CURRENT STATUS AND PERSPECTIVES OF ARTIFICIAL INSEMINATION IN PIGS

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ABSTRACT

Artificial insemination (AI) of pigs has grown exponentially as reproductive technology with more than a dozen of the top pig producers of the world inseminating >95% of the females in production schemes, at either nucleus or multiplication herds. Above 98% of AIs still concern liquid semen, often inseminated just within a day or maximum two from collected and extended, and most often using cervical deposition. Despite this conservative scenario, developments are ongoing. Some relate to the development of new extenders and temperatures of conservation, intending avoidance of the interval 16-20°C towards more convenient cooling temperatures of 5°C. Others focus on diminishing sperm numbers per AI-dose, so that differences in fertility can be determined between boars (often close to a billion sperm per dose). As well, new methods for intrauterine-AI are devised (trans-cervical, deep-intrauterine, double-intrauterine) to deposit low-to-very-low sperm numbers and thus accommodate for further use of selected sperm, either by robustness (colloid-separation) or chromosomal sex, fresh- or frozen-thawed. As re-emergent issues in pig-AI we see the use of alginate-encapsulated sperm for single AI with liquid semen per estrus, as well as re-integration of autologous seminal plasma (or some selected components) to washed sperm to facilitate sperm survival over time and to be crucial signaling to the female. Use of frozen-thawed semen is gaining terrain with the use of selected ejaculate sub-populations, or addition of exogenous chemicals (prostaglandins, oxytocin) or, of new strategies for the accurate induction of ovulation (GnRH agonists) and of single fixed-time AIs. Clouds are still blurring further development. Focused selection of females for ovulation rate and uterine capacity has led to large - and heterogeneous- litters (often yielding high piglet mortality/weakness). Males, selected using such criteria, have only shown modest increases in sperm production and minor positive changes in sperm quality. Male-to-male variation is still a problem to solve, including our incapacity to properly diagnose fertility levels of boars with an apparent similar semen picture; these males still could yield different fertility after conventional AI. New developments in sperm transcriptomics for semen (sperm and seminal plasma) are, however, encouraging and are discussed alongside my critical views of the state-of-the-art in porcine AI.

Keywords**:** AI Methods, Low-sperm Numbers, Sperm Selection, Fixed-time AI, Cryopreservation, Small RNAs

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INTRODUCTION

About 40% of the red meat consumed worldwide is provided by pigs despite decreasing trends in production growth (from 1.8%/year for 2003-2013, 1.4%/year during 2013-2022 to 0.8%/year by the year 2030; OECD-FAO 2013). These figures encompass well the trend foreseen for livestock growth, from 1.2% in 2014 to 0.9% by 2030, and the fact that most production shall continue being headed by Continental China and South-America (Brazil, Argentina, Chile) where feedstuff is still readily available at rentable prices. Worldwide pig meat consumption is, on the other hand, projected to continue increasing; to reach next year 15.3 kg/capita and to keep this amount up to 2030, surpassing beef (OECD-FAO 2013). Although the figures described are less optimistic than some years ago (Delgado *et al.* 2006), pig production still provides efficient fast growth, high carcass merit and meat quality. Increased search for improved feed efficiency (as an indirect way to reduce feed wastage and lower the environmental impact of porcine waste) for larger litters (increased prolificacy) have now been achieved. The latter is a proof that genetic female selection for high ovulation rate (OR) and uterine capacity (UC) was successful. However, drawbacks of this selection strategy are evident; high pig mortality before weaning and low sow robustness resulting in premature culling. Further goals of the industry, as disease resistance and increased survival of growing pigs are also still problematic.

Whether the selection goals mentioned above for females have also increased boar fertility with an impact on prolificacy is yet to be determined. While breeding dams are selected for fertility, prolificacy, good motherhood and longevity, stud boars are generally selected for their genetic potential to produce litters that grow quickly, efficiently and have commercially-attractive carcass types. Application of artificial insemination (AI) has contributed enormously to the propagation of the genetic material of selected stud boars and, probably, shall prevail to do so for the boars that continuously replace the best ones we presently use (Gerrits *et al.* 2005, Robinson and Buhr 2005). Unfortunately, some problems arise; (i) traits related to boar fertility are of low heritability (h^2 ~0.01-0.06), (ii) these traits are strongly affected by genetic and environmental effects of the boar itself, the dam and the offspring and; (iii) female fertility has a greater impact on reproductive performance at herd level, than boar fertility does (Freking *et al.* 2012).

The present review describes the state-of-the-art regarding porcine AI worldwide, including a critical view of its future development encompassing application of sex-sorting, novel AI techniques, and new sperm diagnostic approaches that would reinforce the wider use of genomic selection of sires and the tackling of the emerging problem of large litters in commercial production. It intends to avoid reiteration of the large amount of relevant articles available elsewhere (Rodriguez-Martinez 2007, Roca *et al*. 2011 and references therein).

ARTIFICIAL INSEMINATION: THE MOST SUCCESSFUL REPRODUCTIVE BIOTECHNOLOGY

Porcine AI has passed 80 years of documented action (Serdiuk 1970). It was started by Ivanov in the Soviet Union in the 1920´s, efforts continued by Milovanov in the following decade, designing extenders to handle boar semen at room temperature (Milovanov 1962). The use of boar semen preserved for AI has increased 5-fold over the past 30 years (in Europe >90% of all females are bred via AI with liquid semen), with >99% of the approximately 30 million of registered first AIs´ in the world done with liquid semen while the rest 1% still regards AI with frozen-thawed semen (Riesenbeck 2011, Rodriguez-Martinez 2012). Most AI is done to breed terminal pigs with semen either commercially supplied by specialised boar centres/companies or collected, processed and inseminated on-farm (do-it-yourself, DYS). AI favors genetic improvement by using the semen from selected (either commercially, national or international breeding programmes) boars which is deposited in breeding sows as liquid- or

cryopreserved semen. The firstly named is used for genetic improvement at national or regional level while the second mostly concerns international trade, avoiding movement of live stock.

WHAT IS NEW IN AI WITH LIQUID-STORED BOAR SEMEN?

Today, AI with liquid boar semen yield high fertility and prolificacy (>90% with an average of 13 piglets born alive), consequence of a combination of factors; sperm numbers are high (often >2 billion sperm per dose), AIs are done using newly-collected/extended semen (long live-span) and often, two AIs (even three) are performed during spontaneous estrus. In consequence, although new products such as AI-catheters and extenders are periodically introduced by the industry, the current use of liquid semen for pig AI has not dramatically changed. As example, extension of the lifespan of boar sperm has not reached a breakthrough, despite many extenders can keep sperm alive for two weeks (Roca *et al.* 2011, Broekhuijse *et al.* 2014). However, most producers, particularly those that use DYS, inseminate rather soon after semen collection and extension, and those buying commercial semen AI-doses are also inseminating (whenever possible) on the first or second day following collection. This means that classical extenders, being kept, stored or shipped at moderately reduced temperatures (i.e., 16-20°C) can maintain viability and potential fertilizing capacity for up to 5 days before AI (De Ambrogi *et al.* 2006), and are thus yet widely used, with very few, mostly cosmetic modifications of composition and preparation. Sperm numbers are considered excessive and ought to be dramatically decreased provided that boar-to-boar differences do not shadow current performance. Since decreases to around 1.5 billion sperm/dose can be a threshold to display fertility differences between boars (Tardif *et al.* 1999) producers and the AI-industry keep high sperm numbers/dose as a warrant for prolificacy. Storing high sperm numbers for lengthy periods requires of antibiotics to keep microorganism growth at minimum. Antibiotic use in large scale is connected to development of resistance by bacteria, a growing health problem in animals and human and thus storage at 5ºC, with low antibiotic content is looked upon (Namula *et al.* 2013). Another approach is to "filter" the semen through colloid columns to remove microorganisms (Morrell and Wallgren 2011) while selecting the most robust sperm present (Morrell and Rodriguez-Martinez 2010).

LOW-SPERM AI-DOSE: A TREND TO STAY

Commercial retro-cervical pig AI implies the deposition of 2.5–4x10^9 "fresh" sperm per AI-dose, usually extended to 70-100 mL, with 1-3 AIs per estrus. An ejaculate yields then ~20-25 AI-doses. Decreasing sperm number/dose would facilitate better use of popular boars, in particular those of high genetic merit. Deposition in the proximal-cervix, but particularly at various depths intra-uterine, has proven that lower sperm numbers can be equally effective (Rodriguez-Martinez 2007, Vazquez *et al.* 2008, Roca *et al.* 2011). Several procedures, based on catheter length and type, are today commercially available for the deposition of variable sperm numbers: (i) post-cervical/uterine body AI (Watson and Behan 2002), (ii) Deep intra-uterine AI (diu-AI, Martinez *et al.* 2001, 2002) and (iii) the Double uterine deposition AI (DUDI, Mozo-Martin *et al.* 2012). The (i) post-cervical-AI with 1-1.5x10^9 liquid-extended sperm is technically simpler than Diu-AI or DUDI and can be used in sows or gilts. On the other hand, both DUDI and Diu-AI can drastically further decrease sperm numbers, being optimal for DUDI values of 750x10^6 sperm and for Diu-AI as few as 200x10^6 sperm. The major difference between these techniques, besides DUDI having two deposition points –a medial and a tip located- is the volume of inseminate (DUDI requires 30-50 mL, while 5 mL are enough for Diu-AI). Either technique has farrowing rates and litter sizes comparable to conventional retro-cervical AI, but their future is probably not within the use of liquid semen for commercial production. Catheters are costly, AI more time-consuming and further, restricted to sows. In spontaneous estrus, two AIs are still required, so the gains are yet non-competitive.

EFFECS OF ADDITIVES, INCLUDING SEMINAL PLASMA

In pigs, as in other species, there is a significant sire-effect on fertility, visible after natural mating and accentuated when their semen is extended or further manipulated. The ejaculate contains more than sperm and its seminal plasma (SP) is a rich source of nutrients, buffering salts, peptides, (glyco)proteins and hormones (Rodriguez-Martinez *et al.* 2011, Lopez Rodriguez *et al.* 2013). Some of these substances (i.e. prostaglandins, oestrogens, etc.) can induce uterine contractions and promote sperm transport while others (glycoproteins, cytokines/chemokines, smallRNAs) can influence sperm survival, female ovulation, the female immune system and the resulting fertility (Rodriguez-Martinez *et al.* 2005, 2011). Therefore, holding boar sperm post-collection in SP (Chutia *et al.* 2014) or keeping the amount of homologous SP to proportions of 10-20% v/v has become routine (Roca *et al.* 2011, Rodriguez-Martinez *et al.* 2011, Flowers *et al.* 2013, Rodriguez-Martinez and Peña Vega 2013). Other additives commonly used are analogues of $PGF_{2\alpha}$ in semen doses which mostly have shown low effects, compared to when the analogue is injected to the sow at AI, to increase uterine contractility and/or advance ovulation. The treatment requires, however, proper timing and does not compensate for insufficient sperm numbers or low-quality semen. Beneficial effects are mostly seen in sub-fertile summer season (De Rensis *et al.* 2012).

ENCAPSULATION OF SPERM

Microencapsulation of sperm, wrapping un-extended semen droplets in biodegradable polymers, has been tested since the mid-1980s, when Nebel *et al.* (1985) succeeded in encapsulating bull sperm in sodium alginate and poly-L-lysine capsules, a technique later applied to boar sperm (Nebel *et al.* 1993). Although successful, the technique was considered cumbersome and requested a simplification now achieved as one-step processing (Faustini *et al.* 2012). In brief, the sperm-rich fraction of the ejaculate is suspended in a saturated solution of barium chloride to be thereafter dripped in a viscous sodium alginate solution thus building an alginate wall around sperm, wall that thickens by diffusion of barium ions. Once this suspension is inseminated *ad praxis*, the polymer wall absorbs water from the intraluminal genital tract fluids, swells and degrades (sodium ions replace the cross-linked ions of the wall), releasing –in different amounts since the process is slow and depends on sodium ions availability- the sperm, which apparently survive for longer periods after AI, compared to conventional sperm suspensions. However, this capacity is also a drawback of the methodology, since the timing of sperm release varies with the type of alginate used, the composition of the SP (variable between boars) and particularly, the intrauterine fluid milieu. All this implies uncertainty in sperm release timing. However, research is advancing looking for “intelligent” multi-layered hydrogels so that the sperm release is pulsatile over time, or it is triggered by enzymes present in the capsule by stimulation of the pre-ovulatory LH-peak, either way ensuring enough sperm numbers are available for fertilization when ovulation spontaneously occurs (Kemmer *et al.* 2011). Such development should however accompany reasonable costs, be workable at boars´ collection places, and include low concentrations of fresh- or manipulated sperm (frozen-thawed, sorted, etc.). Time will show.

SPERM SELECTION

The boar ejaculate, as that of other animals, is a heterogeneous suspension of sperm, which differ not only in genomic- transcriptomic- and proteomic terms but also phenotypically (intactness, morphology, motility, life-span etc.). Therefore, researchers have always described presence of sub-populations, from “robust-to-less-robust” sperm (Gil *et al.* 2005, Saravia *et al.* 2007 and references therein). Separation of “robust” sperm, holding intact attributes for fertilization has been a goal, either to rescue the best sperm from low-quality ejaculates or after stressful manipulations (for instance after strong centrifugation, excessive extension, cooling, freezing-thawing, flow cytometry [FC], etc.). Different methods (washing, swim-up, density gradients, etc.) had been used, with varying

degree of performance (Rodriguez-Martinez *et al.* 1997). Today, FC-sorting and colloid density selection are prevailing albeit for different purposes, the first-named (although proven valuable for separation via selective probes) is mostly focused to genomic sorting (sex-sorting) while the second is designed for selection for robustness under more or less practical conditions (simple equipment, high volume and yield, short time). Colloid centrifugation of boar semen is now applied using single columns of silane-coated spheres, suspended in species-specific media which can harvest most robust sperm, separate them from SP or extension/freezing media and also from microorganisms, thus providing additional advantages such as less need for antibiotics (Morrell and Rodriguez-Martinez 2009, 2010). The columns are able to process large volumes, thus facilitating handling of ejaculates (Wallgren and Morrell 2011). Gender selection using high speed FC-sorting of DNA-stained sperm (Beltsville Sperm Sexing Technology), based on the difference in size (and thus emitted fluorescence to a laser beam exposure) between sex chromosomes (Garner *et al.* 2013) is, in pig production, highly desirable since it would allow the production of either male or female crossbred lines, and then ameliorate the expanding banning of male piglet castration. Yet the commercial application of sex-sorted boar semen for AI is shadowed by the well-known low sperm survival due to the high pressure and to the extreme sperm extension applied during the process (Suh *et al.,* 2005), conveying detrimental effects of the absence of SP-components (Caballero *et al.,* 2004, 2006). Moreover, boar sperm can only be routinely separated at low speed, counteracting expected production of doses for conventional AI. Using additives to the sperm-media (mostly SP) and Diu-AI, piglets have been successfully born (Roca *et al.* 2011), increasing expectations.

MOMENT OF AI: THE MOST CRUCIAL TIMING

Everyone would agree that current numbers of sperm/AI/estrus are excessive and are only kept for the sake of warranting a certain level of fertility and prolificacy. Moreover, we lose opportunities in separating most fertile boars from others and facilitating use of popular boars in a more efficient way, a matter we aim to solve by decreasing sperm number/dose or inseminating only once per estrus. However, timing of sperm deposition relative to ovulation is probably the most important variable affecting the outcome of AI, particularly when low sperm numbers or frozen-thawed semen is used (Wongtawan *et al.* 2006, Rodriguez-Martinez and Wallgren 2010, Wallgren 2013). Ovulation always occurs in the last third of standing estrus, imposing accurate estrus detection as pre-requisite to determine onset of standing reflex, a matter difficult to achieve without changing today´s commercial pig production management (Wallgren 2013). An alternative is to synchronize and provoke ovulation with exogenous hormones to use fixed-time AI (Brussow *et al.* 2009, Driancourt 2013, Driancourt *et al.* 2013). Use of fixed-time AI has provided variable results with sex-sorted semen, but also promising ones using both liquid- as well as frozen-thawed semen (Roca *et al.* 2011).

CRYOPRESERVATION OF SEMEN: ANY BREAKTHROUGH?

Today, boar sperm are frozen slowly, with extracellular ice formation, dehydration, a toxic hyper-concentration of intracellular solutes which does not resolve during thawing, jeopardizing cell survival or handicapping vital cell functions post-thaw (rev by Rodriguez-Martinez 2012). Research has concentrated towards various cryo-protectants (CPA) either low-to-medium toxicity at low concentrations (soluble/membrane penetrating CPA as glycerol, dimethyl sulphoxide [DMSO], ethylenglycol [EG], propyleneglycol [PG] etc.) or the use of non-penetrating CPA (such as sucrose or trehalose). Ultra-high speed has also been tested with apparent acceptable results (Saragusty and Arav 2011). Despite efforts made, there are still inherent difficulties in freezing boar sperm as well as a major boar-dependent cryosurvival to current procedures, which most often yields thawed sperm with a shortened life span and lead to lowered prolificacy as major negative output. Interplay with the SP, including that of particular portions of the ejaculate (Rodriguez-Martinez *et al.* 2009) has proven beneficial explored *in vitro* (Saravia *et al.* 2010) and after

Diu-AI (Rodriguez-Martinez and Wallgren 2010, Roca *et al.* 2011) or cervical AI (Okazaki and Shimada 2012, Wallgren 2013). The novelty applied has clearly simplified the processing, making freezing of boar semen a less cumbersome and expensive process, and cost are now similar to processing conventional liquid semen (Gonzalez-Peña *et al.* 2014). In sum, although optimization of freezing-thawing is required at most levels, particular focus should be given towards the sole use of xeno-components, assuring not only cryosurvival but also freedom of modification of the genome and its transcribing capacity.

SPERM DIAGNOSTICS: WHAT´S AHEAD TO TRY?

Obviously, whatever form of semen handling for AI is applied, sperm is to be evaluated, particularly in relation to fertility and prolificacy. Our current arsenal of diagnostic andrological methods basically cover the intactness of all attributes sperm need for successful fertilization and initiation of embryonic development, from the plasmalemma to the nuclear DNA; as well as the interplay with SP-components (Rodriguez-Martinez 2014). Yet it seems insufficient. Semen delivers a series of small regulatory non-coding RNAs (ncRNA, 19-22 nucleotides, Bartel 2009); microRNAs (miRNAs) that are shed both in the SP (Belleannee *et al.* 2012, Wu *et al.* 2012) as well as present in each sperm (McIver *et al*. 2012, Hamatani 2012). miRNAs appear as key controllers of gene expression (primarily inhibiting) by affecting stability and translation of mRNA, and are particularly relevant for embryo development (Kumar *et al*. 2013). For instance, the murine miR-34 (also present in human and stallion sperm), appears essential for 1st cleavage division (Liu *et al.* 2012). miRNAs moreover can, acting epigenetically, play an important role in the acquisition and maintenance of male fertility (Dadoune 2009, Jodar *et al.* 2013). Some miRNAs are species-specific (Curry *et al.* 2009, Govindaraju *et al.* 2012, Krawetz *et al.* 2011, Peng *et al.* 2012, Das *et al.* 2013, Card et al. 2013) while many are conserved over species. Levels of miRNAs relate to some sperm functional attributes in bull (Govindaraju *et al.* 2012), stallion (Das *et al.* 2013) or pig (Curry *et al.* 2009, 2011). In bull sperm, miRNAs show differential expression in relation to sire fertility levels (Govindaraju *et al.* 2012). Cryopreservation (including conventional vitrification i.e. embedding of sperm in a glassy medium without ice crystal formation) of human sperm causes alterations in the essential mRNA transcripts PRMI and PRM2 (Valcarce *et al.* 2013a). Comparative studies in domestic species are yet scarce (Bissonette et al 2009, Govindaraju *et al.* 2012) calling for a wider exploration of the miRNAome to establish molecular biomarkers of sperm quality and fertility after cryopreservation (Valcarce *et al.* 2013b, Jodar *et al.* 2013). Such exploration should include SP and sperm, provided somatic cells and non-viable sperm are removed using sperm selection methods (Morrell and Rodriguez-Martinez 2010).

CONCLUSION

This review provides a critical description of the state-of-the-art regarding porcine artificial insemination (AI) worldwide, and the future application of sex-sorting, novel AI techniques, and new sperm diagnostic approaches that would reinforce the wider use of genomic selection of sires, and the tackling of the emerging problem of large litters in commercial production. Further use of lower sperm numbers per AI/estrus will be pivotal in selecting high fertility boars as well as facilitating the use of sex-sorted and frozen-thawed semen. Inseminating techniques will also push for this development, albeit post-cervical AI (uterine body) will probably dominate in pig production. Owing to the intense pressure on costs and effectiveness, use of hormonally manipulated fixed-AI strategies shall dominate, despite not being the most physiological approach. Finally, there is an urgent need for exploration of transcripts in sperm and SP, in order to reinforce our diagnostic capabilities regarding boar fertility.

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