest has been directed toward the development and improvement of assisted reproductive technologies (ARTs) for swine. Although some ARTs, such as embryo transfer, *in vitro* production of embryos, cloning and transgenic technology, have been successfully attempted, the current low level of efficiency limits their use in applied production systems. However, ARTs in which only spermatozoa are handled may be applicable for the pig industry in a short period of time (Martinez *et al.* 2005). Certain sperm technologies, such as cryopreservation of male gametes and sperm sorting for gender pre-selection, might be of great benefit to the swine industry for improving the efficiency of production. Nevertheless, the low reproductive performance associated with the application of these technologies limits their current use.

Many factors come into play in determining the success and spread of sperm technologies. One of these factors is the fertilising ability of the spermatozoa. Handling spermatozoa during technical procedures is usually associated with a decrease in fertilising ability.

Mammalian fertilisation is a complex process involving a precisely programmed set of events during which the spermatozoa acquire their fertilising ability through a series of molecular and cellular changes that are pre-requisite for successful interaction with a female gamete (Petrunkina *et al.* 2005, Waberski *et al.* 2005). To be able to carry out these tasks, the spermatozoa must maintain many physical and biochemical parameters that allow it to bind to the zona pellucida and penetrate into the oocyte cytoplasm. In addition, the spermatozoon must have an intact nucleus capable of proper de-condensation, nuclear reorganisation and genetic performance

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in order to maintain proper zygotic and embryonic development (Graham & Moce 2005). Spermatozoa are cells with a limited biosynthetic capability (Amann *et al.* 1993), and thus their function can be influenced dramatically by the external environment. Long periods of storage, cryopreservation or sperm sorting by flow cytometry can have a detrimental impact on its function and fertilising ability (Maxwell *et al.* 1998, Maxwell & Johnson 1999, Martinez *et al.* 2001, 2005, Roca *et al.* 2004, 2006, de Graaf *et al.* 2008).

In many cases, if not all, the ultimate objective for the practical application of sperm technologies is to obtain optimal reproductive performance. For this reason, special attention should be given to improve the functionality and fertilising ability of the treated spermatozoa.

Since optimal characteristics are required for successful application of frozen-thawed and sex-sorted boar spermatozoa, the present paper summarises the value of the current available methods for their functional assessment as well as the effects of these technologies on boar sperm functionality. In addition, strategies developed to reduce sperm damage and improve the yields of both sperm technologies in swine production are also reviewed with particular attention to the contributions of the authors.

Current procedures to evaluate optimal characteristics of boar spermatozoa

Sperm technologies such as cryopreservation or flow cytometric sex sorting require extensive *in vitro* handling, which eventually induces dramatic changes in sperm survivability. Extension, cooling, laser illumination, freezing and thawing, among others, not only cause cell death but also subtly damage most of the spermatozoa in the surviving population, leading to reduced sperm lifespan both *in vitro* and *in vivo* (Maxwell *et al.* 1998). These procedures involve different degrees of change in sperm function following physical and biochemical damage to sperm membranes, DNA, cellular signalling mechanisms, and later on are manifest in alterations to the fertilising capacity of these cells.

Spermatozoa in an ejaculate vary in the integrity of the attributes needed for successful fertilisation as well as in their capability to overcome all the events leading up to fertilisation (Rodriguez-Martinez 2007). Similarly, spermatozoa subjected to technologies such as cryopreservation or sex sorting respond differently to the treatment involved in these procedures. Such variation among cells within a given ejaculate complicates the use of laboratory evaluations as accurate estimation tools (Holt & Van Look 2004). Moreover, it may be assumed that standard seminal parameters like motility, morphology, and sperm concentration are insufficient to indicate subtle changes in sperm cells subjected to these treatments. Detection of such changes is currently an issue of great importance (Peña *et al.* 2007, Petrunkina *et al.* 2007). Therefore, functional assessment analysis should be used to determine minimal and early physical and biochemical damage along with failed metabolic activity in treated sperm samples.

These functional tests include sperm kinematics (assessed by computer-assisted analysis), evaluation of early changes in sperm membranes, evaluation of damage to the sperm chromatin structure, and DNA integrity and sperm-oocyte or sperm-oviduct interactions (Holt *et al.* 2007, Silva & Gadella 2006, Petrunkina *et al.* 2007).

Traditionally, computer-assisted sperm analysis (CASA) systems have been introduced for routine motility evaluations at the commercial level due to their ability to objectively identify sperm motility and motions parameters (Didion 2008). However, in common practice, the use of the mean values of motility descriptors simplifies the motility analysis but does not take into account internal variability, thus providing misleading information about sperm quality, which is not revealed by the usual statistical measures (Holt *et al.* 2007). New approaches to the

analysis of data provided by CASA systems involve the identification of sperm subpopulations by means of clustering algorithms. Consequently, these systems offer new insight into the true structure of sperm populations under different experimental conditions and therefore on the relationship between motility, sperm quality and, ultimately, fertility (Cremades *et al.* 2005; Holt *et al.* 2007. See Fig. 1). Recently, by means of CASA systems, it has been possible to find computerised systems to objectively estimate sperm head dimensions with a similar statistical approach. Computer-assisted sperm morphometry analysis (CASMA) can be used to distribute spermatozoa in morphometrically distinct subpopulations within the ejaculate and to disclose their relationship with sperm quality (Saravia *et al.* 2007).

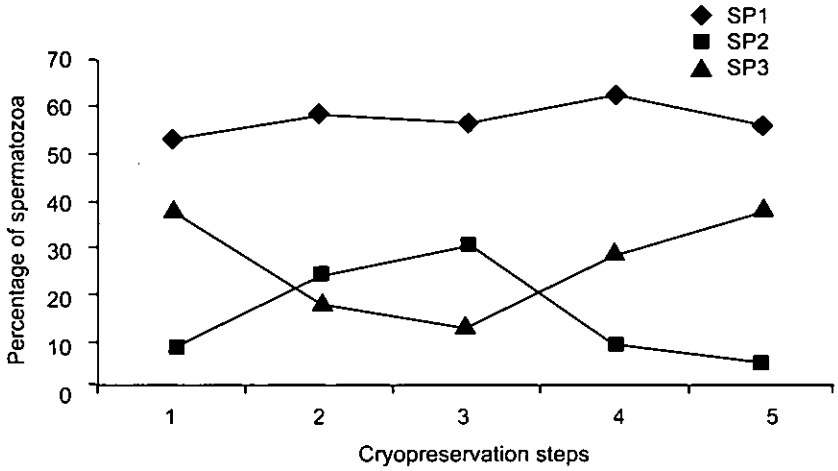

Fig. 1 Behaviour of different motile sperm populations (SP1: cells with progressive and vigorous movement; SP2: progressive cells only; SP3: vigorous cells only, hyperactive-like) identified within one ejaculate by using CASA and statistical analysis during a cryo-preservation procedure. The graphic shows how only those spermatozoa belonging to SP1 are able to maintain optimal motility levels during the freezing and thawing processes. The percentages of motile spermatozoa were recorded at five steps during a conventional boar semen cryo-preservation procedure: 1: Immediately after arrival of the BTS-extended sperm-rich ejaculate fraction (22°C) at the Laboratory of Andrology. 2: After centrifugation and extension of the sperm pellet with LEY extender at 17°C. 3: After adding the LEYGO extender at 5°C. 4: After thawing and re-extension with BTS (1:1, vol/vol) and holding in a water-bath at 37°C for 30 minutes. 5: After holding the re-extended thawed semen in a water bath at 37°C for 150 minutes (modified from Cremades *et al.*, 2005).

During recent years, special attention has been paid to plasma membrane integrity since spermatozoa with disrupted plasma membranes are considered unable to fertilise. Plasma membrane integrity is traditionally assessed after staining cells with membrane-permeable dyes such as SYBR-14, combined with propidium iodide as a counter-stain (Silva & Gadella 2006). Although checking sperm viability is a widespread and highly useful technique for primary sperm quality screening, it cannot discern sub-lethal changes in the sperm cells.

Male gamete structures are extremely sensitive to technological procedures. During these processes, they can suffer a series of events similar to those taking place during sperm capacitation, or as more recently hypothesised, premature ageing or so-called apoptosis-like changes (Peña *et al.* 2009). In either case, spermatozoa affected by these destabilising events

undergo membrane changes that apparently lead to cell death (Peña *et al.* 2009). Some of these changes are linked to early membrane destabilisation and can be evaluated by determining the degree of lipid packing disorder with the lipid dye merocyanine-540 (Harrison & Gadella 2005, Guthrie & Welch 2005) or by investigating membrane asymmetry through changes in the position of certain phospholipids (using Annexin-V) such as phosphatidylserine, which may be trans-located from the inner to the outer leaflet of the plasma membrane after being disturbed (Peña *et al.* 2003). Changes in membrane permeability imply destabilisation of membranes and can be monitored using the impermeant nuclear dye YO-PRO-1. YO-PRO-1 is used to detect early changes in membrane permeability, alone or in combination with other stains (Peña *et al.* 2005).

Cell volume is another informative method for the detection of functional membrane changes in live cell populations. The volume regulation capability of spermatozoa is of considerable importance for sperm survival during several processes, including cryopreservation (Petrunkina *et al* .2005, 2007).

Mitochondrial integrity and functionality are clearly related to sperm viability and motility, and they also play an important role in early sperm changes during technological treatments (Peña *et al.* 2009). Several dyes have been proposed to evaluate the functional status of these organelles, mostly measuring mitochondrial inner membrane potential, such as JC-1, Mitotracker Green or Mitotracker Deep Red (Guthrie & Welch 2006, Bussalleu *et al.* 2005, Hallap *et al.* 2005).

Many other analyses can be performed to investigate sperm functionality, such as measuring the intracellular calcium concentration (Caballero *et al.* 2009), stimulating protein tyrosine phosphorylation (Piehler *et al.* 2006), or searching for the presence of activated caspases (Moran *et al.* 2008), intracellular reactive oxygen species (Guthrie & Welch 2006) or other signalling pathways, all of them intimately related to detrimental changes in sperm functionality.

Several studies have shown that spermatozoa with normal function, including motility, viability, fertilisation rates and initial cleavage rates can have damaged DNA that will result in reduced fertility and an increase in early pregnancy loss (Bathgate 2008). Changes in sperm chromatin structure and DNA integrity have been widely related to infertility in several mammalian species, and it is recommended that chromatin integrity should be studied as an independent complementary parameter for a better assessment of sperm quality. Sperm chromatin structure assays (SCSA), comet assays and the sperm chromatic dispersion test are some of the techniques currently applicable to evaluate boar sperm DNA fragmentation (Fraser & Strzezek, 2005; Hernandez *et al.*, 2006; Lopez-Fernandez *et al.*, 2008)

In recent decades, laboratory assessments of either fresh or processed semen have evolved to become more detailed and have moved from the diagnosis of sperm attributes to determining the fertilising potential of the sample using *in vitro* tests such as zona pellucida (ZP) and oviductal epithelium binding assays, *in vitro* penetration assays or *in vitro* maturation/*in vitro* fertilisation/ *in vitro* production (IVM/IVF/IVP) systems (Rodriguez-Martinez 2003).

In 2005, Waberski *et al.* reviewed the procedures developed to evaluate the functional competence of boar spermatozoa by analyzing *in vitro* sperm-oocyte and sperm-oviduct interactions. In the former case, the ability of spermatozoa to interact with the ZP is evaluated by means of a sperm-zona binding assay, a sperm-zona penetration assay or an *in vitro* fertilisation assay, whereas in the latter case, the main aim is to assess multiple functions of the sperm plasma membrane that must be carried out before fertilisation.

Experiments conducted in our laboratory demonstrate that the evaluation of penetration rates and the average number of spermatozoa per penetrated oocyte using zona intact pig oocytes at the germinal vesicle stage (immature oocytes) in a homologous *in vitro* penetration (hIVP) assay provides a useful way to accurately predict male fertility (Martínez *et al.* 1993,

1996). This assay is simple and rapid as it avoids the *in vitro* maturation of oocytes. Other parameters such as pronuclear formation and early embryonic development can be obtained using IVM/IVF/IVP systems. However, the problems of polyspermic penetration of oocytes and the poor quality of the embryo culture medium remain in the porcine IVF system, resulting in low-efficiency production of viable embryos and making their use for the prediction of fertility difficult (Gil *et al.* 2005).

The sperm-oviductal epithelium binding assay is emerging as an important tool for evaluation of sperm functionality since it evaluates plasma membrane quality for the multiple functions necessary prior to the fertilisation process (Waberski *et al.* 2005). Since binding to the oviductal epithelium stabilises the spermatozoa and enhances their survival in a hostile environment, it is clear that the ability of sperm cells to bind to the oviduct is a very important attribute intimately related to fertilising ability (Petrunkina *et al.* 2007). Along this line, several studies performed using this assay have demonstrated that the binding index was found to be lower in sperm samples coming from sub-fertile sires compared to that of fertile sires (Petrunkina *et al.* 2007, Waberski *et al.* 2005).

How sperm technologies affect boar sperm fertilising ability

Artificial insemination (AI) could be considered the most relevant sperm technology used worldwide in the reproductive management of domestic mammals (Weitze 2000). The manipulation of boar semen for AI generally involves two main steps consisting of dilution and, in most cases, storage of extended spermatozoa. Although this protocol is less stressful for the spermatozoa, some detrimental effects on their fertilising ability may be induced, especially if long storage periods are required (Vazquez *et al.* 1998, de Ambrogi *et al.* 2005).

The situation is different when sophisticated sperm technologies are applied to the boar gametes. Special characteristics of the boar sperm plasma membrane composition determine the extreme sensitivity of spermatozoa to cooling and freeze-thaw processes as well as to procedures that involve high dilution rates such as sex sorting by flow cytometry (de Graaf *et al.* 2008, Vazquez *et al.* 2009). This peculiarity of the male pig gamete is likely one of the most important limiting factors in the commercial use of these technologies for swine production.

Cryopreservation of spermatozoa involves several steps (temperature reduction, cellular dehydration, freezing and thawing) that may compromise sperm viability and normal function (Medeiros *et al.* 2002). Detrimental effects of the cryopreservation procedure include damage to the sperm plasma membrane and other organelles as a result of osmotic stress, cold shock and intracellular ice formation (Guthrie & Welch 2005).

Over the years, great efforts have been dedicated to reducing the negative effects of freezing procedures on boar spermatozoa by introducing several improvements in sperm cryopreservation protocols (Eriksson *et al.* 2001, Carvajal *et al.* 2004, Saravia *et al.* 2005). As result of these investigations, sperm survival rates above 50% have been achieved along with promising fertility results (Roca *et al.* 2006).

Despite the improvements and the hopeful results, post-thaw viability, lifespan and fertility of cryopreserved boar spermatozoa are still reduced as a consequence of cellular injuries that arise during cryopreservation procedures (Medeiros *et al.* 2002) and remain at sub-optimal levels (Sancho *et al.* 2007). These facts together with the great variability in sperm freezability among boars and the large number of spermatozoa required per AI dose has discouraged the extensive use of frozen semen in commercial swine production (Johnson *et al.* 2000).

As noted above, the short lifespan of boar spermatozoa following cooling and freeze-thaw processes is one of the major drawbacks to the successful application of cryopreserved boar

spermatozoa in commercial AI programmes, and it explains the low fertility results obtained (Roca *et al.* 2006). Premature capacitation (Watson 2000) and/or premature ageing (Peña *et al.* 2009) suffered by post-thaw spermatozoa have been pointed out as the main cause for the reduction in sperm lifespan, and thereby in fertilising capability. In either case, the reduction has been related to alterations in the cholesterol content of sperm membranes as well as with excessive formation of reactive oxygen species (ROS) as a consequence of the cryopreservation technique (Roca *et al.* 2004).

As occurs in cryopreservation procedures, flow cytometric sorting of X- and Y- chromosome bearing boar spermatozoa involves several steps that can induce stress, damage or even kill spermatozoa. These steps include DNA staining, exposure to the UV-laser beam, high working pressures, high dilution rates in different extenders (which may involve changes in osmolarity and pH), centrifugation to concentrate the cells and in some cases the further stress of the cryopreservation procedure itself (Bathgate, 2008, Vazquez *et al.* 2008a, Garcia *et al.* 2007). Both the physical effect of the sorting procedure and the high dilution rate cause alterations in the functionality of sorted boar spermatozoa (Parrilla *et al.* 2005). However, the extensive dilution of spermatozoa (from approximately 800 x 10^6 in a sperm-rich fraction to a final concentration of sorted sperm of around 1 x10^6 according to Bathgate, 2008) has been identified as the main determinant factor in the reduction of sorted sperm functionality. This fact might be due to the removal of seminal plasma factors required for the maintenance of sperm plasma membrane integrity and sperm functionality (Maxwell & Johnson, 1999, Centurion *et al.* 2003, Caballero *et al.* 2004, 2008).

Therefore, as a result of all the manipulations needed for sperm sorting by flow cytometry, a stressed cell population with destabilised plasma membranes and showing capacitation-like changes is obtained (Maxwell & Johnson 1999). This status renders the spermatozoa immediately able to fertilise an oocyte whether under *in vivo* or *in vitro* conditions without the need to perform further capacitation treatments (de Graaf *et al.* 2008). Detrimental effects caused by this premature capacitation status include reduced viability, lower fertilising ability and compromised storage capability after sorting (Maxwell & Johnson 1999, Parrilla *et al.* 2005, see Figs 2 and 3). From a practical point of view, the reduction in the lifespan of sorted boar spermatozoa is an important drawback for the application of sexed semen in pig farming because, in most cases, great distances must be travelled between sperm-sorting laboratories and AI facilities (Vazquez *et al.* 2009).

Another important disadvantage of sorting technology when applied to AI in pigs is the reduction in fertility parameters with regard to farrowing rates and litter size (Vazquez *et al.* 2003, Garner 2006, Johnson *et al.* 2005). Whether this alteration in the reproductive parameters is a consequence of the low number of sorted sperm used – which may be unable to produce the minimum number of embryos necessary to maintain the pregnancy – or is due to alterations in sorted sperm DNA – resulting in the poor developmental potential of embryos – remains unclear. Further investigations into this phenomenon are clearly necessary (Bathgate 2008, Vazquez *et al.* 2009).

Strategies for optimising the characteristics of spermatozoa subjected to technological treatments

The handling of spermatozoa for sperm cryopreservation and sperm sexing by flow cytometry usually includes the concentration and the dilution of the spermatozoa, processes that produce a detrimental effect on the sperm cells. Consequently, new approaches are needed to improve these steps.

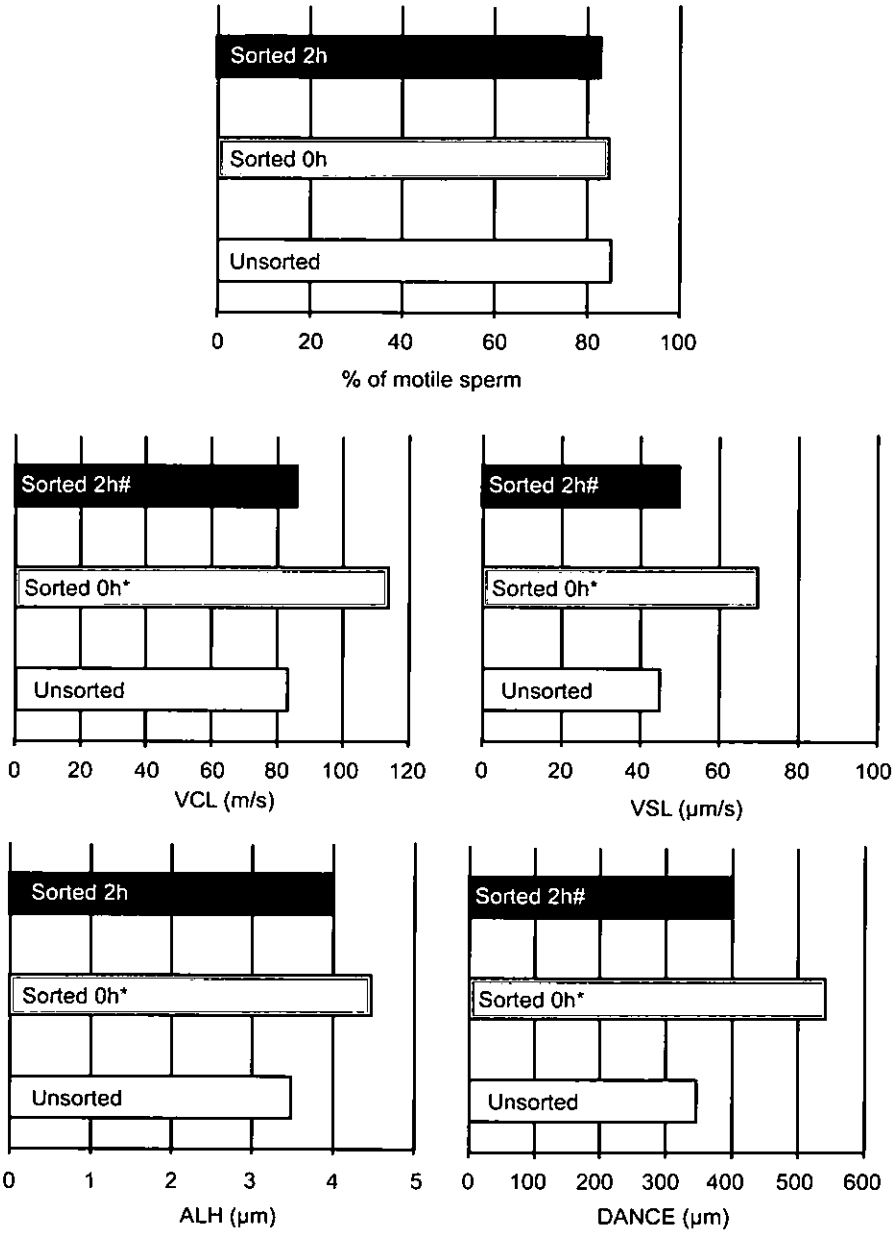

Fig.2 Changes in motion parameters in unsorted and sorted spermatozoa analyzed by CASA at 0 (sorted 0h) and 2 hours (sorted 2h) of storage after sorting in the presence of seminal plasma. The percentage of motile sperm was maintained at around 80% up to 2h of storage. Among the other variables explored, sperm velocity (VCL or VSL) and angularity parameters (ALH and Dance) increased significantly ($p < 0.05$) immediately after sorting (sorted 0h) only to return to unsorted sperm values after 2h of storage in the presence of seminal plasma. VCL: Curvilinear velocity; VSL: Straight-line velocity; ALH: Amplitude of lateral head displacement; Dance: Curvilinear velocity multiplied by amplitude of lateral head displacement. * $P < 0.05$ compared to the unsorted sample, # $P < 0.05$ compared to sorted 0h (modified from Parrilla *et al.*, 2005).

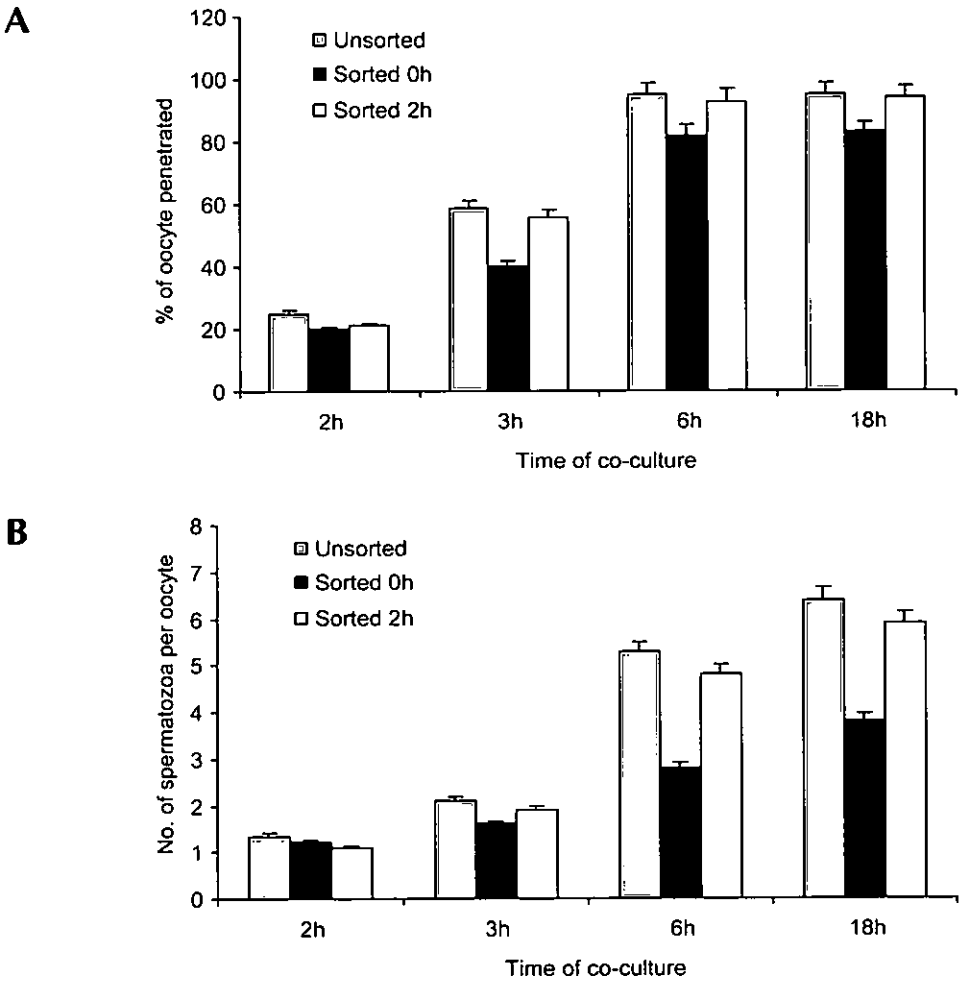

Fig. 3 Effects of flow sorting and flow sorting and storing in the presence of seminal plasma (SP) on time-course (2, 3, 6, 18 hours) penetration rates (A) and the number of spermatozoa per oocyte (B). Sorted 0h: Spermatozoa sorted and co-incubated just after sorting; Sorted 2h: Spermatozoa sorted and stored for 2h in presence of SP. The histogram shows how spermatozoa sorted and stored for 2h in the presence of SP had higher penetration rates and number of sperm per oocyte. This difference may be because the flow sorting procedure induces a scrambling in the plasma membrane components, affecting glycoproteins related to oocyte recognition and penetration. Incubation with SP for 2h might reorganize the plasma membrane, restore the fertilizing capability and increasing the percentage of penetrating spermatozoa. * indicates significant differences ($p < 0.05$) between histogram bars (modified from Parrilla *et al.*, 2005).

Methodologies for sperm concentration

Although centrifugation is the usual procedure used for concentration of spermatozoa, it has been described as a process with detrimental effects on sperm cell membranes, reducing its

fertilising capacity (Katkov & Mazur 1998, Carvajal *et al.* 2004). Moreover, the induced damage appears to be worse when centrifugation is applied to "weak" spermatozoa, such as those that have been sex-sorted (Garcia *et al.* 2007). Sedimentation, as an alternative to centrifugation, can be use to concentrate boar spermatozoa, improving the *in vivo* fertilising ability of sex sorted boar spermatozoa (Garcia *et al.* 2007).

Implementation of additives to the sperm extenders

Improving the sperm environment through the use of an optimal medium is a principal strategy to overcome the low sperm quality obtained after application of sperm technologies. Addition of seminal plasma, certain proteins from the seminal plasma and antioxidant substances to the media surrounding the male reproductive cell have been used with cryopreserved and sex- sorted boar sperm with promising results (Maxwell & Johnson 1999, Roca *et al.* 2004, Centurión *et al* .2003, Garcia *et al.* 2007).

Seminal plasma

Seminal plasma (SP) is the biological fluid in which the spermatozoa are bathed. It is well known that SP contains factors that influence both the spermatozoa and the female genital tract (Mann & Lutwak-Mann 1982, Rozeboom *et al.* 2000). Most sperm technologies include the removal or extreme dilution of SP, a process that has been associated with a decrease in sperm quality and the acquisition of capacitation-like changes that lead to a shortening of sperm lifespan (Maxwell & Johnson 1999). Therefore, a common counter-measure to alleviate these detrimental effects has been the addition of a certain proportion of SP to sperm extenders (Maxwell & Johnson, 1997). However, there is considerable variability in the results reported in the literature regarding the effect of SP on frozen-thawed spermatozoa (Caballero *et al.* 2004, Maxwell *et al.* 2007, de Graaf *et al.* 2008). Initial research in sperm cryopreservation showed that pre-freezing spermatozoa in their own SP protected against cold shock (Pursel *et al.* 1973). On the other hand, Kawano *et al.* (2004) showed an improvement in sperm cryosurvival when SP was removed immediately after collection. Similar results have been reported regarding the addition of seminal plasma to post-thaw extenders with either improved membrane status (Vadnais *et al.* 2005) or diminished viability of the cryopreserved spermatozoa (Ericksson *et al.* 2005).

The application of SP to sex-sorted spermatozoa has been more straightforward. The addition of a certain proportion of SP (usually 10%) to the collection media of sex-sorted spermatozoa improves sperm viability (Maxwell & Johnson 1999). This protective effect is associated with a decrease in the capacitation-like changes observed in the sex-sorted spermatozoa (Maxwell & Johnson 1997, Parrilla *et al.* 2005. See Figs 2 and 3). Although the beneficial effect of SP on sex-sorted sperm is somewhat consistent, as demonstrated by its inclusion in the collection media of most modern boar sperm sorting protocols (Grossfeld *et al.* 2005), such an effect is, however, boar-dependent (Caballero *et al.* 2004). These differences are associated with the variability observed in the SP composition among males as well as between ejaculates or fractions of the same ejaculate (Caballero *et al.* 2004, Rodríguez-Martínez *et al.* 2005, 2008, Saravia *et al.* 2009). Furthermore, a similar donor-dependent effect has been observed regarding sperm freezability, where the addition of SP from boars with good freezability to freezing extenders improved sperm cryosurvival (Hernández *et al.* 2007). These differences are prompting researchers to study the effects of specific components of the SP, such as SP proteins.

Seminal plasma proteins

In boars, the bulk of SP proteins (> 90%) belongs to the spermadhesin family, a group of 12–16 kDa glycoproteins that bind to the sperm surface. Depending on their binding capability, spermadhesins can be classified into heparin-binding (AQN-1, AQN-3, AWN) and non-heparin-binding spermadhesins (PSP-I/PSP-II heterodimer) (Töpfer-Petersen *et al.* 1998). The PSP-I/PSP-II heterodimer exerts a protective effect on highly diluted spermatozoa (Centurión *et al.* 2003). Based on immunolocalisation studies, PSP-I/PSP-II heterodimer has been seen to bind to the acrosomal cap of the spermatozoa. The binding of the heterodimer to the sperm surface seems to stabilise the plasma membrane of the spermatozoa (Caballero *et al.* 2006), delaying capacitation-like events, such as the increase in intracellular calcium and remodelling of the surface proteins of the sperm membrane (Caballero *et al.* 2006, 2009). This protective effect points to the PSP-I/PSP-II as a candidate additive for extending the viability of technologically treated spermatozoa. In this way, the PSP-I/PSP-II heterodimer has been tested as an additive for sex-sorted spermatozoa, showing a similar protective effect as that observed with SP (García *et al.* 2007). Experiments carried out in our laboratory using laparoscopic insemination demonstrated that the fertilising ability of sex-sorted spermatozoa collected in the presence of the PSP-I/PSP-II heterodimer increased when the PSP-I/PSP-II heterodimer was combined with sedimentation (instead of centrifugation) to concentrate the sperm sample (García *et al.* 2007).

Antioxidants

Sperm technologies often include procedures that are associated with the generation of ROS, which are related to defective sperm function (Muiño-Blanco *et al.* 2008). The use of antioxidants has been proposed to diminish the generation of ROS. Addition of catalase and superoxide dismutase reduced post-thaw ROS generation, improving sperm motility, viability and the ability of frozen-thawed spermatozoa to produce embryos *in vitro* (Roca *et al.* 2005). A beneficial effect of antioxidants was also seen with regard to the quality of flow-cytometrically sorted frozen-thawed bull spermatozoa (Klinc & Rath 2007), suggesting that the addition of antioxidants could be complementary for improving the fertilising ability of sex-sorted spermatozoa. Unfortunately, there are no references regarding the effects of antioxidant addition to the media used in sex sorting protocols for boar sperm.

Other strategies to optimise sperm treatment technology outputs

Application of sperm cryopreservation or sperm sex sorting in pigs generally implies the use of a small or very small number of weak spermatozoa. Together with the particular characteristics of the anatomy and reproductive physiology of the sow, this fact should be taken into account for the development and optimisation of protocols that allow the achievement of optimal fertility results after *in vivo* fertilisation.

Different strategies have been proposed in recent years to obtain optimal fertility results from insemination with a reduced number of boar sperm. These new procedures are mainly related to the site of semen deposition. In an attempt to deposit the spermatozoa as close as possible to the location of fertilisation by post-cervical insemination (intrauterine insemination, Watson & Behan 2002), deep intrauterine insemination (Martinez *et al.* 2001) and laparoscopic intra-oviductal insemination (García *et al.* 2007, Vazquez *et al.* 2008a, 2008b) procedures have been described and successfully used in combination with cryopreserved (Roca *et al.* 2003) and sex-sorted spermatozoa (Vazquez *et al.* 2003). In the case of sex-sorted spermatozoa, it

has also been demonstrated that, in addition to the insemination procedure, the effect of the time of insemination relative to ovulation has an important influence on the success of the insemination procedure (reviewed by Vazquez *et al.* 2008a, 2008b).

Concluding remarks

Although it is clear that optimal sperm characteristics are those that allow the spermatozoa to fertilise an oocyte with subsequent embryo development, better knowledge of the characteristics of spermatozoa subjected to different technologies and an accurate prediction of fertilising ability by the application of the most appropriate sperm assessment assays are essential for optimising the yields of these techniques.

Reduced viability, lifespan and low fertility after the application of boar spermatozoa subjected to different sperm technologies have been widely described by several authors. These are the main drawbacks to overcome before these technologies are ready for application at commercial level. Important achievements have been made in cryopreservation and sex sorting protocols by modifying sperm handling procedures as well as through the addition of different substances. The consequent improvements in sperm functionality have been described by several different laboratories.

Furthermore, it should not be forgotten that fertilisation is a process that not only depends on sperm factors but also largely affected by aspects of the AI procedure and the physiology and management of the female. AI methods that allow deposition of the spermatozoa as close as possible to the site of ovulation and also as close to the ovulation time as possible are currently the most powerful tools for optimising the *in vivo* fertility of boar spermatozoa that have been subjected to cryopreservation or flow sorting.

As a general conclusion, it should be noted that important progress has been made in sperm technologies and many promising results have been achieved, which will be helpful for introducing these technologies on to pig farms. However, further research efforts should be pursued in order to facilitate a fully successful introduction of these sperm technologies to the swine production industry.

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