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Review Article

Sperm Encapsulation from 1985 to Date: Technology Evolution and New Challenges in Swine Reproduction

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Contents
In the last 30 years, encapsulation technology has been applied to different species to minimize the loss of spermatozoa after artificial insemination. In particular, the vehiculation of boar sperm cells in barium alginate membrane has proved a valid strategy to reduce the risk of polyspermy and optimize *in vivo* fertilizing yields. Controlled release of male gametes into the female genital tract has reduced the minimum fertilizing dose of spermatozoa. Notwithstanding these results, encapsulation has not yet reached commercial application, largely due to the additional costs of production. However, encapsulation could be useful in advanced reproductive technology, such as sex sorting, to store sorted boar semen. The controlled release of flow cytometrically sorted spermatozoa could be a promising strategy to reduce the number of cells necessary for each insemination and hence allow the widescale use of sex sorting in this species.

Introduction

Assisted reproductive technologies (ARTs) are extensively used in the zootechnical field, but have not been as widely applied in swine as in other species (Okere and Nelson 2005). Nowadays, 90% of swine in Europe are bred using the artificial insemination (AI) technique to minimize the risk of disease transmission and introduce genetic selection (Maes et al. 2008). With respect to natural mating, AI offers a number of advantages: cost reduction, hygiene improvement, preventive determination of semen quality and better fertility (Okere and Nelson 2005). Nevertheless, AI efficiency is limited by difficulties related to swine reproductive physiology: the sow is characterized by a seasonal variation in the length of oestrus, the interval from oestrus to ovulation and the duration of ovulation (Belstra et al. 2004), while boar sperm cells are highly susceptible to dilution and cooling procedures so that AI is routinely performed with liquid-stored semen at 15–20°C for 1–5 days (Johnson et al. 2000; Corcini et al. 2011).

In addition to the reproductive physiology-related problems, traditional swine AI procedures present several limitations. After deposition of diluted semen in the female reproductive tract, many sperm cells are removed by uterine backflow or by leucocyte phagocytosis (Roca et al. 2006, 2011). As a result, AI commonly involves two or three sequential inseminations with 2–3 billion sperm cells. To maximize reproductive yields, ongoing research aims to reduce the sperm number per AI and at the same time determine the optimal timing of insemination (Garcia et al. 2007). In recent years, many researchers have proposed encapsulation technology to allow the controlled release of sperm cells into the female genital tract during the oestrous cycle.

The Development of Sperm Encapsulation Technology

Cell encapsulation in a semipermeable membrane dates back to about 50 years ago. The first scientific publication demonstrated that encapsulation of living cells preserved their biological properties both *in vitro* and *in vivo* (Chang 1964). In the zootechnical field, the encapsulation of male gametes was first proposed to develop a controlled release of spermatozoa. In the last 30 years, many researchers have applied encapsulation to seminal material of various animals including bovine, porcine and canine species.

In 1985, Nebel and collaborators devised a 'three-step procedure' to encapsulate bovine seminal material in calcium alginate and poly-L-lysine capsules: the seminal material was dispersed in a sodium alginate solution, and the cellular suspension obtained was extruded in a buffer solution supplemented with calcium chloride. Contact between the cell-containing droplets and calcium ions ensured the gelification of alginate; the beads obtained were covered with poly-L-lysine, and then the alginate matrix was dissolved using a sodium citrate solution as a chelating agent of bivalent cations. *In vitro* and *in vivo* studies have demonstrated that this technology does not damage bovine spermatozoa despite a high percentage of sperm agglutination suggesting that sperm underwent some level of capacitation while in the encapsulated state (Nebel et al. 1996; Vishwanath et al. 1997).

Nebel et al.'s technology applied to boar semen determined the premature loss of motility both *in vitro* and *in vivo* (Esbenshade and Nebel 1990). As boar sperm cells are highly susceptible to dilution, the encapsulation procedure proposed by Nebel et al.

(1985) was not adequate for swine as it led to precocious capacitation and the loss of fertilizing ability of treated spermatozoa. It is well known that calcium ions play a fundamental role in the activation of sperm cells and induce their capacitation (Gadella and Luna 2014). In addition, the dissolution of alginate matrix provoked the dilution of seminal plasma proteins inhibiting the protective and stabilizing effect of the sperm plasma membrane (Medeiros et al. 2002).

A few years later, Conte et al. (1999) proposed a different encapsulation technology to overcome these problems. Their 'one-step method' limited the manipulation and dilution of seminal material to prevent the loss of viability and motility of sperm cells. Ejaculate was added to barium chloride solution to reach a variable Ba^{++} concentration, and the cellular suspension obtained was extruded into a sodium alginate solution through a hypodermic needle. When the semen drop came into contact with the alginate, the barium ions diffused out of the drop and reacted with the alginate chains promoting polymer gelification around the liquid nucleus. This technology presented several advantages. Not only was the ejaculate not diluted, it was also protected from the outside through the barium alginate semipermeable membrane. Furthermore, the seminal material underwent a virtual dilution: nutrients, metabolites and catabolites could diffuse through the membrane, whereas the seminal plasma proteins remained in the nucleus protecting the plasmatic membrane of the encapsulated spermatozoon (Conte et al. 1999). At the same ion concentration, barium yielded a stronger alginate gel than calcium (Gombotz and Wee 1998; Goh et al. 2012; Huang et al. 2012), thereby decreasing the chemical stress on the sperm cells during encapsulation and inhibiting premature capacitation (Munoz-Garay et al. 2001; Villani et al. 2008).

In Vitro and *In Vivo* Results of Boar Semen Encapsulation

Our research group has extensively studied encapsulation technology. The properties of Ba^{2+}-alginate capsules were initially investigated in a formulative study (Torre et al. 2002a), and results demonstrated that the process variables, such as ion and alginate concentration, were directly correlated to weight, gel thickness, total and core diameter of capsules. The stability of encapsulated boar semen was evaluated at 38°C for 24 h: the encapsulation procedure did not negatively influence cell metabolism, and it enhanced acrosome integrity (Torre et al. 2002b). An *in vitro* evaluation of enzymatic activity showed that encapsulation protected boar spermatozoa from the loss of glucose-6-phosphate dehydrogenase, cytochrome c oxidase and lactate dehydrogenase. Instead, enzyme leakage was evident in diluted semen and seems to be correlated with membrane damage (Faustini et al. 2004).

Vigo et al. (2009) conducted the first large scale *in vivo* study comparing the traditional AI procedure with diluted semen (2/3 inseminations) and AI with encapsulated sperm (only one insemination). All inseminations were conducted with refrigerated seminal material doses each containing 2.5 billion spermatozoa. Data obtained from more than 4000 sows were expressed in terms of positive pregnancy diagnosis, successful delivery and number of spermatozoa used for each AI. The positive pregnancy diagnosis and successful delivery results did not differ between the two treatment groups, but a significantly lower mean number of spermatozoa were used was for the capsule group (2.90 ± 0.91 billion spermatozoa) with respect to the traditional AI group (4.91 ± 0.54 billion sperm).

Another problem significantly reducing the reproductive yield in swine species is the high incidence of polyspermy (Gardner and Evans 2006). For this reason, we studied the effect of *in vitro* sperm encapsulation on polyspermy as a function of storage time at 18°C (Faustini et al. 2010). An *in vitro* fertilization assay was performed considering diluted and encapsulated sperm as two treatment groups. The preservation of boar spermatozoa in barium alginate capsules significantly reduced ($p = 0.033$) the risk of polyspermic oocytes (incidence risk ratio: 0.766 with respect to diluted sperm).

Sex Sorting and Sperm Encapsulation: New Challenges in Boar Reproduction

In the last 20 years, increasing interest has focused on sex pre-selection in swine reproduction with a view to optimizing the insemination schedule and farm management (Vazquez et al. 2009). Meat producers prefer female breeding lines so the most widely used breeding strategy is the castration of young male boars. However, the European Union has legislated for the abolition of castration from January 2018 to improve animal welfare (Council Directive 2008/120/EC). Sperm sexing by flow cytometry is one of the potential alternatives to castration of male piglets. This technique is based on the separation of X- and Y-chromosome-bearing spermatozoa on the basis of DNA content using flow cytometric sperm sorting (Johnson 1995).

Although sex sorting is commercially used in dairy cattle production, the high susceptibility of boar sperm cells to flow cytometry-induced stress precludes the routine use of this technology in swine reproduction (Johnson et al. 2005; Spinaci et al. 2010). However, the most limiting factor for routine sperm sexing for common AI procedures in swine is the low number of boar sperm cells obtained after the sorting procedure. Advances in low-dose AI (Johnson et al. 2005) are required to harness flow cytometry and improve the reproductive efficiency of swine.

Data published by our research group (Vigo et al. 2009) demonstrated that the vehiculation of boar seminal material in barium alginate capsules allowed the controlled release of cells in the sow reproductive tract and the consequent reduction of sperm dose for

each AI intervention. Despite the optimal results obtained in recent years, encapsulated seminal material is not commercially available, probably due to the high production costs. The encapsulation of boar sorted seminal material is a dual challenge: the advantages introduced by the controlled release of cells (dose reduction) could also solve the problem of the low number of spermatozoa obtained from sex-sorting procedures. We investigated the effect of encapsulation technology on plasma membrane and acrosomal integrity of pig sexed sperm cells during liquid storage at 15°C for 72 h (Spinaci et al. 2013a). The results showed that the encapsulation process did not produce a significant supplemental injury to the membranes during storage. The percentage of sorted sperm cells with intact plasma membranes was approximately 55% and 52% for liquid-stored and encapsulated sorted sperm, respectively, while acrosomal integrity was 80% for the sorted control group and approximately 75% for sorted encapsulated spermatozoa.

As the sex-sorting process is known to destabilize the plasma membrane of boar sperm cells (Maxwell and Johnson 1997; Spinaci et al. 2006; Bucci et al. 2012), three different capacitation-related parameters were investigated to evaluate the activation of encapsulated spermatozoa: chlortetracycline (CTC) staining, tyrosine phosphorylation and Hsp70 immunolocalization (Spinaci et al. 2013b).

CTC staining disclosed a progressive increase in B-pattern cells (typical of capacitated spermatozoa) during storage, whereas no significant differences were observed between diluted (control) and encapsulated sorted spermatozoa. Considering protein tyrosine phosphorylation, our results showed that encapsulation caused a reduction ($p < 0.05$) of A-pattern cells (uncapacitated spermatozoa) only in unsorted semen, whereas no differences were found between liquid-stored and encapsulated sorted spermatozoa. Regarding Hsp70 immunolocalization, the encapsulation process seems to protect the sorted semen: the percentage sperm cells displaying the uncapacitated pattern was higher in the capsule group (24.3%) with respect to the control group (2.8%).

The *in vitro* characterization of boar spermatozoa, in terms of plasmatic and acrosomal membrane integrity and capacitative patterns, does not accurately predict the fertilizing potential of sperm cells. For this reason, we performed an *in vitro* fertilization assay after 24, 48 and 72 h of storage of both diluted and encapsulated sorted semen (Spinaci et al. 2013a). The percentage of penetrated oocytes was lower in the capsule group (approximately 25%) with respect to diluted semen (approximately 47%). The protective effect of encapsulation significantly reduced the polyspermy rate (9% and 29% for capsule and control groups, respectively), so that no differences were found between the two treatment groups in terms of normospermic oocytes (approximately 16% and 18% for capsule and control, respectively).

These results demonstrated that encapsulation technology does not induce any additional damage to the quality of sorted spermatozoa during 72 h of storage, nor does it produce any negative effect on sperm *in vitro* fertilization yield.

Conclusions

To date, semen encapsulation has only been used on a laboratory scale. From an economic standpoint, production costs are not very high, but they prevent the commercial development of these systems as the commonly used AI procedures with diluted boar seminal material yield acceptable results at lower cost.

Advanced reproductive technologies such as cryopreservation and sex sorting are not routinely used in swine. Freezing and thawing procedures subject the spermatozoa to stress resulting in a loss of viability, while sex sorting of semen produces fewer spermatozoa per unit time. One method proposed to lower the number of spermatozoa needed for AI is deep intrauterine insemination (Martinez et al. 2001). However, this technique still requires too high number of spermatozoa, and skilled personnel are needed to avoid damaging the uterine mucosa and compromising subsequent fertility. Laparoscopic insemination into the uterine horn has been proposed to allow the use of a very low number of sperm, but this procedure is often related to a high rate of polyspermy (Vazquez et al. 2006) and its cost is too high for widescale application (del Olmo et al. 2014).

In this setting, encapsulation technology could be an optimal strategy to overcome many problems. The results obtained by our research group demonstrated that the encapsulation of boar spermatozoa yields a good pregnancy rate *in vivo* with a single insemination and a low dose of sperm cells (Vigo et al. 2009). At the same time, encapsulation technology does not induce any additional damage to sorted spermatozoa (Spinaci et al. 2013a,b). This experimental evidence suggests that encapsulation technology could enhance the *in vivo* fertilizing potential of sorted boar spermatozoa. The controlled release of male gametes into the female reproductive tract could optimize the *in vivo* performance of a low number of sperm obtained after sex sorting.

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Conflict of interest

The authors declare that there is no conflict of interest.

Author contributions

All authors have been involved in designing the study, analysing the data and drafting of the article.

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