Storage of sexed boar spermatozoa: Limits and perspectives

STORAGE OF SEXED BOAR SPERMATOZOA: LIMITS AND PERSPECTIVES

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Abstract

Despite the great potential application of sex-sorted spermatozoa in swine, the technology is not practiced in the pig industry due to technical factors and species-specific issues. The susceptibility of boar spermatozoa to stresses induced by the sorting procedure, the relative slowness of the sex-sorting process together with the high sperm numbers required for routine artificial insemination in pig are some of the main factors limiting the commercial application of this technology in pig. This review briefly describes the damage to spermatozoa during sex-sorting focusing on an additional limiting factor: the increased susceptibility of sexed boar spermatozoa to injuries induced by liquid storage and cryopreservation that, in turn, impairs sperm quality leading to unsatisfactory results *in vivo*.

Strategies to extend the lifespan of sex-sorted boar spermatozoa and to improve their fertilizing ability after liquid storage or cryopreservation need to be implemented before this technology can be used in pig farms. In this regard, encapsulation in barium alginate membranes could be a promising technique to optimize the *in vivo* use of sexed boar spermatozoa, by protecting, targeting and controlling the release of sperm into the female genital tract.

Key words: Liquid and frozen sexed semen; Pig; Sperm sexing; Sperm encapsulation

1. Introduction

Flow-cytometric sperm-sorting based on X and Y sperm DNA difference is currently the only accurate method to pre-determine the sex of offspring before fertilization [1].

Even if the sexing technique has already reached a commercial level in the bovine species [2], the use of sexed semen in the swine industry is far from being a routine procedure. Reproductive management in pig production would benefit from sex preselection by accelerating genetic progress and allowing the production of preselected female livestock [3]. Moreover, female production through use of sexed semen may be an alternative to the castration of male piglets to prevent the distasteful “boar taint” [4]. Castration is regarded as an infringement of animal welfare and in response to these growing concerns, several leading players within the pig and pork industry have agreed to a plan to voluntarily end the practice of surgically castrating pigs in the EU by 1 January 2018.

In the research field, sex-sorting in association with sperm mediated gene transfer (SMGT) could be strategically useful to shorten the time for producing homozygous transgenic pigs [5] as organ donors for xenotransplantation, as valuable models for biomedical studies and in the use of transgenic swine as bioreactors [6-8].

2. Factors limiting the large scale use of sexed boar spermatozoa

Even if flow cytometric sorting of pig spermatozoa could have great potential for application, the technique is currently under research and it is still unknown if the use of sex-sorted semen through routine pig artificial insemination (AI) will be economically feasible [3].

As indicated by the flow cytometric sorting index elaborated by Garner et al. [2], boar spermatozoa are approximately as easy to sort as ram and bull sperm, due to both the relatively high difference in the DNA content between X and Y chromosome bearing sperm (3.6%) and the flattened, oval heads that tend to be readily oriented in a sperm sorter using

hydrodynamics. Therefore, the effectiveness of the sexing technology in this species, is not an issue [9]. However the efficiency of the sex sorting procedure in the porcine species can be influenced by inter- and intra-boar variability in the sortability of spermatozoa due to differences in the ability of ejaculates to exhibit well-defined X and Y peaks in the split on a flow cytometry histogram [10,11]. Alkmin et al. [10], analyzing 67 ejaculates from different boars, found that around 15% failed to exhibit a well-defined split in the first ejaculate (bad sperm sorters)(inter-boar variability). Analyzing five ejaculates from three of the bad sperm sorter boars, the percentage of the ejaculates not exhibiting a well-defined split ranged between 20 and 70% (intra-boar variability) [10]. Such variability in pig, unlike other species (dog, horse) [12,13], is not influenced by the percentage of non viable spermatozoa in the semen samples but is closely related to ejaculate sperm concentration. Ejaculates are diluted to achieve the optimal sperm concentration for Hoechst 33342 (Ho) staining; during the staining step samples from ejaculates with low sperm concentration would have a high proportion of seminal plasma that may alter Ho entrance into the sperm cell thereby affecting the effectiveness of DNA staining [10, 11].

2.1. Sperm sorter output

Although there has been significant progress in the throughput of sperm sorters (about 20 million sperm per hour), one of the major limiting factors for the broad use of sexed semen in pig farms is the unreasonable sorting time (about 100 h) necessary to obtain an adequate number of sexed spermatozoa for conventional AI (2-3 billion spermatozoa/insemination dose). In an attempt to overcome this problem, offspring were produced by a combination of reproductive technologies such as surgical insemination, deep-intrauterine insemination (DIUI), IVF-ICSI and embryo transfer (ET) (Table 1). Fresh sexed boar spermatozoa have been successfully utilized for low dose insemination protocols by non-surgical DIUI depositing as few as 70-140x10^6 bulk-sorted [14] or 50x10^6 sex-sorted spermatozoa in the

anterior third of the uterine horn of sows [15,16]. However the relatively high number of sexed spermatozoa needed and the reduced fertility rates limit the use of sexed semen in DIUI on a large routine scale [17]. Laparoscopic insemination with a very low number of sex-sorted sperm (3-6 x10^6 spermatozoa) has been demonstrated to produce satisfactory fertility at the farm level with a farrowing rate of around 80% [18]. Owing to its high cost, routine use of this insemination technique is not feasible in the pig industry, but could at best be confined to niche situations such as elite breeding units or nucleus herds [17,18]. In addition, the fertility outcome using these techniques is strictly dependent on proper timing of semen deposition and hormonal treatments for accurate prediction of ovulation [17].

In vitro techniques such as in vitro fertilization (IVF) or intracytoplasmic sperm injection (ICSI), that greatly reduce the number of sperm required, combined with surgical or non-surgical embryo transfer offer a more efficient use of sorted sperm, and have proved a feasible, albeit expensive, alternative to employing sexed sperm in pig [19-22].

2.2. Damage to boar spermatozoa during sorting

Another factor limiting the application of sperm-sorting technology in the pig is the susceptibility of boar spermatozoa to stress induced by the sorting procedure that seems to be more severe than that in the bull and ram [23,24]. Sex-sorting-induced damage has been extensively documented and reviewed, thus it is only described briefly focusing on the increased susceptibility to storage for pig spermatozoa sorted by flow cytometry.

Chemical, physical and electrical insults during the sex-sorting process (Hoechst 33342 staining, variations in temperature, high pressure, exposure to the UV laser beam, electrical charging of droplets containing spermatozoa, projection into the collection tube, high dilution, centrifugation) can induce the death of some sperm cells. However, those sperm that survive such processing can undergo sublethal modifications that, in turn, can shorten sperm lifespan after sorting and reduce their fertilizing ability [25-32]. The stressors

associated with the sex-sorting procedure seem to primarily affect the sperm surface. After sorting Heat shock protein 70 (Hsp70) has been reported to be relocated, without consumption of the protein as evidenced by Western blotting, from the equatorial subsegment towards an equatorial line and this lateral movement suggests the beginning of a capacitation-like process [30,33]. Likewise, changes in chlortetracycline (CTC) labeling patterns in boar spermatozoa after sex-sorting suggest a destabilization of the sperm surface and reflect a capacitation-like state of the sperm membrane [25,34]. This is not surprising as processing steps for sorting (dilution, promotion of protein release from the sperm surface by mechanical forces, presence of BSA in media) can mimic the condition utilized in vitro to induce sperm capacitation [35]. The induction of a capacitation-like process due to the sorting procedure is confirmed by the data on sperm motility patterns obtained immediately post-sorting [29] and by the need to reduce the number of spermatozoa for IVF to fifty compared to unsorted sperm in order to avoid polyspermic fertilization [20]. However non-membrane parameters considered to be markers of the capacitation processes, such as actin cytoskeleton polymerization and protein tyrosine phosphorylation, seem to be less affected by the sex-sorting process and sexed sperm do not completely reflect the changes detected during capacitation in vitro. This suggests that the evolution of capacitation-like changes in sexed spermatozoa probably follows a different pathway to that of true capacitation [34].

3. Storage of sex-sorted boar spermatozoa

Storage of sexed boar semen is necessary in order to ship it from sorting facilities to recipient females for use on a wider scale. Moreover, an adequate liquid storage protocol for sexed boar spermatozoa is required when producing sexed boar sperm for DIUI as the time needed for each insemination dose might be longer than 10h [23].

Sorted spermatozoa can be either chilled at 15°-17°C or frozen, however this last method is still unsatisfactory in the pig as shown by the promising *in vitro* results but poor *in vivo* outcomes reported by the few studies investigating the survivability of sex-sorted, frozen-thawed boar sperm [36-40]. Boar sperm are known to be highly sensitive to cold shock leading to membrane damage [41]. This susceptibility is exacerbated in sexed sperm due to the modification induced by the sorting procedure and the need to cool and freeze diluted samples [4]. So far pig industry has made very limited use of unsorted frozen boar semen (1%) [42] and it is questionable whether sexed frozen boar semen could meet commercial demand in the future, even with optimization of the procedures.

3.1 Cryopreservation of sexed boar spermatozoa

So far preservation methods for sorted spermatozoa have differed only marginally from the procedures used for unsorted semen, and few studies have been performed to adjust the standard boar sperm cryopreservation procedures to the specific requirements of sex-sorted boar spermatozoa. A suitable modification of the freezing method was proposed by Parrilla et al. [36] who observed an improvement in post-thaw motility of sex-sorted spermatozoa frozen at low concentrations with the use of final glycerol concentrations (0.5–1%) lower than those used in standard boar sperm cryopreservation procedures (TM: 10.1%, 21.3%, 27.8% in the presence of 3%, 1%, 0.5% glycerol respectively).

Even if the quality of sexed-frozen-thawed semen appears promising, cryopreservation leads to a boar dependent impairment of sperm parameters such as motility, viability, malondialdehyde generation, and DNA fragmentation, more so than liquid storage [37]. As a consequence, extremely poor results have been achieved *in vivo* using sex-sorted, frozen-thawed spermatozoa (Table 1). Johnson et al. [38] obtained the first piglets after surgical insemination with frozen-thawed sex-sorted spermatozoa. Ten sows were inseminated with sorted-frozen sperm and four litters were born but the average litter size was nearly half that

of controls due to, as concluded by the authors, a reduced developmental potential of embryos obtained with boar spermatozoa that had undergone these biotechnical procedures [38]. This hypothesis seems to be confirmed by the studies of Bathgate et al. [39,40] who obtained only one litter (of 5 piglets) after non surgical DIUI of 12 sows with 50 $x10^6$ motile sex-sorted, frozen-thawed boar spermatozoa while in a subsequent study all pregnancies were lost after achieving an apparent conception rate of nearly 70%.

The use of sex-sorted, frozen–thawed sperm in combination with IVF and ET could permit a more efficient use of sex-sorted sperm, due to the large reduction of sperm numbers required, and thereby offers one solution to commercialization of this technology in pig. Bathgate et al. [43] demonstrated that pre-sexed porcine embryos could be successfully produced in vitro using sex-sorted frozen–thawed sperm and these embryos are capable of initiating pregnancies when non-surgically transferred into recipients at the eight-cell stage. However they failed to produce piglets of a pre-determined sex suggesting a poor developmental potential of embryos.

One factor that may contribute to the poor outcome achieved with sex-sorted, frozen–thawed boar spermatozoa is a deterioration at the DNA level. Boar spermatozoa are considered to have a very stable chromatin structure [44]. The level of DNA damage observed in sex-sorted fresh [45] and frozen-thawed boar spermatozoa [37] seems to be limited (less than 5%). Moreover the sorting procedure has been reported to improve DNA quality in boar, bull and stallion [37,45-48]. However, the positive effect of the sorting procedure on stallion sperm DNA integrity was lost after thawing suggesting that the freezing process reduces the DNA quality of sex-sorted sperm. Boar sperm cryopreservation has been reported to destabilize the nucleoprotein structure through an increase in the number of disrupted disulphide bridges between cysteines in sperm nucleoproteins and to induce, probably as a consequence, an increase in DNA fragmentation that does not appear

189 immediately after thawing but only after 2 to 4 h of post-thawing incubation [49-51]. Similar
190 or more severe sperm chromatin damage in sex-sorted frozen–thawed boar sperm could
191 explanain the low fertility achieved using spermatozoa that underwent such biotechnical
192 procedures. Subtle sperm chromatin damage can cause reproductive failure occurring with
193 the activation of the embryonic genome without any influence on membrane, motility and
194 fertilizing parameters of spermatozoa or the cleavage rate of oocytes [52].

195 To date, no studies have been performed on the mRNA expression pattern of pig embryos
196 derived from sex-sorted spermatozoa. In bovine and ovine species differential expression
197 of developmentally important genes has been observed between embryos derived from
198 unsorted and sex-sorted sperm [53,54]. In addition, morphological abnormalities have been
199 documented in bovine blastocysts produced with flow-cytometrically sex-
200 sorted spermatozoa [55].

201 The high incidence of pregnancy loss is also observed after DIUI and laparoscopic
202 insemination with non-frozen sex-sorted spermatozoa [14,18] suggesting that DNA
203 alterations of sexed spermatozoa may negatively affect the developmental potential of
204 embryos. However, the low number of viable embryos and foetuses, which per se impairs
205 pregnancy in this species [56,57], might contribute to the pregnancy loss observed with both
206 frozen and unfrozen sex-sorted spermatozoa [14,18,58].

207 Taken as a whole the results obtained to date demonstrate that it is still not economically
208 feasible to incorporate frozen-thawed sexed boar semen into the commercial production of
209 pigs, although it has considerable application in breeding programs.

210 *3.2. Liquid storage of sexed boar spermatozoa*

211 Sexed boar semen can be stored in liquid form at 15-17°C even if sorted spermatozoa lose
212 their fertilizing ability with prolonged intervals from sorting to insemination. In fact, the sorting

procedure seems to increase boar sperm sensitivity to storage not only in cryopreserved but also in liquid state.

Sexed sperm motility and membrane integrity begin to differ significantly, compared to unsorted sperm, after 10 h of storage and subsequently worsen with increasing storage time [10,29,59,60]. Moreover, while modifications on sperm tyrosine phosphorylation patterns immediately after sorting are scarce [34], overall percentage of sexed spermatozoa displaying an uncapacitated pattern after 72 h storage was reduced [61]. This suggests a progressive modification towards a capacitation-like state of sexed spermatozoa during liquid storage as also confirmed by CTC results. However a high sperm quality (viability above 70%) was recently reported for sex-sorted spermatozoa from boars classified as "good sperm sorters" after 48-120 hours of storage at 15°-17°C [10,11]. The commercial application of sex-sorted spermatozoa in swine AI programs may therefore be feasible in the future provided that strategies to extend the lifespan and fertilizing ability of sex-sorted spermatozoa could be developed. The selection of semen donors seems to be very important for sorting and for further storage in liquid or frozen form as differences in the ability of spermatozoa from individual boars to withstand semen handling associated with these technologies have been reported. Moreover, the response of spermatozoa to a specific semen-processing technique does not predict the response of spermatozoa from the same boar to other semen-processing techniques [10,37].

While the sorting procedure does not negatively affect the *in vitro* functional competence of boar spermatozoa compared to unsorted sperm [20,60,62], a significant reduction of fertilizing ability has been recorded starting from 5 h after sorting [29]. Spinaci et al. [60] observed that fertilization was negatively affected when IVF was performed with sorted boar spermatozoa stored in liquid state at 15-17°C for 24 h using a low sperm:oocyte ratio (100:1) and a gamete co-incubation of 5 h. However, no differences on fertilization parameters were

observed when gamete co-incubation was performed for a shorter time (1h) using a higher sperm:oocyte ratio (5000:1). Even though these results confirmed the partial loss of fertilizing ability of sexed boar spermatozoa after liquid storage, they suggest the in vitro outcome can be improved by optimizing the parameters of IVF procedure.

Few studies have been performed in vivo using liquid stored sexed boar spermatozoa. High penetration and monospermy rates were obtained by laparoscopic insemination with deposition directly into the oviductal ampulla using 0.3 x 10^6 sexed spermatozoa stored 16-18 h at 17°C but all the putative zygotes were collected and sows were not allowed to farrow [59]. Acceptable pregnancy rates (around 90%) and farrowing rates (around 80%) have been obtained by laparoscopic insemination using 3-6 x 10^6 sexed spermatozoa stored at 22°C for a maximum of 12 h but no further information was given on the storage time of the single insemination dose [18].

3.3. Use of additives during storage of sexed boar spermatozoa

Different additives have been tested in the attempt to improve the quality of stored sexed spermatozoa. The addition of seminal plasma to the collection medium during sorting or in the medium after centrifugation has been demonstrated to stabilize the sperm surface and to reverse the capacitation-like status acquired by boar spermatozoa emerging from the flow cytometer by counteracting the removal of beneficial seminal plasma components due to the high dilution [27,30]. For this reason, seminal plasma is routinely included in the collection medium in boar sperm sorting protocols [16,27-30] and has been added to the liquid storage medium of sexed boar spermatozoa [29,60,63].

A beneficial effect on membrane integrity, motility and fertilizing ability was obtained by Garcia et al. [59] when sexed spermatozoa were stored for 18h in the collection medium containing PSP-I/PSP-II spermadesins from seminal plasma in order to overcome the variability of the protein content in the different crude seminal plasma in boars [64]. In fact,

while heparin-binding spermadesins from boar seminal plasma have been reported to have a detrimental effect on the *in vitro* function of spermatozoa diluted to a level mimicking sex-sorting, non heparin-binding proteins, PSP-I/PSP-II spermadhesins, showed the opposite effect [65]. The protective action of PSPI/PSP-II was largely preserved in its isolated PSP-II subunit suggesting its potential use as a supplement for highly diluted boar spermatozoa [66].

Recent research demonstrated that when boar spermatozoa were collected in 2% egg yolk medium in the absence of seminal plasma, the addition to sheath fluid of EDTA, Ca^{2+} chelating agent known to prevent plasma membrane destabilization [67], preserved sexed sperm quality and fertility and maintained good sperm characteristics after prolonged post-sorting liquid storage [11,31].

On the other hand, the flow sorting process increased the susceptibility of spermatozoa to the harmful effect of ROS [68,69], and the high content of unsaturated fatty acids in the plasma membrane makes boar spermatozoa particularly sensitive to peroxidative damage [70]. Different antioxidants have been tested during liquid storage of sex-sorted boar spermatozoa to minimize the adverse effects of oxidative stress and to improve sexed sperm quality, but the results obtained were sometimes limited or unsatisfactory. While ascorbic acid-2-glucoside increased the viability of sorted boar spermatozoa maintained at 37°C for 4.5 h, [71], pyruvate, catalase and mercaptoethanol failed to improve the quality of sex-sorted porcine semen either fresh or after frozen storage [72]. Vallorani et al. [63] tested the effect of different antioxidants added to the medium of sexed boar spermatozoa during 24h liquid storage at 15°C, reporting a positive effect of epigallocatechin-3-gallate (EGCG) or superoxide dismutase (SOD) plus seminal plasma (but not Na pyruvate plus catalase) on sexed boar sperm viability (58.2%. vs. 58.7% vs 51.6% in SP+EGCG vs. SP+SOD vs. control group respectively), while acrosome status, caspase activation and Hsp70 pattern

were not influenced. It is worth pointing out that the antioxidant protection of sexed sperm seems to differ among species: Vallorani et al. [63] observed a detrimental effect of EGCG on bovine sorted spermatozoa after liquid storage, while SOD, Na pyruvate and catalase had a positive impact. The protective effect of Na pyruvate and catalase on sexed bull spermatozoa was also demonstrated after freeze-thawing [69] while the pre-sorting incorporation of catalase into the sorting protocol failed to improve post-thaw ram sperm quality [73]. Therefore, it is not possible to predict whether the beneficial effect exerted on spermatozoa in one species could be present in another species making it necessary to test each substance in each species.

3.4. Sexing stored boar spermatozoa

As sperm-sorting requires a long time to obtain a sperm population large enough to be used *in vivo*, the possibility of sorting semen after liquid storage could be of great interest to obtain the highest number of sexed spermatozoa from each ejaculate, particularly if it is of high value. Moreover, semen can be collected in AI centres located far from the sorting facilities and ejaculates may have to be shipped overnight to the sorting laboratory. The feasibility of such procedures was demonstrated by Alkmin et al. [11] who observed that a holding time as long as 24h before sorting does not negatively affect the ability to exhibit well-defined X- and Y-chromosome-bearing boar sperm peaks.The seminal plasma content during pre-sorting storage seems to influence sperm sortability as a higher number of ejaculates stored in presence of 0-10% seminal plasma exhibited a better sorting efficiency than those stored with 50% seminal plasma. Spinaci et al. [62] demonstrated that, after one day of storage, the whole sorting procedure does not reduce the percentage of viable cells with active mitochondria compared to fresh-sorted semen. Additionally, no significant differences between semen sorted as fresh (52 blastocysts/331 oocytes) or after 24h of storage at 17° C (66 blastocysts/ 476 oocytes) were observed in terms of in vitro blastocyst yield. Blastocysts

were also obtained with semen sorted after 48 and 72h of storage. Moreover holding boar spermatozoa 24 h at 15-17°C before sorting did not negatively influence the ability of sex-sorted spermatozoa to tolerate liquid storage up to 120 h at 15-17°C in terms of viability, motility plasma membrane fluidity and intracellular generation of reactive oxygen species [11].

Even if positive results have been obtained, new strategies to prolong the lifespan of sex-sorted boar spermatozoa and to improve their fertilizing ability after liquid storage/cryopreservation are needed before the commercial application of sexed semen in the pig industry.

4. Encapsulation as a possible strategy for storing sex-sorted boar spermatozoa

4.1 The evolution of sperm encapsulation technology

In the zootechnical field, encapsulation technology has been developed to control the release of sperm cells into the female genital tract. About thirty years ago, Nebel et al. [74] first encapsulated bovine spermatozoa in alginate and poly-L-lysine capsules and demonstrated that encapsulation could be applied to bovine male gametes with minimum effect on sperm quality. Furthermore, the capsules protected bovine spermatozoa from phagocytosis and promoted the bioadhesion of polymeric matrix to the uterine cervix preventing sperm retroflux.

The encapsulation technology proposed by Nebel et al. [74] was not adequate for boar spermatozoa as the dilution of sperm cells during the last phase of the encapsulation procedure and the use of calcium as an alginate gelation agent induced a precocious sperm capacitation [75]. To overcome these problems a different encapsulation technology was proposed by Conte et al. [76]. A $BaCl_2$ solution was blended with boar seminal material and

the cell suspension was added dropwise to a sodium alginate solution. Barium ions, diffusing out of the drop, reacted with alginate chains which gelled forming a barium alginate semipermeable membrane around a nucleus of the ejaculate. This technology overcame two major limits of Nebel et al.'s procedure: dilution of seminal plasma proteins and precocious sperm capacitation. The barium alginate membrane protected spermatozoa from outer stimuli, allowing the diffusion of nutrients, metabolites and catabolites, at the same time entrapping seminal plasma proteins in the nucleus thereby protecting the plasma membrane of encapsulated boar spermatozoa. In addition, substituting Ca^{2+} with Ba^{2+} avoided cell activation and premature capacitation [77].

By modifying some technological parameters, Torre et al. [78] demonstrated the possibility of obtaining capsules with different characteristics. In particular, capsule weight and volume were directly correlated to the gelling ion concentration. In addition, raising the Ba^{2+} concentration increased the thickness of the alginate membrane and hence reduced the velocity of sperm release. A different study demonstrated that storage of boar semen in barium alginate capsules, both at 18°C and 38°C, enhanced the quality of spermatozoa in terms of acrosomal membrane integrity and secondary anomalies [79]. The same research group reported that the encapsulation process does not affect motility and plasma membrane integrity of swine spermatozoa and, moreover, an in vitro fertilization assay confirmed the penetration potential of boar sperm cells [80].

In order to optimize the velocity and percentage of boar spermatozoa released, Chou and Wang [81] performed artificial insemination using capsules with different thicknesses. To prevent precocious capacitation, due to the use of calcium, the capsules were suspended in a medium containing fructose and fructose-6-phosphatase able to inhibit sperm activation. The functionality of encapsulated boar spermatozoa during 72 h of storage at 18°C was confirmed by the study of their in situ enzymatic activity [82]. In particular, three enzymes were considered an index of sperm integrity: lactate dehydrogenase (LDH),

cytochrome oxidase (COX) and glucose 6-phosphate dehydrogenase (G6PDH). LDH activity is correlated to acrosomal membrane status. Specifically, the acrosomal reaction reduces intracellular LDH, while the release of COX, located in mitochondrial membrane, is generally considered a sign of cell damage. Instead, when the cytoplasmic membrane is damaged, G6PDH is the first enzyme released. The microdensitometry analyses showed an overall higher enzymatic activity for encapsulated boar spermatozoa with respect to controls. After 72h storage at 18°C, LDH activity decreased in diluted semen but not in the encapsulated spermatozoa. The results obtained in terms of COX activity demonstrated that the encapsulation process preserved sperm cells by lipid peroxidation and higher values of G6PDH activity were maintained by encapsulated semen during 24h storage.

Based on these results, Vigo et al. [83] conducted the first large scale in vivo trial evaluating the fertilizing potential of boar spermatozoa vehiculated in barium alginate capsules. About 4,000 sows were divided into two treatment groups: the first group was artificially inseminated through a traditional procedure using 5 billion spermatozoa per dose for 2 or 3 interventions, whereas the second group was inseminated using a single insemination (5 billion of spermatozoa) with encapsulated semen. The same results were obtained with control and encapsulated semen in terms of successful delivery (96.1% vs. 95.6%) and mean number of live born piglets (11.9 vs. 11.6). The encapsulation treatment did not influence pregnancy rates and at the same time significantly reduced the number of spermatozoa necessary for fertilization. These results suggest that encapsulation technology could be a valid strategy to enhance the in vivo performance of boar sperm cells. The barium alginate capsules reduce the loss of spermatozoa after insemination due to the protective effect from phagocytosis and retroflux. At the same time, the controlled release of male gametes into the female genital tract reduces the number of sperm for a good fertilization rate.

390 *4.2 Encapsulation of sex-sorted boar spermatozoa*

391 On the basis of the positive results reported above, studies have been conducted to assess
392 the impact of the encapsulation process on sexed boar sperm to evaluate the feasibility of
393 using barium alginate capsules as an alternative method for storing sex-sorted pig
394 spermatozoa and potentially controlling their release into the sow genital tract. The results
395 obtained demonstrated that the encapsulation technique does not damage sorted boar
396 semen. During 72 h storage, no differences were observed between diluted and
397 encapsulated sorted spermatozoa in terms of membrane and acrosome integrity [84].
398 Although the sorting procedure reduced sperm viability, encapsulation limited the damage
399 to sorted spermatozoa. The difference in membrane integrity was 27% between diluted
400 unsorted and sorted spermatozoa while the discrepancy was only 11% between the
401 encapsulated unsorted and sorted group. The evaluation of capacitation status, by CTC
402 staining and immunolocalization of tyrosine phosphorylated proteins, revealed that the
403 encapsulation process does not induce any further capacitation-like modification to sorted
404 boar spermatozoa. In fact, no differences between liquid stored and encapsulated sexed
405 spermatozoa were recorded in the percentage of cells displaying different CTC and tyrosine
406 phosphorylation patterns at 72h of storage [61]. Moreover, encapsulation during 72h storage
407 seems to protect sorted semen based on the percentage of sperm cells displaying the Hsp70
408 immunolocalization pattern typical of fresh semen being higher in the encapsulated sorted
409 group (24.3 %) compared with liquid stored semen (2.8 %) [61].

410 The *in vitro* fertilization assays performed after 24h, 48h and 72h of storage at 15°C to
411 assess the impact of the encapsulation process on the fertilizing potential of sorted
412 spermatozoa, confirmed the progressive and time-dependent reduction of the fertilizing
413 ability of flow-cytometrically sorted spermatozoa [84]. While the combination of sorting and
414 encapsulation techniques did not lead to a reduction of membrane integrity (plasmalemma
415 and acrosome), a decrease in penetration rates was observed after storage. Despite this,

sorted spermatozoa stored in barium alginate capsules showed a total fertilizing efficiency (normospermic oocyte/total inseminated) similar to that achieved with diluted sorted sperm. This demonstrated that the handling associated with encapsulation technology does not induce any additional damage to the quality of sorted spermatozoa during 72h storage and does not negatively affect their in vitro fertilization yield.

These encouraging results suggest that encapsulation in barium alginate capsules could be an alternative method for storing sex-sorted boar spermatozoa. Moreover encapsulation may be a promising technique to maximize the *in vivo* use of sexed spermatozoa in the pig, by protecting, targeting and controlling the release of sexed semen into the female genital tract and lowering numbers of sperm required.

5. Concluding remarks

More than 20 years after the production of the first litters of pigs from sexed semen [85], the application of sex sorting in the porcine production system still presents several major challenges. Many efforts have been made to increase the sorting efficiency in terms of yield and sperm quality. In parallel, the development of new instruments and insemination strategies has reduced the number of sexed sperm needed per dose. At this point, further research aimed at optimizing the liquid storage and cryopreservation of sex-sorted boar sperm should be performed to achieve field application of sexed spermatozoa in the pig. Particular attention should be paid to protocols and the use of additives that could permit safe and prolonged storage for spermatozoa either in the liquid state or frozen form. From the genetic point of view, the selection of males of high genetic value should be coupled with the selection of boars producing spermatozoa that are not only easy to sort but also have a reduced sensitivity to storage in cryopreserved or in liquid state.

In this context, encapsulation technology could be a possible future strategy, preferably in association with deep insemination techniques, to increase the fertility of stored sexed boar spermatozoa and to control their release into the sow uterus.

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685 1991:26:195–204.

686 Table 1. Production of piglets from flow-cytometrically sorted spermatozoa in combination with
687 other reproductive technologies (in chronological order).
688

Insemination technique	Type of sorted spermatozoa	Sperm number	N° sows inseminated/ ET recipients	Farrowing rate (%)	Average litter size	Piglets of predicted sex (%)	Reference
Surgical AI (oviduct)	Fresh	3 x 10^5	18	50	7.8	71	Johnson (1991) [85]
IVF, ET	Fresh	4 x 10^5/ml	2	100	5	100	Rath et al., 1997 [19]
IVF, ET	Fresh	2 x 10 ^{4}/ml (57-67 sperm/oocyte)	28	28.6	4.1	97	Abeydeera et al., 1998 [21]
IVF, ET	Fresh	35 sperm/oocyte	21	28.6	5.8	97	Rath et al., 1999 [20]
Surgical AI (oviduct)	Frozen-thawed (sorted)	0.4 x 10^6	10	40	6.8	-	Johnson et al. 2000) [38]
DIUI	Fresh (bulk sorted)	70-140 x 10^6	91	39.1-46.6	8.7-9.2	-	Vazquez et al., 2003 [14]
DIUI	Fresh	50 x 10^6	1	100	11	100	Rath et al., 2003 [15]
ICSI, ET	Fresh		4	100	3.3	100	Probst et al., 2003 [22]
DIUI	Frozen-thawed	50 (motile) x 10^6	12	8	5	40	Bathgate, 2004 [39]
DIUI	Fresh	50 x 10^6	12	33.3	7.5	97	Grossfeld et al., 2005 [16]
Surgical AI (oviduct and uterine horns)	Fresh	3-6 x 10^6	109	78.9 – 80.7	9.2 – 10.8	92	Del Olmo 2014 [18]

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