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1 **STORAGE OF SEXED BOAR SPERMATOZOA: LIMITS AND PERSPECTIVES**

2

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21 **Abstract**

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

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

37

38 **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 $70\text{-}140 \times 10^6$ bulk-sorted [14] or 50×10^6 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 ($3-6 \times 10^6$ 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

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

133

134 **3. Storage of sex-sorted boar spermatozoa**

135 Storage of sexed boar semen is necessary in order to ship it from sorting facilities to recipient
136 females for use on a wider scale. Moreover, an adequate liquid storage protocol for sexed
137 boar spermatozoa is required when producing sexed boar sperm for DIUI as the time
138 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

164 of controls due to, as concluded by the authors, a reduced developmental potential of
165 embryos obtained with boar spermatozoa that had undergone these biotechnical
166 procedures [38]. This hypothesis seems to be confirmed by the studies of Bathgate et al.
167 [39,40] who obtained only one litter (of 5 piglets) after non surgical DIUI of 12 sows with 50
168 $\times 10^6$ motile sex-sorted, frozen-thawed boar spermatozoa while in a subsequent study all
169 pregnancies were lost after achieving an apparent conception rate of nearly 70%.

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

178 One factor that may contribute to the poor outcome achieved with sex-sorted, frozen–
179 thawed boar spermatozoa is a deterioration at the DNA level. Boar spermatozoa are
180 considered to have a very stable chromatin structure [44]. The level of DNA damage
181 observed in sex-sorted fresh [45] and frozen-thawed boar spermatozoa [37] seems to be
182 limited (less than 5%). Moreover the sorting procedure has been reported to improve DNA
183 quality in boar, bull and stallion [37,45-48]. However, the positive effect of the sorting
184 procedure on stallion sperm DNA integrity was lost after thawing suggesting that the freezing
185 process reduces the DNA quality of sex-sorted sperm. Boar sperm cryopreservation has
186 been reported to destabilize the nucleoprotein structure through an increase in the number
187 of disrupted disulphide bridges between cysteines in sperm nucleoproteins and to induce,
188 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 explain 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 fetuses, 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

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

215 Sexed sperm motility and membrane integrity begin to differ significantly, compared to
216 unsorted sperm, after 10 h of storage and subsequently worsen with increasing storage time
217 [10,29,59,60]. Moreover, while modifications on sperm tyrosine phosphorylation patterns
218 immediately after sorting are scarce [34], overall percentage of sexed spermatozoa
219 displaying an uncapacitated pattern after 72 h storage was reduced [61]. This suggests a
220 progressive modification towards a capacitation-like state of sexed spermatozoa during
221 liquid storage as also confirmed by CTC results. However a high sperm quality (viability
222 above 70%) was recently reported for sex-sorted spermatozoa from boars classified as
223 “good sperm sorters” after 48-120 hours of storage at 15°-17°C [10,11]. The commercial
224 application of sex-sorted spermatozoa in swine AI programs may therefore be feasible in
225 the future provided that strategies to extend the lifespan and fertilizing ability of sex-sorted
226 spermatozoa could be developed. The selection of semen donors seems to be very
227 important for sorting and for further storage in liquid or frozen form as differences in the
228 ability of spermatozoa from individual boars to withstand semen handling associated with
229 these technologies have been reported. Moreover, the response of spermatozoa to a
230 specific semen-processing technique does not predict the response of spermatozoa from
231 the same boar to other semen-processing techniques [10,37].

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

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

242 Few studies have been performed in vivo using liquid stored sexed boar spermatozoa. High
243 penetration and monospermy rates were obtained by laparoscopic insemination with
244 deposition directly into the oviductal ampulla using 0.3×10^6 sexed spermatozoa stored 16-
245 18 h at 17°C but all the putative zygotes were collected and sows were not allowed to farrow
246 [59]. Acceptable pregnancy rates (around 90%) and farrowing rates (around 80%) have
247 been obtained by laparoscopic insemination using $3-6 \times 10^6$ sexed spermatozoa stored at
248 22°C for a maximum of 12 h but no further information was given on the storage time of the
249 single insemination dose [18].

250 *3.3. Use of additives during storage of sexed boar spermatozoa*

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

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

263 while heparin-binding spermadesins from boar seminal plasma have been reported to have
264 a detrimental effect on the *in vitro* function of spermatozoa diluted to a level mimicking sex-
265 sorting, non heparin-binding proteins, PSP-I/PSP-II spermadhesins, showed the opposite
266 effect [65]. The protective action of PSPI/PSP-II was largely preserved in its isolated PSP-II
267 subunit suggesting its potential use as a supplement for highly diluted boar spermatozoa
268 [66].

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

274 On the other hand, the flow sorting process increased the susceptibility of spermatozoa to
275 the harmful effect of ROS [68,69], and the high content of unsaturated fatty acids in the
276 plasma membrane makes boar spermatozoa particularly sensitive to peroxidative damage
277 [70]. Different antioxidants have been tested during liquid storage of sex-sorted boar
278 spermatozoa to minimize the adverse effects of oxidative stress and to improve sexed sperm
279 quality, but the results obtained were sometimes limited or unsatisfactory. While ascorbic
280 acid-2-glucoside increased the viability of sorted boar spermatozoa maintained at 37°C for
281 4.5 h, [71], pyruvate, catalase and mercaptoethanol failed to improve the quality of sex-
282 sorted porcine semen either fresh or after frozen storage [72]. Vallorani et al. [63] tested the
283 effect of different antioxidants added to the medium of sexed boar spermatozoa during 24h
284 liquid storage at 15°C, reporting a positive effect of epigallocatechin-3-gallate (EGCG) or
285 superoxide dismutase (SOD) plus seminal plasma (but not Na pyruvate plus catalase) on
286 sexed boar sperm viability (58.2%. vs. 58.7% vs 51.6% in SP+EGCG vs. SP+SOD vs.
287 control group respectively), while acrosome status, caspase activation and Hsp70 pattern

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

297 3.4. Sexing stored boar spermatozoa

298 As sperm-sorting requires a long time to obtain a sperm population large enough to be used
299 *in vivo*, the possibility of sorting semen after liquid storage could be of great interest to obtain
300 the highest number of sexed spermatozoa from each ejaculate, particularly if it is of high
301 value. Moreover, semen can be collected in AI centres located far from the sorting facilities
302 and ejaculates may have to be shipped overnight to the sorting laboratory. The feasibility of
303 such procedures was demonstrated by Alkmin et al. [11] who observed that a holding time
304 as long as 24h before sorting does not negatively affect the ability to exhibit well-defined X-
305 and Y-chromosome-bearing boar sperm peaks. The seminal plasma content during pre-
306 sorting storage seems to influence sperm sortability as a higher number of ejaculates stored
307 in presence of 0-10% seminal plasma exhibited a better sorting efficiency than those stored
308 with 50% seminal plasma. Spinaci et al. [62] demonstrated that, after one day of storage,
309 the whole sorting procedure does not reduce the percentage of viable cells with active
310 mitochondria compared to fresh-sorted semen. Additionally, no significant differences
311 between semen sorted as fresh (52 blastocysts/331 oocytes) or after 24h of storage at 17° C
312 (66 blastocysts/ 476 oocytes) were observed in terms of in vitro blastocyst yield. Blastocysts

313 were also obtained with semen sorted after 48 and 72h of storage. Moreover holding boar
314 spermatozoa 24 h at 15-17°C before sorting did not negatively influence the ability of sex-
315 sorted spermatozoa to tolerate liquid storage up to 120 h at 15-17°C in terms of viability,
316 motility plasma membrane fluidity and intracellular generation of reactive oxygen species
317 [11].

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

322

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

324

325 *4.1 The evolution of sperm encapsulation technology*

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

333 The encapsulation technology proposed by Nebel et al. [74] was not adequate for boar
334 spermatozoa as the dilution of sperm cells during the last phase of the encapsulation
335 procedure and the use of calcium as an alginate gelation agent induced a precocious sperm
336 capacitation [75]. To overcome these problems a different encapsulation technology was
337 proposed by Conte et al. [76]. A BaCl₂ 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),

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

374 Based on these results, Vigo et al. [83] conducted the first large scale in vivo trial evaluating
375 the fertilizing potential of boar spermatozoa vehiculated in barium alginate capsules. About
376 4,000 sows were divided into two treatment groups: the first group was artificially
377 inseminated through a traditional procedure using 5 billion spermatozoa per dose for 2 or 3
378 interventions, whereas the second group was inseminated using a single insemination (5
379 billion of spermatozoa) with encapsulated semen. The same results were obtained with
380 control and encapsulated semen in terms of successful delivery (96.1% vs. 95.6%) and
381 mean number of live born piglets (11.9 vs. 11.6). The encapsulation treatment did not
382 influence pregnancy rates and at the same time significantly reduced the number of
383 spermatozoa necessary for fertilization. These results suggest that encapsulation
384 technology could be a valid strategy to enhance the in vivo performance of boar sperm cells.
385 The barium alginate capsules reduce the loss of spermatozoa after insemination due to the
386 protective effect from phagocytosis and retroflux. At the same time, the controlled release
387 of male gametes into the female genital tract reduces the number of sperm for a good
388 fertilization rate.

389

4.2 Encapsulation of sex-sorted boar spermatozoa

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

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

416 sorted spermatozoa stored in barium alginate capsules showed a total fertilizing efficiency
417 (normospermic oocyte/total inseminated) similar to that achieved with diluted sorted sperm.
418 This demonstrated that the handling associated with encapsulation technology does not
419 induce any additional damage to the quality of sorted spermatozoa during 72h storage and
420 does not negatively affect their *in vitro* fertilization yield.
421 These encouraging results suggest that encapsulation in barium alginate capsules could be
422 an alternative method for storing sex-sorted boar spermatozoa. Moreover encapsulation
423 may be a promising technique to maximize the *in vivo* use of sexed spermatozoa in the pig,
424 by protecting, targeting and controlling the release of sexed semen into the female genital
425 tract and lowering numbers of sperm required.

426

427 **5. Concluding remarks**

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

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

443 **6. Acknowledgements**

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445

7. References

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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	3×10^5	18	50	7.8	71	Johnson (1991) [85]
IVF, ET	Fresh	$4 \times 10^5/\text{ml}$	2	100	5	100	Rath et al., 1997 [19]
IVF, ET	Fresh	$2 \times 10^4/\text{ml}$ (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)	0.4×10^6	10	40	6.8	-	Johnson et al. 2000) [38]
DIUI	Fresh (bulk sorted)	$70\text{-}140 \times 10^6$	91	39.1-46.6	8.7-9.2	-	Vazquez et al., 2003 [14]
DIUI	Fresh	50×10^6	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	$50 \text{ (motile)} \times 10^6$	12	8	5	40	Bathgate, 2004 [39]
DIUI	Fresh	50×10^6	12	33.3	7.5	97	Grossfeld et al., 2005 [16]
Surgical AI (oviduct and uterine horns)	Fresh	$3\text{-}6 \times 10^6$	109	78.9 – 80.7	9.2 – 10.8	92	Del Olmo 2014 [18]

689