UNIVERSIDAD DE MURCIA

FACULTAD DE VETERINARIA

Rendimiento productivo de la inseminación post-cervical en la especie porcina. Estudio de la selección espermática en el tracto genital de la hembra a través del análisis del reflujo

Productive output of post-cervical insemination in porcine. Study of sperm selection in the female genital tract through backflow analysis

D. IVÁN HERNÁNDEZ CARAVACA

2015

UNIVERSIDAD DE
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AUTORIZA:

La presentación de la Tesis Doctoral titulada "Rendimiento productivo de la inseminación post-cervical en la especie porcina. Estudio de la selección espermática en el tracto genital de la hembra a través del análisis del reflujo", realizada por D. Iván Hernández Caravaca, bajo mi inmediata dirección y supervisión, y que presenta para la obtención del grado de Doctor por la Universidad de Murcia.

Murcia, a 22 de Mayo de 2015.

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Tesis Internacional

Esta tesis doctoral ha sido propuesta para la Mención de "Doctor Internacional" en virtud de las estancias de investigación realizadas y de los informes de dos expertos extranjeros.

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27 de Enero de 2015

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Certifica que:

D. Iván Hernández Caravaca ha realizado estancias en Boehringer-Ingelheim Health Management Center perteneciente al area de investigación y desarrollo de Boehringer Ingelheim Vetmedica (Ames, Iowa, USA) durante dos periodos de tiempo: del 12 de septiembre al 12 de Octubre del 2011 y desde el 29 de Abril hasta el 29 de Junio de 2013 (tres meses en total).

Durante estas estancias D. Ivan Hernández Caravaca realizó un excelente trabajo.

Su integración en nuestro equipo fue muy buena siendo un placer para todos los miembros de nuestro laboratorio tenerlo entre nuestro equipo.

Y para que conste, a los efectos oportunos, firmo el presente documento,

Atentamente,

Arturo Oropeza Mendoza

Prof. Arturo Oropeza
Technical manager Boehringer Ingelheim Vetmedica Inc.

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Dear sir/madam,

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The project of Doctoral Thesis entitled "**Productive output of post-cervical insemination in porcine. Study of sperm selection in the female genital tract through backflow analysis**" presented by Iván Hernández Caravaca under the research supervision of Dr. María José Izquierdo Rico and Dr. Francisco Alberto García Vázquez evaluated positively for the next public defense and evaluation by a jury to obtain the academic degree of Doctor by the University of Murcia with the mention "International Doctorate".

Informa:

El proyecto de Tesis Doctoral titulado "**Rendimiento productivo de la inseminación post-cervical en la especie porcina. Estudio de la selección espermática en el tracto genital de la hembra a través del análisis del reflujo**" presentado por Iván Hernández Caravaca bajo la dirección de los doctores María José Izquierdo Rico y Francisco Alberto García Vázquez es evaluado positivamente para ser defendido en acto público ante un tribunal para obtener el grado académico de Doctor por la Universidad de Murcia con mención de "Doctorado Internacional".

Yours faithfully

Luis Vieira

25th May 2015.

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Yours faithfully

W. V. Holt

William V. Holt PhD

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1. Summary/Resumen

The porcine industry is continuously growing in all areas related to it, whether it is from a health, economic, production or reproductive point of view. In the reproduction area, numerous research groups are constantly trying to maximize the reproduction efficiency in this species. Under study, to, are pure science aspects such as signalling during sperm capacitation or the determination of proteins involved in the process of fertilization, or aspects more related with applied science such as improvements in the production of *in vitro* embryos, sperm freezing, spermatozoa sexing or improvements in the techniques of assisted reproduction such as artificial insemination. With respect to this last point, artificial insemination is a very commonly used technique on farms, where more than 80% of the insemination that takes place worldwide is artificial, while only a very reduced percentage involves natural insemination (rural, little industrialized areas).

Although artificial insemination is extensively used at a farm level, its efficiency has not yet been maximized, so there is still a wide margin for improvement. This area related to artificial insemination can be divided into two: 1) the implementation of improved insemination techniques *per se*; 2) new knowledge related to the interaction of spermatozoa with the female genital tract. In relation to insemination techniques there have been recent improvements in new insemination techniques with the objective of reducing the insemination dose, so that one ejaculate can be used to inseminate a greater number of sows. Among these techniques is post-cervical insemination, which consists of depositing semen in the corpus uteri, thereby reducing the sperm concentration to be used per insemination and sow. On the other hand, it is known that of the millions of spermatozoa which are deposited in the female genital tract, only a few thousand are able to reach a site close to where fecundation takes place, which implies an enormous loss of sperm during the passage of the spermatozoa through the female genital tract. Some of the mechanisms through which this reduction in the spermatozoa population occurs are known but we still do not know if it is as random or selective process that depends on the characteristics of the ejaculate, or more specifically, on the intrinsic characteristics that each spermatozoon has.

Bearing all this in mind, the main objective of this work was to study the viability of applying post-cervical insemination on the farm (applied science), as well as to the study the influence of different spermatozoa populations - with different characteristics - on the selection that takes place in the sow uterus (pure science); for this we analyzed spermatozoa which were expelled in the reflux after insemination and those which did reach the utero-tubal junction.

In **article 1**, the objectives were first (*experiment 1*) to compare the reproductive parameters [return to oestrus (%), abortions (%), gestation (%), births (%) and size of litter] and economic performance in sows subjected to cervical ($3x10^9$ spermatozoa in 80 ml) and post-cervical ($1.5x10^9$ spermatozoa in 40 ml and $1x10^9$ spermatozoa in 26 ml) insemination. In second place (*experiment 2*), to evaluate the volume (% initial dose) and sperm concentration (% initial dose) in the reflux of the 3 above mentioned insemination groups, as well as the sperm quality (motility %, progressive motility, viability %, morphology % and decondensation of the chromatin) compared with the initial insemination dose, checking whether the uterus has some sort of selective effect by eliminating the less apt spermatozoa.

The results of *experiment 1* showed that the use of post-cervical insemination produces a very similar (in the case of insemination with $1x10^9$ spermatozoa in 26 ml) or even higher (if inseminating with $1.5x10^9$ spermatozoa in 40 ml) reproductive yield than cervical insemination, the yield being much higher when inseminating sows with 2-3 or ≥6 births. At the same time, a detailed economic study of post-cervical insemination on the farm showed that this method produced substantially greater benefits than traditional insemination.

In relation to *experiment 2*, we were able to observe that the volume (%) as well as the number of spermatozoa (%) in the reflux was higher when using cervical insemination compared to post-cervical. On the other hand, the sperm quality was lower in those spermatozoa collected from the reflux (regardless of the insemination technique used) compared to the initial insemination quality.

In **article 2**, we evaluated the influence of different levels of sperm motility in the insemination dose on the percentage of sows with reflux, the volume (%), sperm concentration (%), and type of spermatozoa (%) (based on the motility characteristics) collected from the reflux at different times after post-cervical insemination. For this, the females were inseminated with $1500x10^6$ spermatozoa in 25 ml. Each insemination comprised two parts: (1) $750x10^6$ of non-dyed spermatozoa in 12.5 ml with high motility (>70%) and (2) $750x10^6$ of dyed spermatozoa (Hoechst) in 12.5 ml and different levels of motility (low, 7.50% motile; medium, 42.50%; and high, 75.00%). Spermatozoa were dyed for identification after collection of the reflux, and the reflux was collected at different times post-insemination (0-15, 16-30 and 31-60 min).

The results showed that there were no differences in the % of sows which had reflux, regardless of the insemination dose received (low, medium or high motility). In the same way,

there were no observable differences as regards the volume (%) and number of spermatozoa (%) between the different experimental groups at different times of collection. However, the % of spermatozoa of medium or low motility collected in the reflux was higher than those of higher motility. This observation was made 16 minutes after insemination, indicating the expulsion of random spermatozoa in the first moments following insemination (0-15 min), while at 16-60 minutes sperm elimination was a selective process, whereby spermatozoa with a low motility were expelled.

In **article 3**, we evaluated the morphometric differences between spermatozoa collected in the reflux and utero-tubal junction and those that formed the initial insemination dose. The purpose of study was to analyze whether the spermatozoa which were eliminated during reflux or reached a place close to the fecundation site in the sow uterus presented any particular morphometric characteristics. With this purpose, the study was divided into two experiments. In *experiment 1*, we analyzed parameters related to the size of the sperm head (length, width, area and perimeter), the shape (shape factor, ellipticity, elongation and regularity) and the length of the flagellum. These morphometric parameters were measured in the spermatozoa collected from reflux at different times (0-15, 16-30 and 31-60 min) after insemination and were compared with the data obtained from the initial insemination dose. We also evaluated whether the site of deposition influenced the morphometry of the spermatozoa found in the reflux. In *experiment 2*, we compared the flagellum length of the spermatozoa collected from the reflux and utero-tubal junction with the same parameter of the spermatozoa in the insemination dose.

The results of *experiment 1* showed that the reflux was formed of sperm populations with a certain size and shape, mainly those with a small head and flagellum. It was also demonstrated that the uterine fluid, acrosome alteration and osmorality were not involved in these morphometric changes. On the other hand, we observed that the sperm deposition place was related with the size of the spermatozoa collected during reflux. In *experiment 2*, the data obtained showed that spermatozoa which reached the utero-tubal junction had the same length as those in the initial insemination dose.

In **article 4**, we considered the hypothesis that the spermatozoa with morphoanomalies could be eliminated or modified by the uterine environment after deposition in the female genital tract. For this, the study was divided into two experiments. In *experiment 1*, we assessed whether the uterus acted as a barrier for spermatozoa with morphoanomalies by analysing sperm morphology in the reflux (60 minutes post- insemination) and in spermatozoa

which succeeded in reaching the utero-tubal junction (24 hours post- insemination) and compared the findings with the initial insemination dose. In *experiment 2*, we evaluated whether the composition of the uterine fluid could be involved in the morphological modification of spermatozoa. For this experiment, we used epididymal spermatozoa (high level of morphoanomalies and absence of seminal plasma) and ejaculate (low degree of morphoanomalies and presence of seminal plasma), which were incubated up to 24h in the presence and absence of uterine fluid (collected from sows during late follicular phase).

The results of experiment 1 showed a higher % of spermatozoa with morphoanomalies in the reflux than in the seminal dose, while practically the whole spermatozoa population which colonized the utero-tubal junction had a normal morphology. In experiment 2 it was seen that the uterine fluid had no effect on the morphological changes that occurred in the ejaculated spermatozoa, although when the uterine fluid was incubated with epididymal spermatozoa, there was a drastic decrease in the number morphoanomalies especially in the distal cytoplasmic droplets.

In conclusion, this PhD thesis shows that post-cervical artificial insemination is a viable technique for use in farms, where the advantages are clear from both reproductive and economic points of view. Indeed, its implementation and application on farms is firmly established. Furthermore, we demonstrate that ejaculations are heterogeneous populations with a diverse motility, morphology and morphometry, and that these particularities - characteristics of each spermatozoon - influence any interaction with the genital tract of the female once deposited. Most spermatozoa are eliminated during their journey to the site of fecundation in what seems to be a selective process of discrimination or due to modifications in some of the characteristics of each individual spermatozoon as it interacts with the uterus.

La industria porcina se encuentra en un continuo crecimiento en todas las áreas que la rodean, ya sea desde un ámbito sanitario, pasando por el económico y productivo y terminando con los aspectos reproductivos. En el caso del área reproductiva, numerosos grupos de investigación se encuentran en un continuo avance con el fin último de maximizar la eficiencia de la reproducción en esta especie, ya sea desde investigaciones en ciencia básica tales como estudios de señalización durante la capacitación espermática o determinación de diversas proteínas involucradas en el proceso de fecundación, o en ciencia aplicada como la mejora de la producción *in vitro* de embriones, congelación de semen, sexaje de espermatozoides o mejoras en las técnicas de reproducción asistida como la inseminación artificial. Si nos paramos en éste último punto, la inseminación artificial es una técnica ampliamente aplicada en granja, donde más del 80% de las inseminaciones a nivel mundial son artificiales, mientras que solo en un reducido porcentaje se sigue realizando la inseminación natural (zonas rurales y poco industrializadas).

A pesar de que la inseminación artificial se encuentra altamente extendida a nivel de granja, todavía no se ha maximizado su eficiencia, por lo que el margen de mejora en este aspecto es todavía considerable. Este campo de mejora relacionada con la inseminación artificial puede dividirse en dos vertientes: 1) Implementación de mejoras en la técnica de inseminación *per se*; 2) nuevos conocimientos relacionados con la interacción de los espermatozoides en el tracto genital de la hembra. En relación a la técnica de inseminación, en los últimos años se han avanzado en nuevos dispositivos de inseminación con el fin último de reducir las dosis de inseminación, de tal manera que un solo eyaculado pueda servir para inseminar un mayor número de hembras. Entre estos dispositivos se encuentra la inseminación post-cervical que consiste en la deposición del semen en el cuerpo del útero, con la consiguiente reducción en la concentración espermática a utilizar por inseminación y cerda. Por otro lado, es sabido que de los millones de espermatozoides que se depositan en el tracto genital de la hembra, únicamente unos miles son capaces de llegar al lugar próximo a la fecundación, lo que supone una gran pérdida espermática a lo largo del trayecto que el espermatozoide tiene que recorrer en el interior del tracto genital de la hembra. Se conocen algunos mecanismos por los cuales se produce esta reducción en la población espermática pero se desconoce si se trata de un proceso aleatorio o selectivo dependiendo de las características del eyaculado, o más concretamente, de las características intrínsecas que cada espermatozoide posee.

Con todo esto, el objetivo principal de este trabajo fue el estudio y rentabilidad de la aplicación de la inseminación post-cervical en granja (ciencia aplicada), así como el estudio de la influencia de distintas poblaciones espermáticas con diferentes características en su selección en el útero de la cerda (ciencia básica); para ello se analizaron los espermatozoides que fueron expulsados en el reflujo tras la inseminación y aquellos que llegaron a la unión útero-tubárica.

En el **artículo 1**, los objetivos fueron en primer lugar (*experimento 1*) comparar los parámetros reproductivos [retorno a estro (%), abortos (%), gestación (%), partos (%) y tamaño de la camada] y rendimientos económicos en aquellas cerdas sometidas a una inseminación cervical ($3x10^9$ espermatozoides en 80 ml) o post-cervical ($1.5x10^9$ espermatozoides en 40 ml y $1x10^9$ espermatozoides en 26 ml). En segundo lugar (*experimento 2*), evaluar el volumen (% dosis inicial) y concentración espermática (% dosis inicial) en el reflujo de los 3 grupos de inseminación anteriormente descritos así como la calidad espermática (motilidad %, motilidad progresiva, viabilidad %, morfología % y descondensación de la cromatina) comparándola con las dosis inicial de inseminación, comprobando si el útero ejercía algún efecto selectivo mediante la eliminación de aquellos espermatozoides menos aptos.

Los resultados del *experimento 1* mostraron que la aplicación de inseminación post-cervical tiene unos rendimientos reproductivos similares (en el caso de inseminar con $1x10^9$ espermatozoides en 26 ml) o superiores (si se insemina con $1.5x10^9$ espermatozoides en 40 ml) que la inseminación cervical, siendo los rendimientos mayores cuando se inseminan cerdas con 2-3 o $\geq$6 partos. Al mismo tiempo se realizó un estudio económico detallado de la aplicación de la inseminación post-cervical en granja, siendo éste sustancialmente beneficioso al aplicar dicha técnica en comparación con la inseminación tradicional.

En relación al *experimento 2*, se pudo observar que tanto el volumen (%) como el número de espermatozoides (%) en el reflujo eran superiores cuando se utilizaba la inseminación cervical en relación a la post-cervical. Por otro lado, la calidad espermática se encontraba reducida en aquellos espermatozoides recogidos en el reflujo (independientemente de la técnica de inseminación utilizada) comparados con la dosis seminal inicial.

En el **artículo 2**, se evaluó la influencia de diferentes niveles de motilidad espermática en la dosis de inseminación sobre el % de cerdas con reflujo, el volumen (%), concentración espermática (%) y tipo de espermatozoides (%) (basados en sus características mótiles) recolectados en el reflujo a diferentes tiempos tras la inseminación post-cervical. Para ello, las hembras se inseminaron con $1500x10^6$ espermatozoides en 25 ml. Cada inseminación estaba compuesta por dos partes: (1) $750x10^6$ de espermatozoides no teñidos en 12.5 ml con alta

motilidad (>70%) y (2) $750x10^6$ de espermatozoides teñidos (Hoechst) en 12.5 ml y con diferentes grados de motilidad (baja: 7.50% mótiles; media: 42.50%; y alta: 75.00%). Los espermatozoides fueron teñidos para identificarlos claramente tras su recogida en el reflujo. El reflujo se recolectó a diferentes tiempos tras la inseminación (0-15, 16-30 y 31-60 min).

Los resultados mostraron que no había diferencias en el % de cerdas que presentaban reflujo independientemente de la dosis de inseminación recibida (motilidad baja, media o alta). De la misma manera no se observaron diferencias en relación al volumen (%) y número de espermatozoides (%) entre los diferentes grupos experimentales a los distintos tiempos de recogida. Sin embargo, el % de espermatozoides de media o baja motilidad recolectados en el reflujo eran mayores que si presentaban una motilidad alta. Este hecho se observó a partir de los 16 minutos tras la inseminación, indicando un proceso de expulsión de espermatozoides aleatorio en los primeros momentos tras la inseminación (0-15 min) mientras que entre los 16-60 minutos, la eliminación espermática se correspondía a un proceso selectivo descartando en mayor medida espermatozoides con una capacidad móvil disminuida.

En el **artículo 3**, se evaluó la diferencia morfométrica entre los espermatozoides recolectados en el reflujo y en los que se encontraron en la unión útero-tubárica con aquellos que conformaban la dosis seminal de inseminación. El propósito del estudio se basó en analizar si aquellos espermatozoides que eran eliminados en el reflujo o que alcanzaban el lugar próximo a la fecundación en el útero de la cerda, presentaban unas características morfométricas determinadas. Con este propósito, este estudio se dividió en dos experimentos. En el *experimento 1*, se analizaron diferentes parámetros relacionados con la dimensión de la cabeza espermática (longitud, anchura, área y perímetro) y de la forma (*shape factor*, elipticidad, elongación y regularidad) así como la longitud del flagelo. Dichos parámetros morfométricos se midieron en espermatozoides recolectados en el reflujo a diferentes tiempos (0-15, 16-30 y 31-60 min) tras la inseminación y se compararon con los datos obtenidos de la dosis seminal inicial. También se evaluó si el lugar de deposición influía en la morfometria de aquellos espermatozoides que se encontraban en el reflujo. En el *experimento 2*, se comparó la longitud del flagelo entre los espermatozoides recolectados en el reflujo, los que llegaron a la unión útero-tubárica y los de la dosis seminal.

Los resultados en el *experimento 1* mostraron que el reflujo está formado por poblaciones espermáticas con una determinada dimensión y forma, siendo los de un tamaño de cabeza y longitud del flagelo menor los que tienden a ser encontrados en el reflujo. Además se comprobó que ni el fluido uterino, ni la alteración de acrosomas ni la osmolaridad estaban implicados en los cambios morfométricos.

Por otro lado, se observó que el lugar de deposición espermático influía en el tamaño del espermatozoide que se recolectaba en el reflujo. En el *experimento 2*, los datos obtenidos muestran que los espermatozoides que alcanzan la unión útero-tubárica presentan la misma longitud del flagelo que aquellos presentes en la dosis inicial de inseminación.

En el **artículo 4**, barajamos la hipótesis de que los espermatozoides con morfoanomalías podían ser descartados o modificados por el ambiente uterino tras la deposición en el tracto genital de la hembra. Para ello, este estudio se dividió en dos experimentos. En el *experimento 1*, se evaluó si el útero actuaba como una barrera de espermatozoides con morfoanomalias, mediante el análisis de la morfología espermática en el reflujo (60 minutos tras la inseminación) y en aquellos espermatozoides que alcanzaban la unión útero-tubárica (24 horas tras la inseminación) en comparación con la dosis seminal inicial. En el *experimento 2*, se evaluó si la composición del fluido uterino tenía influencia en la modificación morfológica de los espermatozoides. Para este experimento se utilizaron espermatozoides epididimarios (alto grado de morfoanomalías espermáticas y sin presencia de plasma seminal) y eyaculados (bajo grado de morfoanomalías y presencia de plasma seminal), que fueron incubados hasta 24 h en presencia o ausencia de fluido uterino (recolectado de hembras en fase folicular tardía).

Los resultados del *experimento 1* mostraron un mayor % de espermatozoides con morfoanomalías en el reflujo que en la dosis seminal y prácticamente la totalidad de la población espermática que colonizaba la unión útero-tubárica presentaban una morfología normal. Por otro lado, en el *experimento 2*, se observó que el fluido uterino no tenía ninguna influencia en cambios morfológicos de los espermatozoides eyaculados, sin embargo, cuando el fluido uterino se incubó con espermatozoides epididimarios se produjo una drástica reducción de las morfoanomalías, principalmente de gotas citoplasmáticas distales.

En conclusión, la presente tesis doctoral muestra por un lado, que la inseminación artificial post-cervical es una técnica viable para su uso en las granjas. De hecho las ventajas obtenidas tanto a nivel reproductivo como económico son claras, siendo su implementación y aplicación a nivel de campo una realidad. Por otro lado, se ha comprobado que los eyaculados son poblaciones heterogéneas con diversas características de motilidad, morfología y morfometría, y que estas particularidades propias de cada espermatozoide, influyen en su interacción con el tracto genital de la hembra una vez que son depositados. La mayor parte de los espermatozoides son eliminados en su trayecto hacia el lugar de fecundación, y esa discriminación parece ser un proceso selectivo o debido a modificaciones de algunas de las características propias del espermatozoide que sufren en su interacción con el útero.

2. Introduction

2.1. BOAR SPERMATOZOA: PRESENT AND PROSPECTIVE REMARKS

Fertility failure can be caused by the male or the female, if the male is at fault, then the quality of the spermatozoa immediately becomes suspect (Flowers, 2013). As a result, many investigations have been conducted to identify the characteristics of sperm related with optimal fertilization. Recognizing the properties of spermatozoa would provide valuable insight for the development of useful semen quality tests (Holt and Van Lock, 2004). In this part of the introduction, first we follow the sperm journey from the testis to the moment of ejaculation before examining the value of sperm function tests used in field conditions and in specialized laboratories. Finally, we point new knowledge for developing functional sperm tests that could be used in the near future to predict how fertile an ejaculate is.

2.1.1. The spermatozoon: an overview

Mammalian spermatozoa are produced through spermatogenesis inside the male gonads (testicles) via meiotic division. They are very specialized cells designed to reach the oocyte and transmit the paternal genome to the next generation.

During passage of mammalian spermatozoa through the epididymal duct, the functionally incompetent germ cell produced by the testis is matured and stored. In this time (around 1–2 weeks in most species), the spermatozoon undergoes many changes that prepare it for the diverse tasks required of it (Cooper, 2011). Sperm maturation takes place in the caput and corpus, while the cauda stores the mature spermatozoa until ejaculation occurs (Bonet et al., 2012). During the maturation process, the sperm suffer different modifications such as cytoplamic droplet displacement (Figure 1). During normal spermatogenesis, most of the round spermatid´s cytoplasm is phagocytized by Sertoli cells and only a small cytoplasmic residue ("the cytoplasmic droplet") remains attached to the elongated spermatid after release from the germinal epithelium. As mentioned, one characteristic morphological change to spermatozoa during epididymal transit is the caudal migration of the cytoplasmic droplets (Cooper, 2011), which are displaced from the connecting piece (proximal droplet) to the Jensen´s ring (distal droplet) of the axoneme and its detachment (Bedford, 1975) (Figure 1). The fact that very few ejaculated spermatozoa have droplets in rams, boars, bulls and goats suggests that they are removed around the time of ejaculation (reviewed by Cooper 2011). The presence of a cytoplasmic droplet in ejaculated sperm can be used as an indicator of immature sperm (Gómez

et al., 1996; Keating et al., 1997; Amann et al., 2000; Thundatil et al., 2001) and is also associated with infertility in boars (Waberski et al., 1994; Kuster et al., 2004).

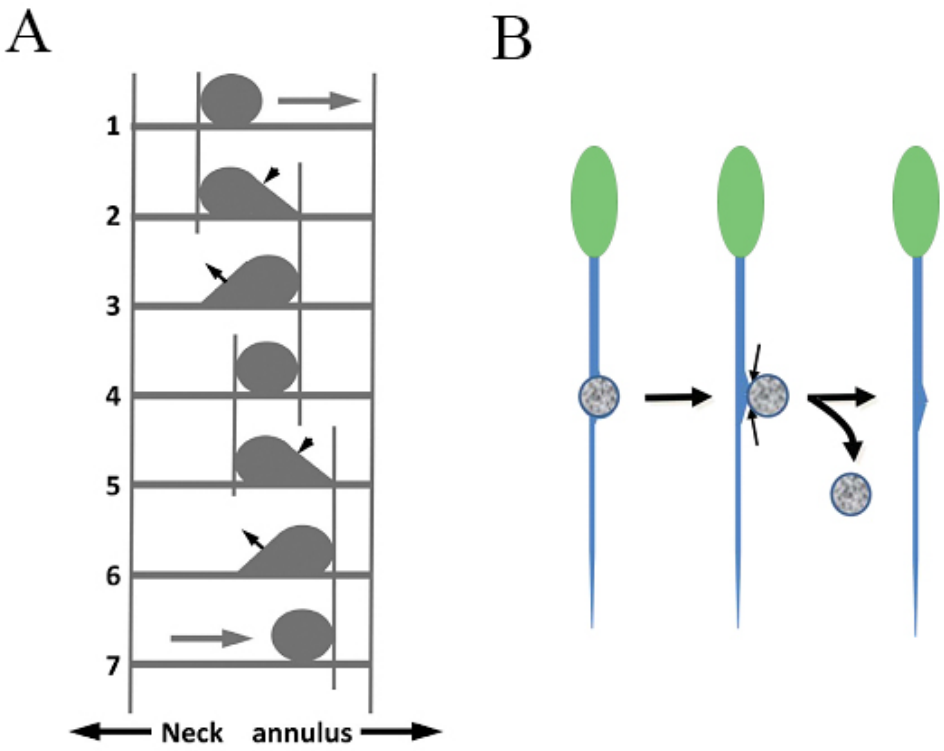

Figure 1. Sperm cytoplasmic droplet displacement. (A) Schematic representation of the mode of migration of a cytoplasmic droplet along the midpiece within the epididymal lumen. (B) Schematic diagram of the mechanisms of sperm cytoplasmic droplet loss. Images modified from Cooper (2011).

Other known modifications that take place in the spermatozoa during epididymal maturation, besides cytoplasmic droplet displacement, are the stabilization of nuclear chromatin, changes in distribution of membrane glycoproteins, redistribution of proacrosin/acrosin from the apical sperm cap to the acrosomic vesicles or changes in the sperm movement pattern (reviewed by Bonet et al. 2012).

Once matured the spermatozoa are stored in the cauda epididymis and wait to be expelled at the time of ejaculation. Ejaculation in boar takes place over 20–30 min, and spermatozoa from the distal cauda epididymis and vas deferens are mixed with fluids of the male accessory sex glands (seminal vesicles, prostate and bulbourethral glands or Cowper´s glands). This non-cellular fraction of the ejaculate is called seminal plasma and is composed of organic and inorganic substances, including proteins or energy substrates which play an

important role in their interaction with the ejaculated sperm and are involved during capacitation events. After that, the spermatozoa come into contact with different fluids in the female tract such as cervical mucus, uterine and oviductal fluid.

The overall structure of a mature sperm cell is usually divided into (i) the head region with the acrosome cap, the nucleus, and the nuclear envelope; (ii) the middle piece containing proximal centrioles, a segmented column, a large number of mitochondria, dense fibers and the annulus; and (iii) the long tail region with the flagellum and a fibrous sheath that is divided into the principal piece and the end piece domain (Figure 2) (De Jonge et al., 2006; Holland and Ohlendieck, 2014).

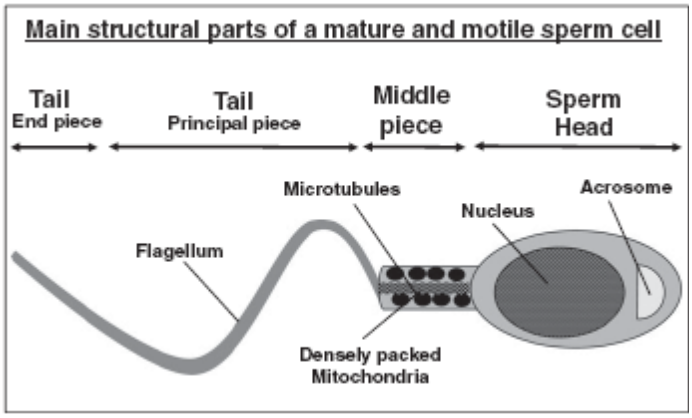

Figure 2. Structure of mature sperm cells (modified from Holland and Ohlendieck, 2014).

2.1.2. Sperm subpopulations

Given that the spermatozoa are highly differentiated cells, it can be assumed that a spermatozoon possesses a high level of perfection. However, spermatozoa within any ejaculate exhibit considerable heterogeneity in many different respects (Holt and Van Lock, 2004). Holt (1996) described for the first time the presence of a specific subpopulation structure in boar ejaculates when sperm motility was analyzed, a finding that was extrapolated to other mammalian species such as gazelle, horse, dog, rabbit, deer, bovine, and ovine (reviewed by Rodríguez-Gil,, 2013). In addition to motility, other sperm subpopulations related to morphology, morphometry or mitochondrial activity have been described (Rubio-Guillén et al., 2007; Ramió-Lluch et al., 2011). These findings support the initial hypothesis of Holt (1996) about the presence of different sperm subpopulations within mammalian ejaculates. However, the biological significance of spermatozoon heterogeneity within an ejaculate is still unknown.

Satake et al. (2006) described how individual spermatozoa respond to bicarbonate stimulation related to the ability for capacitation, which is an essential step involved in fertilization. A further interpretation of ejaculate heterogeneity and complexity is that males may have evolved the rich diversity of sperm-based differences as a response to female tendencies to prevent all but the fittest genotypes from ever reaching the eggs (Holt and Fazeli, 2010). However, boar sperm quality analysis would have to be modified in order to introduce the subpopulation concept to obtain adequate information about the quality of the analyzed ejaculate (Rodríguez-Gil, 2013).

2.1.3. Sperm assessment

The selection of boars with high fertility has an enormous economic impact on farms. Sperm are usually examined after acquisition of the ejaculate and before insemination, in an attempt to predict the fertility of the male. Predicting the fertility of spermatozoa is one of the most relevant aspects in the field of porcine reproduction (Gadea, 2005). Nowadays, there are several methods available to assess a sperm sample although no one method *per se* is indicative of success, while a combination of different traits of the spermatozoa could give us an idea of the potential of the sample. Below, we explain the main techniques used for male gamete analysis and newly developed methods that could well form part of routine sperm evaluation in the near future.

2.1.3.1. Sperm analysis in field conditions

In most porcine AI centers, once the ejaculated is obtained it is subjected to a classical semen analysis (spermiogram) before insemination. A spermiogram is based on several simple analyses that can be carried out quickly and at a low cost. The main tests involved in the analysis of semen are of volume, ejaculate concentration, motility and progressive motility, and morphology.

- ***Volume***: Measured in a graduated beaker (commonly expressed in ml).
- ***Concentration***: Sperm concentration is commonly controlled in a haemocytometer counting chamber (Bürker, Neubauer or Thoma) although automatic counters based on photometric analysis are increasingly used (expressed as spermatozoa/ml).
- ***Motility***: Percent motility (%) and progression (scale from 0 to 5; where 5 represents fast, linear and progressive sperm) are determined by placing a small drop of the sample (~10 µl) on warm glass slides (38ºC), which are then examined by light microscopy

(100X magnification). It is recommendable that the same person test the samples to avoid any difference in the evaluation. Another important point to take into account is that slight variations in temperature during analysis could influence the final result.

- ***Morphology****:* Sperm morphology is determined by mixing semen samples in a 2-4% buffered glutaraldehyde solution and placing 10 µl of the fixed solution on a glass slide covered with a coverslip (24x24 mm) or directly in a Bürker counting chamber. The microscopic examination can be performed at 200X, 400X and 1000X magnification. Spermatozoa are classified into one of the following categories: normal morphology (normal), cells with attached proximal cytoplasmic droplet, cells with distal droplet, tail defects (folded and coiled tails) and others (double tail or head, isolated head or tail, tail abaxial implantation, micro- or macro-cephalic heads). A high percentage of abnormal spermatozoa with cytoplasmic droplets in the ejaculate indicates defective sperm maturation (Briz et al. 1995). In normal conditions, 80-85% of normal sperm is considered as a standard value in boar (revised by Bonet et al., 2012).

Table 1 represents the minimal values to consider an ejaculate as optimal for insemination.

Table 1. Optimal boar semen parameters.

	Range
Volume (ml)	100-300
Total number of sperm ($x10^9$)	10-100
Motility (%)	70-90
Morphologically normal sperm (%)	80-85

The ***sperm osmotic resistance*** is another test than can be carried out in AI centers where seminal doses are produced. This test consists of submitting the male gametes to osmotic shocks and then evaluating their resistance to the disruption of sperm membrane and related acrosome integrity (Bonet et al. 2012) in order to detect whether an intact membrane is biochemically active, which is essential for sperm capacitation. This test is commonly known as the hypoosmotic swelling test (HOS). It is a simple test and easy to perform, inexpensive and repeatable (Gadea, 2005); however it is not used as a routine test in porcine.

Sperm ***viability*** can be assessed in a simple manner. The frequently methods used are specific stains for optic microcopy such as eosin-nigrosin (Dott and Foster, 1972). The basis of

these methods is that the plasma membranes of viable spermatozoa are impermeable to dyes, whereas in non-viable spermatozoa the membrane remains permeable (Bonet et al., 2012).

The ***filtration*** of sperm could be an alternative for improving the quality of an ejaculate. The objective of this technique is to pass the semen samples through chromatographic resins that increase semen quality (Ramió-Lluch et al., 2009). It is based on filtration by gravity and on the fact that dead and abnormal sperm are retained in the resin whereas the normal and live sperm pass through it (Graham and Graham, 1990). The resin most commonly used for the filtration of sperm is *Sephadex* beads. Several studies have been performed in boar sperm, in which the authors have observed that filtration increases the percentage of viable and normal sperm cells (Busalleu et al., 2008). Similar results have also been observed in other species such as bull and dog (Anzar and Graham, 1993; Mogas et al., 1998). The filtered sperm are resuspended in a semen extender and are kept at the storage temperatures of cooled semen (16°C) for use in a maximum of 24 h post-filtration (Ramió-Lluch et al., 2009).This technique is particularly interesting in field conditions when the good genetics of an animal that exhibits poor sperm quality needs to be maintained. Furthermore, the use of this technique requires a minimal and affordable infrastructure.

Another issue that needs to be taken into account is ***contamination*** of the ejaculate. Different pathogens can be transmitted via semen. Some types of bacteria such as *Brucella Suis* or *Leptospira* are especially dangerous and have to be under strict control on farms. The amount of bacterial contamination during semen collection/processing and storage (i.e., incubation) time contributes to the spermicidal effects (Althouse et al., 2000), so that fertility is compromised in addition to the transmission risks to the females through insemination. The evaluation is carried out under the microscope, for example at the same time that motility is evaluated. Moreover, bacterial contamination can induce sperm ***agglutination*** (Yeste et al., 2008; Bussalleu et al., 2011), a condition observed when a spermatozoon binds to another spermatozoon by head-to-head, head-to-tail or tail-to-tail contact (Bonet et al., 2012).

Not only the bacteria but also the viruses have to be checked to avoid the sexual transmission of these pathogens. Viruses such as PRRS, PCV2 and Parvovirus can be found in semen and, as a consequence, be transmitted to the sows by AI. Nowadays, PCV2 and Parvovirus are controlled by vaccines. However, semen should be checked by PCR to avoid the presence of PRRS, especially if it is obtained from an external source. Ideally, insemination doses have to be free of PRRS virus, avoiding the introduction of a new strain in the sow herd or preventing dissemination of the resident virus when the semen is produced in the same farm.

2.1.3.2. Sperm analysis in specialized laboratories

The tests mentioned above are simple tests and usually are insufficient to measure in a precise way the quality of an ejaculate. In an attempt to improve these analyze different techniques and methods can be applied in specialized laboratories, which can help with fertility prediction. Among others can be mentioned fluorescent dyes to test acrosome integrity and reaction, viability or mithocondrial activity using flow cytometry or fluorescent microscope, computer-assisted semen analysis (CASA) to test motion parameters and morphometry, and *in vitro* fertilization tests. The problem is that most of them are expensive and time-consuming, and cannot be used under farm conditions (Gadea et al., 2004).

In the 1940s, scientists started to look for an objective way to analyze the motility of spermatozoa in an ejaculate because, until that moment and still in most analyses at present, this quality is subjectively analyzed and depends, to a large degree, on the particular laboratory and the experience of the technician who tests the sample. This is basically why such techniques as ***CASA*** (Dott and Foster, 1979) provide the opportunity to carry out an objective examination of each sample. CASA is a computerized system connected to a digital camera, which visualizes and digitizes the image of sperm cells and analyzes the sperm concentration, % of motile spermatozoa and sperm velocity parameters (Figure 3 and Table 2), with a high degree of repeatability (Feitsma et al., 2011). The main advantage of CASA is its objectivity, but this is only reached when it is operated properly and by trained laboratory technicians and if the fields to be observed are chosen at random (Feitsma et al., 2011). Significant correlations between the basic parameters of CASA and fertility have been described for several species, including pigs (Holt et al., 1997; Vyt et al., 2008). CASA, then, has the potential to become a useful tool for optimizing semen dose production.

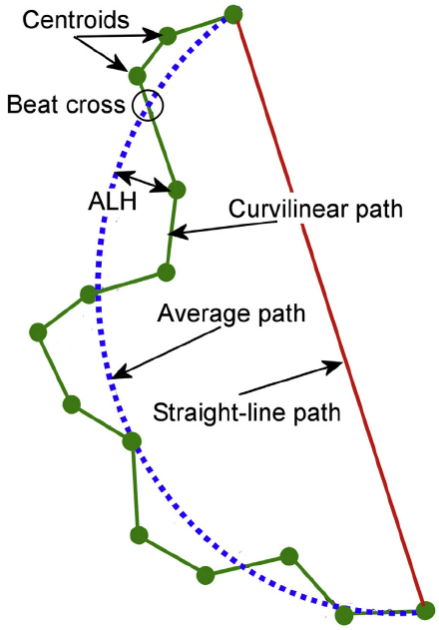

Figure 3. Illustration showing CASA terminology related with motion parameters (Amann and Waberski, 2014).

Table 2. Sperm motion parameters provided by CASA system (modified from Bonet et al. 2012).

Abbreviation	Parameter	Units	Description
Mot	Motility	%	Percent of sperm with movement
PMot	Progressive motility	%	Percent of sperm with progressive or linear movement
VSL	Straight line velocity	µm/s	Average velocity measured in a straight line from the beginning to the end of a track. VSL measures the speed of a spermatozoon in a forwards progression
VAP	Average path velocity	µm/s	Average velocity of the smoothed path of the sperm head
VCL	Curvilinear velocity	µm/s	Average velocity measured over the actual point-to-point track followed by the sperm head
ALH	Amplitude of lateral head displacement	µm	Amplitude of lateral turn regarding intermediate piece
BCF	Beat cross-frequency	Hz	The number of points where the curvilinear path intersects the average path
LIN	Percentage of linearity	%	VSL/VCL
STR	Percentage of straightness	%	VSL/VAP
WOB	Motility parameter “wobble”	%	VAP/VCL

In addition, one of the CASA system modules provides the possibility to measure cellular morphometric characteristics (Figure 4): ***Automated Sperm Morphometric Analysis*** (***ASMA***). This system affords very detailed information on sperm head dimensions and tail length. However, ASMA appeared to be a more complex and time-consuming process, partly because it requires an additional step (i.e. staining of the semen sample before analysis) and for the evaluation at a higher magnification level which reduces the number of evaluated spermatozoa per microscopic field (Rijsselaere et al., 2004, 2012).

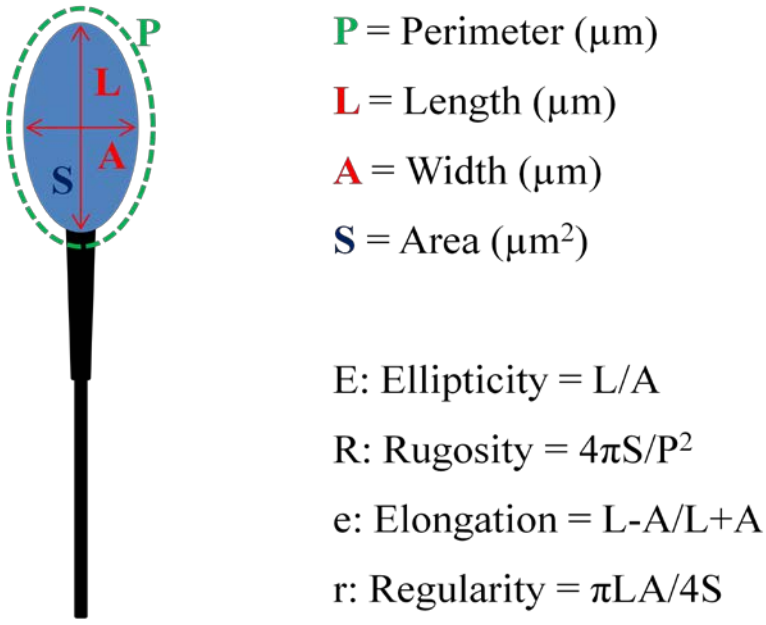

Figure 4. Illustration showing CASA terminology (ASMA module) related with morphometric parameters.

Another objective semen analysis technique is ***flow cytometry***. The principle of this technique is that a sperm cell suspension is introduced in fluid stream through a laser excitation source (Broekhuijse et al., 2012), whereby spermatozoa are fluorescently labeled and analyzed (thousands per second), allowing the assessment of different semen quality characteristics related to male fertility (reviewed by Gadea, 2005; Broekhuijse et al., 2012). Among these characteristics are sperm membrane integrity, acrosome intactness, acrosome responsiveness, chromatin structure, DNA damage and the potential of the inner mitochondrial membrane. Most fluorochrome dyes can also be used in a ***fluorescent microscope*** instead of a flow cytometer.

Among all the sperm tests that have been developed, ***in vitro fertilization*** (IVF) tests might be the most suitable for assessing overall sperm function during fertilization (Gadea, 2005). When IVF is performed, parameters such as the number of sperm attached to the *zona pellucidae*, number of sperm penetrating the oocyte, penetration rate, pronuclear formation or embryo development can be evaluated. However, no clear relation has been established between the functional sperm parameters and the IVF tests (Hammitt et al., 1989; Gadea, 2005).

The ***in vitro sperm-mucus penetration test*** is a sperm function test which measures the ability of sperm in the semen to swim up into a column of cervical mucus or substitute (e.g. methyl cellulose). If it can be proven to be as good as semen analysis in assessing progressive sperm motility, then arguably, its additional benefit as a test of functional competence may make it a suitable and cheaper alternative to the present combination of semen analysis and sperm separation procedures (Ola et al., 2003). The most commonly used parameter evaluated in this test is the vanguard distance of 30 mm measured after incubation at 37°C for 90 min (Galli et al. 1991; Rickard et al. 2014); although other studies have used swim-up sperm count per high power field at 10, 20 and 30 mm in a flat capillary tube as the diagnostic criteria for this test (Aitken et al., 1992; Ivic et al., 2002).

2.1.3.3. Future remarks in sperm analysis

Many research groups are working on new techniques to identify the characteristics of spermatozoa which make them capable of fertilization. The possibility of applying new tools in the laboratory to evaluate the fertility capacity of ejaculates is a very important issue in the biology of reproduction. Some of these investigations are described below:

Holographic imaging of spermatozoa*:* As mentioned above, motility and morphology evaluations are performed in separate analyses using two dimension images provided by the microscope. The possibility to add a third dimension in sperm analysis could provide a better comprehension of spermatozoa behavior and its relation with the fertility capacity of the spermatozoa (Guerrero et al., 2011). The first holographic image of a spermatozoon was published by Mico et al. (2008) and since then new approaches in this area have been developed (Su et al., 2012; Di Caprio et al., 2014). This type of image could offer us an integral analysis of motility, morphology, morphometry and volume of the sperm at the same time. Although this field is still under development, simple and low cost methods have been developed to transform conventional microscopes in apparatus providing holographic images (Lee and Park, 2014; Mico et al., 2014). So, in the near future andrology laboratories might integrate conventional techniques with the information obtained from this type of microscopes.

Spermatozoa molecular markers: Advances in molecular biology techniques will allow us to develop simpler sperm function assays in the near future (Oehninger et al., 2014). Different molecular markers of the maturating process, such as proteins, enzymes or glycoproteins, have been found in the cytosol or sperm membrane using Western blot and immunocytochemical techniques (Bonet et al., 2012). The distribution pattern of carbohydrate residues of sperm membrane (e.g. galactose, glucose, mannose or fucose) (Fabrega et al., 2012), fertilin (Fabrega et al., 2011), acrosin (Puigmulé et al., 2011) or some heat-shock proteins (Casas et al., 2010) has been reported as a putative indicator of sperm maturation.

Proteomic analysis in spermatozoa: The proteomic analysis of gametes is not only crucial for establishing elementary aspects of the structure, function, and maturation of oocytes and spermatozoa, but also for the illumination of pathophysiological mechanisms of female or male infertility (reviewed by Holland and Ohlendieck, 2014). The development of new knowledge in proteomic technology could help us to predict the fertility of boar ejaculates with greater accuracy. Recent proteomic applications using 2-D gel electrophoresis (2-DE) and mass spectrometry (MS) have been employed to identify the proteins present in spermatozoa in several species (Park et al., 2013; Swegen et al., 2014; Zhou et al., 2014; Kasvandiket al., 2015). Studies comparing protein profiles between fertile and subfertile spermatozoa have been performed to investigate male fertility. Park et al. (2012) identified eight proteins that presented at least a three-fold difference in expression between normal and subfertile bull spermatozoa. Among these proteins, five were more highly expressed in normal spermatozoa, while the other three were more highly represented in the spermatozoa of subfertile bulls. New biomarker candidates based on proteomic studies might be useful to improve diagnostic, prognostic, and therapeutic aspects of infertility.

2.2. AN OVERVIEW OF SWINE ARTIFICIAL INSEMINATION: THE SPERMATOZOA JOURNEY WITHIN THE UTERUS*

*This part of the introduction has been modified from the manuscript: *Soriano-Úbeda C, Matás C, García-Vázquez FA. An overview of swine artificial insemination: retrospective, current and prospective aspects. Journal of Experimental and Applied Animal Science 2013;1:67-98.* The authors authorize its use in the present thesis.

In the last two decades assistant reproductive technology (ART) has grown exponentially due to the development of new biotechnologies both in humans and animals. Artificial insemination (AI) is included among ART methods. This technique, although not as new as others, is still considered to be one of the most revolutionary techniques applied in farm animals. It can be defined as a method of assisted reproduction that involves the deposition of sperm unnaturally in the female tract for the purpose of fertilization. Although the use of AI in most countries with intensive pig production has increased greatly in the past two decades, AI in swine cannot be considered a new technique. The first AI attempts were recorded in the 14th century and its introduction in porcine dates from the beginning of 20th century (see section *History of AI*). Nowadays, more than 90% of pigs are artificially inseminated in the European Union and North America, reaching 98% in some countries (Feitsma, 2009).

This method presents great advantages over natural mating. In this respect, the following advantages can be emphasized: genetic gains with the use of genetically superior males and purchased semen allows genetic diversity, which can be used to optimize crossbreeding systems on smaller farms and increased genetic progress. Additionally, the number of boars can be reduced on the farm since good males can be used more extensively than those used for natural service, because AI increases the number of inseminations per ejaculate. Furthermore, this technique presents less risk of disease transmission than natural service mating systems. However, AI requires a high level of management on the part of the farmer. The technician making the AI should provide special attention to the handling of semen as regards environmental changes during transport, including temperature and the risk of dilution that will affect viability. Also AI should be carried out at the right time and the farmer must make an accurate determination of the onset of estrus. This fact is essential for obtaining a high rate of conception and litter size (Maes et al., 2011).

The main goal of the sperm when they are deposited in the female tract is to reach the oocyte and fertilize it. Millions of sperm are placed in the female, but only some 'privileged' spermatozoa arrive at the fertilization site. During this long journey through the uterus in search of the oocyte, the sperm are subjected to different environments and obstacles so that only the most 'capable' spermatozoa are chosen; however, the exact mechanism by which spermatozoa are selected in the uterus is still not well understood. This information leads one to think of genetic material wasted on the journey and the inefficiency of the traditional AI technique. That is why in the last decade new insemination devices have been developed with the goal of reducing the number of sperm deposited placing them deeper in the female tract and, as a consequence, closer to site of fertilization. Besides adopting new devices, the porcine industry is trying to maximize sperm use by the application of new methodologies, such as improving the composition of liquid storage, releasing the sperm progressively in the uterus (encapsulated sperm) or including new quality sperm assays which could permit optimization of the ejaculate.

This part of the thesis introduction summarizes several factors concerning AI, starting with an overview of some physiological aspects including the female reproductive tract and sperm transport, as well as sperm losses during insemination and uterus sperm selection. Strategies developed to reduce the number of sperm during the AI process, are also reviewed.

2.2.1. History of AI

Although most people assume AI to be a recent development, it was first used in the 14th century. The legend says that the first AI successfully performed was in the equine species, when an Arab chieftain stole ejaculated semen from the vagina of a recently mated mare belonging to a rival. The semen, theoretically of better quality, was diluted in camel milk and inseminated in the new mares (reviewed by Allen, 2005). Later, Leeuwenhoek in 1678 using his own created microscope was the first person to observe a sperm, something that he called "animalcules" or "spermatick worms" (Clarke, 2006). The following century, an Italian priest, Spallanzani (1784), examined semen from mammals, fish and amphibians and managed to perform the first successful documented insemination in a dog, obtaining three pups 62 days later. Moreover, experimenting with frogs he demonstrated that previous contact between oocytes and spermatozoa is essential to obtain a tadpole. Perhaps, this was the first experiment of *in vitro* fertilization in the world. He also observed for the first time that the spermatozoa could be inactivated by cooling and reactivated later. Apparently, as early as 1776 he put a sample of collected human semen in the snow and discovered that the spermatozoa were still

motile when they were returned to body temperature. The first known human insemination was made in 1790 by a Scottish surgeon, John Hunter, who collected semen from a merchant with hypospadias in a hot syringe. Following the instructions from the doctor he successfully injected it into his wife's vagina, who became pregnant. The first published reference to donor insemination was made by Paolo Mantegazza in 1887 (Alfredsson et al., 1983), a pathology professor who established the first semen bank for veterinary and possibly, for human use (Traina, 1980). Heape (1897) and others in several countries reported successful AI based on studies with rabbits, dogs, and horses. AI was first established as a practical procedure in 1899 by Ivanov in Russia. By 1907, Ivanov had already studied AI in domestic farm animals, dogs, foxes, rabbits and poultry. He was the first to develop semen extenders and trained technicians to select superior stallions and multiply their progeny through AI (reviewed by Foote, 2002). Some of his investigations, especially in horses, were included in a paper published in 1922 in the Journal of Agricultural Science (Ivanov, 1922). Later, Milovanov (1964) established projects for sheep and cattle breeding. The investigations carried out in Russia on AI encouraged other countries to take this technology to the rest of Europe. AI in Asia started in Japan with Nishikawa in 1912 (Nishikawa 1962, 1964) and in the United States in the 1930s. At that moment the procedures developed in assisted reproduction in animals became a worldwide practice (Salisbury et al., 1978) (Figure 5).

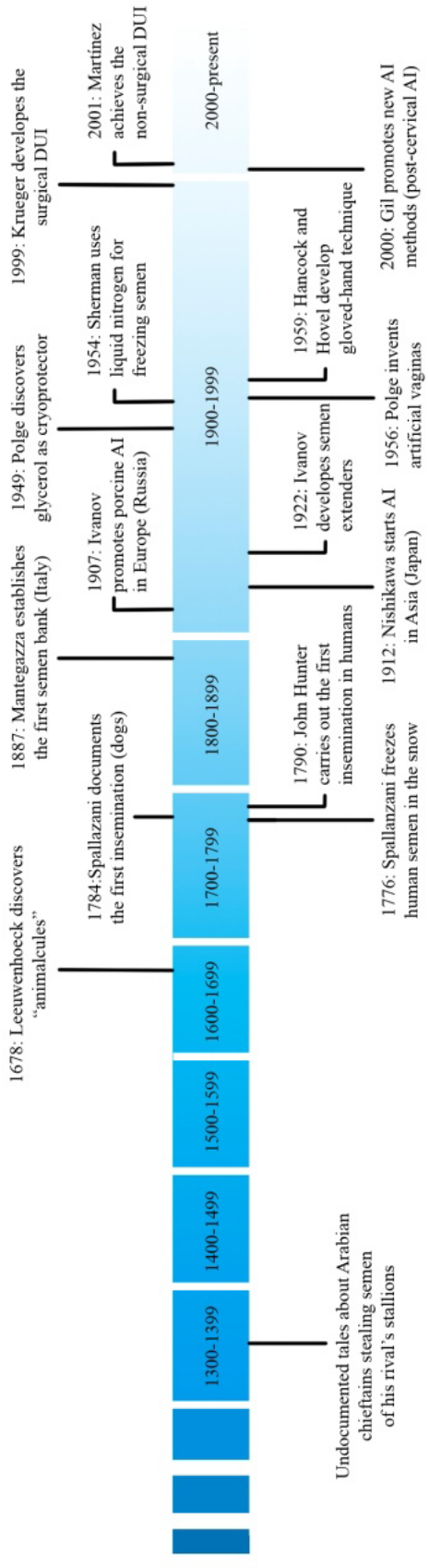

Figure 5. Relevant historical events in the development of AI technology.

Porcine AI also started in Russia with Ivanov in the early 1900s (Ivanov 1907, 1922). The technique quickly spread to the United States (McKenzie, 1931), Japan (Niwa, 1958) and Western Europe (Polge, 1956). In the mid 20th century extensive AI technology in swine lead to standardization of the protocols used by farmers and technicians to carry out the process. The boar were trained on mounting dummies (Polge, 1956) impregnated with sow odor enabling the semen to be extracted from the boar without requiring the presence of a sow. In addition, artificial vaginas helped improve the work of collecting semen and safeguard sample hygiene and quality (reviewed by Althouse and Lu, 2005). The first artificial vaginas were very similar to those currently used, providing a means of applying pressure to the glans (McKenzie, 1931; Polge, 1956). The gloved hand technique was developed later by Hancock and Hovel (1959) (See Figure 5).

Another advance in porcine AI was the use and development of semen extenders and frozen semen. The first diluters as a method to store semen were developed in Russia (Ivanov, 1922; Milovanov, 1938) and in the United States (Philips and Lardy, 1940) with the main objective being to use less sperm cell per insemination. They were based on glucose solutions with sodium potassium tartrate or sodium sulfate and peptone, keeping the concentration of electrolytes low and enabling storage of semen during long enough for shipment and later use in the field. At that moment, the recommended storage temperature was 7º to 12º C; however, Ito et al. (1948) recommended storage at 15º to 20º C, as is used at present. The most widely used semen dilution medium is Beltsville Thawing Solution (BTS), which was developed by the laboratories of The United States Department of Agriculture (USA) by Pursel and Johnson in 1975. BTS increases the storage period of fresh semen up to 48 h while maintaining the same level of fertility of sperm. This and rapid transportation of the dose represent a very important commercial advantage for producers of pig semen.

Between the 1970's and 90's the results of AI in pig production improved very strongly. The greater knowledge of the reproductive physiology of both sow and boar, knowledge of the estrous cycle of the sow and the optimal time of insemination, the training of technicians responsible for inseminating and the correct use of diluted semen have led to similar results to those obtained with natural reproduction. The swine industry has endeavored in recent years to find ways to optimize AI, making more efficient use of semen and using males of high genetic value. The development of new insemination methods has the goal of reducing the number of spermatozoa needed, and some of these techniques are currently being applied under farm conditions.

2.2.2. An overview of the porcine female reproductive tract

The swine female reproductive tract (Figure 6) is a long organ compared with other species, including human, cows or even mares. From cranial to caudal, it is composed of a pair of ovaries to generate oocytes and hormones such as progesterone and estrogen. Each ovary is surrounded by a thin membrane called the infundibulum belongs the oviduct, which acts as a funnel to collect oocytes and redirect them to the female duct. The oviduct is about 15-25 cm long and acts as the fertilization site, being divided into four functional segments: the infundibulum (as we already mentioned), the ampulla, the isthmus and utero-tubal junction (Hunter et al., 1998) (Figure 6). The utero-tubal junction is the connection between the oviduct and uterine horns. Uterine horns have a length of 50-100 cm in non-pregnant sow. They act as a duct for sperm to reach the oviduct and are the site of fetal development. The uterine body, which is small compared with some other species, is located at the junction of the two uterine horns. The cervix is a muscular junction between the vagina and uterus and this has two regions: a uterine region characterized by the presence of glandular acini, and a vaginal region with a large vascular network. Both regions showed a mixed secretory activity by epithelial cells, which produce sulfated mucins (mucous secretion), intermingled with abundant glycogen-rich cells (serous secretion). (Rodríguez-Antolín et al., 2012). This is the site of semen deposition during natural mating and traditional AI (Figure 6). It is dilated during heat (oestrus) but constricted during the remainder of the estrous cycle and during pregnancy. The vagina extends from the cervix to the vulva and serves as a passageway for urine and the piglets at birth.

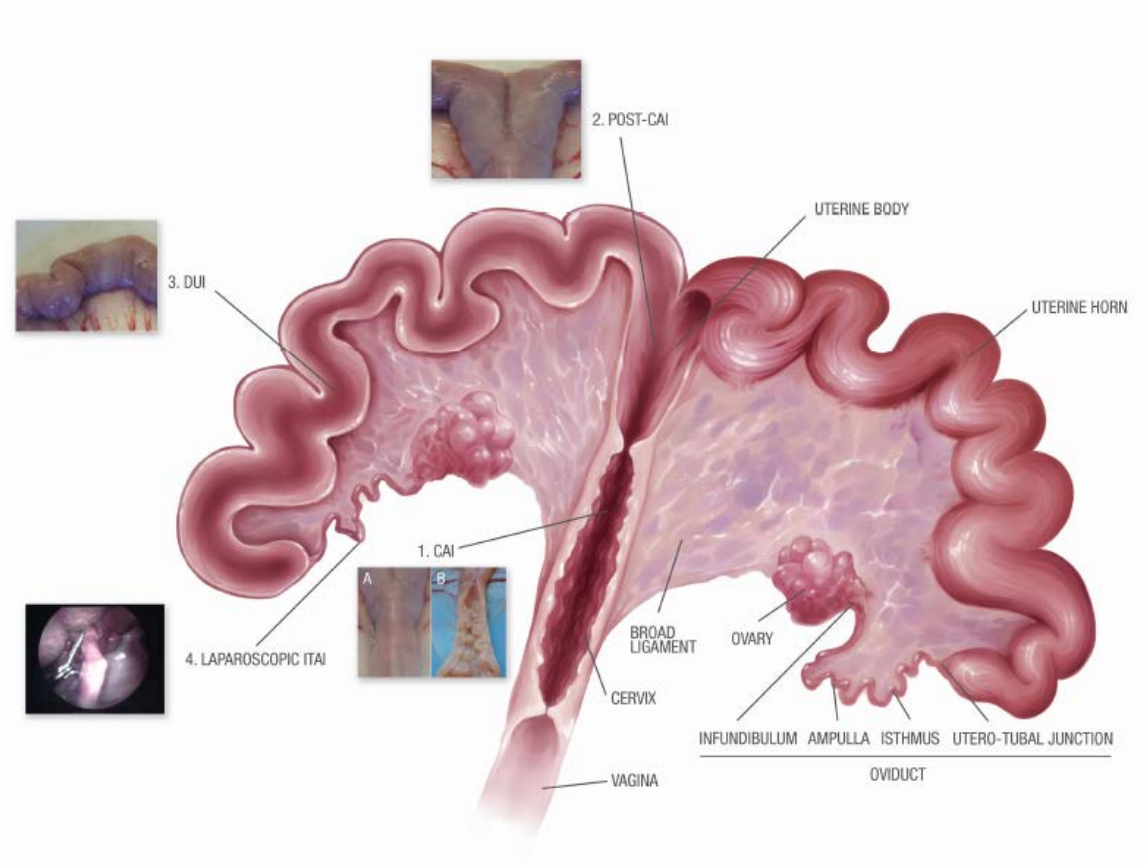

Figure 6. Anatomy of the sow´s uterus and sperm deposition sites during AI. The insemination sites are shown in several real images: 1) Cervical AI (CAI) (1A and 1B: external and internal view of the cervix, respectively); 2) Post-cervical AI (Post-CAI) (external view uterine body); 3) Deep intrauterine insemination (DUI) (external view of the uterine horn); 4) Intraoviductal AI (ITAI) (view of the laparoscopic approach of the oviduct).

2.2.3. Sperm transport through the female reproductive tract

The process of sperm transport from the cervix (site of insemination) to the ampullar-isthmic place (site of fertilization) is complex and involves dynamic interactions between spermatozoa and the female genital tract. This interaction ensures the arrival of fertilization-competent spermatozoa within the functional lifespan of the ovulated egg. Among the factors regulating the transport of sperm in the female tract are included mating behavior, the seminal plasma, the spermatozoa, the female reproductive tract (musculature, secretions, epithelial cell surfaces) the products of ovulation (oocyte, oocyte investing layers, follicular fluid) and immunocompetent elements of the female reproductive tract (revised by Drobnis and Overstreet, 1992).

Semen deposition: from cervix to utero-tubal junction*:* Billions of spermatozoa are deposited into the cervix (during mating or traditional AI), but only thousands are found in the oviduct. Approximately 1-3x10^5 spermatozoa reach the utero-tubal junction and about 1-3x10^3 reach the sperm reservoir in the caudal part of the isthmus (Mburu et al., 1996).

Sperm transport to the site of fertilization is thought to be a combination of both passive and active transport. Passive transport is more important in the initial phase of sperm transport, from the site of deposition to the proximal uterus and the utero-tubal junction (Scott, 2000). The passive part of sperm transport is probably due to the flow of fluid caused by gravity and by contractile movement of the uterine horns, and requires a minimal volume of inseminate during AI (Baker et al., 1968). Although contractions of the myometrium are vigorous during oestrus, and should assist transport and redistribution of the semen between the two uterine horns, an initial distribution of semen in the uterus may be achieved as a result of the force of ejaculation and the volume of fluid involved (Hunter, 1982). Thus, the high volume of semen deposited during natural mating (or in some cases during AI) may favor displacement of a portion of the ejaculate to the region of the utero-tubal junction, which is bathed in a sperm suspension by the completion of mating (Hunter, 1982). Besides, the biochemical constituents of seminal plasma, such as prostaglandins, can stimulate smooth muscle activity of the female reproductive tract and thereby assist the distribution of semen or spermatozoa within the tract (Robertson, 2007). The mechanical stimulus of mating may also enhance visceral contractions and sperm distribution, although the mere presence of a boar during insemination is enough to stimulate uterine activity through the release of oxytocin (Langendijk et al., 2005).

After mating, sperm are transported to the oviduct of pigs faster around the time of ovulation than after mating earlier in estrus (Hunter, 1991). The spermatozoa should arrive in the oviducts within minutes of mating or AI. This rate of transport is much faster than sperm swimming speeds (active transport); consequently, it is attributed to muscular contractility of the female tract and attendant changes in intraluminal pressures (see Hunter, 2012). However, these rapidly transported spermatozoa, will not contribute to the fertilizing population in the oviduct (Overstreet and Cooper, 1978). Later, motile sperm will gradually pass through the utero-tubal junction to establish a tubal population capable of fertilizing.

Active sperm transport, resulting from the intrinsic movement of sperm cells is probably important because it acts to keep sperm in suspension in fluids of the female tract, thereby reducing the scope for adhesion to the endometrium and for migration of sperm cells from the proximal uterus into the utero-tubal junction and the oviduct (Langendijk et al., 2005). In a

previous report (Gaddum-Rosse, 1981) it was shown that neither immotile spermatozoa nor a dye solution were observed to pass through the utero-tubal junction, and it was concluded that sperm motility is important, and probably essential for sperm entry into the oviducts. There is some evidence that sperm pass through the utero-tubal junction into the isthmus via self-propulsion (see Hunter, 2012).

***Sperm reaching the storage reservoir**:* The oviduct plays a significant role in the reproductive process of mammals providing a suitable environment. This site has a selective binding capacity, choosing the most competent sperm population for fertilization based on certain characteristics related to morphology, motility, membrane integrity, or cytosolic calcium levels and training status of tyrosine phosphorylation of proteins (revised by Holt and Fazelli, 2010). In the caudal part of the isthmus, spermatozoa bind to epithelial cells and can be stored with no a reduction in their fertilizing ability until just before ovulation. For this reason this part of oviduct is named sperm reservoir (SR) (Suarez et al., 1991).

***Factors involved in the formation of sperm reservoir**:* The binding of sperm to oviductal epithelial cells in order to create an SR involves carbohydrate interactions present in the oviductal epithelial cells and lectin-like proteins on the sperm head (Suarez, 2002). This ligand-receptor interaction is species-specific. For example, in pig the molecules involved in this process seem to be galactosyl and mannosyl residues (Ekhlasi-Hundrieser et al., 2005), while in hamsters, sperm binding to oviductal epithelium is mediated by sialic acid (DeMott et al., 1995) and by galactose in horses (Dobrinski et al., 1996). On the sperm side, spermadhesins AQN1 and AWN which bind to the sequences Galb1,3GalNAc and Galb1,4GlcNAc (Dostálová et al., 1995), have been shown to contain carbohydrate-binding affinities, enabling them to interact with the epithelial cells. Whatever the case the binding is a reversible process involving different sugars in all species studied (Dobrinski, 1996; Suarez, 1998). The relative contribution of other factors such as mucus, the chemical properties of oviductal fluid or temperature gradients may contribute in varying degrees to the formation of the reservoir.

Another important aspect to take into account is the oviductal fluid (OF). OF has an ionic concentration, pH, osmolarity or macromolecular content that vary according to the time of the estrous cycle and oviductal region. In the middle of the cycle the difference between the pH of the ampulla and isthmus ranges between 0.3 and 0.7 units, increasing a further 0.4 units at the time of ovulation (Nichol, 1997). These variations may be of great significance, since an alkaline pH may influence sperm motility and training in the proximity of the female gamete.

OF also varies as regards the number of proteins (Killian, 2004) and content of sulfated (hyaluronic acid) and non-sulfated glycosaminoglycans (GAG) (Tienthai et al., 2000).

***Sperm release from the oviductal storage reservoir**:* The mechanisms that induce sperm release from the porcine reservoir are still poorly known but it has been showed that the pattern of sperm release from the SR and their progression along the isthmus during the period around ovulation is sequential and probably continuous, rather than occurring in a bulk (Mburu et al., 1996). Spermatozoa are gradually released from epithelial binding and, undergoing progressive hyperactivation, proceed along the isthmus to the site of fertilization.

A loss of binding sites on the oviductal epithelium and/or changes in sperm (capacitation and hyperactivation) could be responsible for the release of sperm from the reservoir. This sperm release is due not only to a reversible loss of epithelial binding proteins in sperm plasma membrane (AQN1) (Töpfer-Petersen et al., 2008) but also to the modification of glycan residues in the epithelium by oviductal glycosidases, such changes in the epithelium being the consequence of the switch from follicular oestradiol to progesterone secretion around ovulation (Hunter, 2012). The non-sulfated glycosaminoglycan hyaluronan, a major component of the porcine cumulus extracellular matrix, which increases around ovulation, has also been suggested to participate in sperm capacitation and release from the SR (Brüssow et al., 2008). Gradients in temperature could be another factor promoting release. During and after ovulation, an increase of temperature in the storage region would facilitate activation and the release of maturing spermatozoa (Hunter, 2009).

Besides, there is evidence that sperm changes associated with capacitation are responsible for releasing sperm. During capacitation, there are some modifications in the plasma membrane, including a combination of shedding extrinsic proteins. The modification or loss of these proteins could be involved in sperm release from the oviductal ephitelium. Remodeling of the sperm surface and of the molecular architecture within the sperm plasma membrane is viewed as one feature of the response to a peri-ovulatory influx of Ca^{2+} ions into bound spermatozoa (see Flesch and Gadella, 2000; Gadella and Harrison, 2000; Petrunkina et al., 2001) and this influx of Ca^{2+} initiates the sperm hyperactivation. In mouse, change in sperm beating increases flagellar bend amplitudes, usually on one side of the flagellum, which causes the flagellum to beat asymmetrically (Suarez and Ho, 2003). The power of the increased bend amplitude can provide the force necessary to overcome the attraction between sperm and epithelium. It has been showed that only hyperactivated sperm become detached from the epithelium (Suarez et al., 1992; DeMott and Suarez, 1992).

The durability of the sperm in the SR depends on the time of the estrous cycle and varies between 36 and 48 h (Hunter, 1984). Disorder in sperm transport might result in a lack of spermatozoa at the fertilization site or in large numbers of spermatozoa, which might give rise to a polyspermic situation (Hunter and L'Eglise, 1971).

***Sperm looking for the oocyte**:* Once the sperm are released from the reservoir they are in search of their objective, the oocyte. Apparently, sperm are equipped with a mechanism for turning towards the oocyte in response to thermotactic and chemotactic factors. Because hyperactivation occurs in the caudal isthmus, which lies a considerable distance from the site of fertilization, sperm may already be hyperactivated when they come under the influence of taxis signals. A temperature difference of up to 2° C between the cooler tubal isthmus and the warmer tubal ampulla has been detected in rabbits and there are indications that capacitated rabbit sperm tend to swim towards warmer temperatures (Bahat et al., 2003). Once in the tubal ampulla, and close to the oocyte, chemotactic mechanisms may guide sperm closer to the oocyte. Among substances that have been identified as potential chemoattractants is progesterone, which is released during ovulation (present in follicular fluid) and is produced by the cumulus cells that surround the oocytes (Chang and Suarez, 2010; Uñates et al. 2014). It has been postulated that $[Ca^{2+}]_i$ increases during sperm chemotaxis (inducing turning swimming with asymmetric flagellar bending) (for review, see Yoshida and Yoshida, 2011). Other components in OF have been identified as chemoattractants, such as natriuretic peptide precursor, which modifies sperm pattern motility and enhances $[Ca^{2+}]_i$ levels, whose receptor has been recently demonstrated in mouse spermatozoa (Bian et al., 2012). Temperature also seems to play a role in the levels of $[Ca^{2+}]_i$. Temperature stimulation activates the release of the internal sperm Ca^{2+} store, affecting flagellar bending (Bahat and Eisenbach, 2010).

After fertilization, any sperm remaining in the female reproductive tract may be phagocytosed by isthmic epithelial cells or may be eliminated into the peritoneal cavity, where they are phagocytosed (see Suarez and Pacey, 2006).

In summary, after AI, sperm ascend the female genital tract and with the help of the contractions of the uterus (passive transport) and sperm motility itself (active transport) arrive at the site of fertilization. Of the total number of sperm that are deposited in the cervix, only a small proportion is able to reach the oviduct, bind to epithelial cells and form the SR. In this place, sperm remain until the time of ovulation, when they are released sequentially by different

factors, which involve oviductal epithelium, the intraluminal fluid and sperm activity (reviewed by Coy et al. 2012).

2.2.4. Sperm losses in the uterus during and after AI

As mentioned, only a few sperm of those deposited reach the oviduct. Most of the sperm are lost during insemination and on their way through the uterus. Two of the main mechanisms known to be involved in sperm losses are the influx of leukocytes into the lumen of the uterus and backflow.

The uterus acts as an immunological organ, changing according to the oestrus cyclic stage. These changes affect the leukocyte populations within the endometrium (Taylor et al., 2009). When the female is in the oestrus stage, a massive migration of leukocytes (mainly poly-morphonuclear neutrophils-PMNs) into the sub-epihelial stroma takes place (reviewed by Taylor et al., 2009). Contact of the semen constituents with the uterus and cervical tissues induces a series of immunological reactions and mechanisms (Schuberth et al., 2008). After natural mating or AI the PMN influx into the uterine lumen and activated PMNs bind to spermatozoa and phagocytose them. Given that in some aspects semen is a foreign material for the female organism, it seems logical to interpret many of the immune responses as actions to eliminate such material (Schuberth et al., 2008). Inflammation seems to be a normal process to remove spermatozoa and bacteria, producing an ideal environment for embryo implantation (Troedsson, 1997; Rozeboom et al., 1998).

The influx of leucocytes into the lumen is enhanced within a few hours after AI, and PMN are cleared from the uterine lumen within 24 to 36 h following AI (Rozeboom et al., 1999). As a consequence of the influx, phagocytosis by PMNs substantially decreases the number of sperm after insemination, although the mechanism/s and the stimulus involved are still unknown. Several factors, including sperm, seminal plasma (SP) or seminal extender, may be involved in the leukocyte influx.

Rozeboom et al. (1999) demonstrated that spermatozoa in the absence of SP induce a great influx of PMN into the uterus. These results agree with other reports showing spermatozoa to be chemotactic mediators of PMN migration via complement activation (Clark and Klebanoff, 1976; Troedsson et al., 1995). In contrast, SP has been shown to be an essential protector of spermatozoa in an inflamed uterine environment (Katila, 2012), reducing

chemotactic and phagocytotic activity of PMN (Rozeboom et al., 1999) and supporting *in vitro* data (Taylor et al., 2009; Li et al., 2012). Moreover, other authors (Rodríguez-Martínez et al., 2010) reported that the major SP glycoproteins (spermadhesins) induce migration of PMN into the uterine cavity of the sow, initiating the endometrial-related cascade of transient and long-lasting immunological events in oestrous sows. Therefore, semen extenders may substitute the role of SP as a vehicle and provider of nutrition (Katila, 2012). But artificial extender components cause a rise in leukocyte numbers *in vivo*, probably due to irritation of the uterine epithelium (Taylor et al., 2009).

Other factors that can influence PMN influx into the lumen are the ovulatory status, dose volume, number of sperm or extender composition. Taylor et al. (2009) observed differences in PMN migration into the uterus between pre-ovulatory and post-ovulatory inseminations. Furthermore, a reduction in the inseminate volume and the addition of caffeine and $CaCl_2$ to the inseminate dose increased the number of non-phagocytosed spermatozoa in the uterus of sows 4 h after insemination (Matthijs et al., 2003). In the same way a reduction in the number of inseminated sperm decreases the relative number of non-phagocytosed spermatozoa (Matthijs et al., 2003).

In species such horse, pig and cattle the onset of PMN chemotaxis by sperm is rapid and the duration of PMN infiltration relatively short. It has been hypothesized that PMN takes part in sperm cell selection, removing superfluous, non-motile or damaged spermatozoa (Tomlinson et al., 1992). Whether sperm cell phagocytosis is a selective or random process is still questionable (Schuberth et al., 2008). As already mentioned, this ensures effective removal of sperm and bacteria and the subsequent return of the endometrium to a normal state, ready to receive the embryo (reviewed by Katila, 2012).

As mentioned, the sperm in the genital tract are reduced to a low percentage of the inseminated number of spermatozoa within only 4 h of insemination (Matthijs et al., 2003). One of the main factors involved in spermatozoa loss, rather than PMN influx, is the backflow of semen. During natural mating, approximately one-third of the spermatozoa in the ejaculate is lost through backflow within 2 h after mating (Viring and Einarsson, 1981). An increase in uterine contractility could be one of the main factors that causes the backflow. Seminal plasma has been shown to stimulate uterine motility *in vitro* (Einarsson and Viring, 1973). The most likely reason for this is the estrogen content of seminal plasma (Langendijk et al., 2005). After insemination, the estrogens in the ejaculate cause an immediate release of prostaglandin by the endometrium (Claus, 1990). Intrauterine infusion of estrogens and prostaglandin has been

shown to increase uterine motility in sows (reviewed by Langendijk et al., 2005) and, as a consequence, the stimulation of contractions can also increase the reflux of semen (Langendijk et al., 2002a). Willenburg et al. (2003) also observed an increase in the backflow amount during AI when prostaglandin was added to the insemination dose. In another study (Langendijk et al., 2002b) the increase of uterine contractions was attained artificially by the intrauterine infusion of cloprostenol raising the backflow during the insemination and consequently reduced the number of sperm cells in the oviducts (Langendijk et al., 2002a). Beside the increased number of uterine contractions, the magnitude of contractility after stimulation and the timing of stimulation related to the time of insemination could affect semen backflow (Langendijk et al., 2005).

During traditional cervical AI sperm loss in the backflow has been reported to be 25-45% (Steverink et al., 1998; Matthijs et al., 2003), reaching 70% of the dose volume (Steverink et al., 1998). Zerobin and Spörri (1972) observed that contractions in the caudal part of the uterus (cervical deposition) obstructed the infusion of semen. An increased frequency of contractions probably delays the influx of semen into the caudal part of the cervix and even increases the risk of backflow (Langendijk et al., 2005). Moreover, other factors such as ovulation time or sow age may be important in backflow quantity. Table 3 summarizes the backflow data collected from different reports.

Table 3. Backflow data collected during and after insemination, reported by different authors.

Authors	Sperm deposition	Sperm Dose (x 10^9)	Dose volume (ml)	Backflow collection time (h)	Backflow volume (%)	Sperm (%) in backflow	Sperm treatment
Araujo et al. 2009	Cervical	3.0	100	2	85.8	26.0	
	Intrauterine	1.0	100	2	83.2	16.4	
	Intrauterine	1.0	50	2	83.0	1.1	
	Intrauterine	0.5	100	2	87.8	16.1	
	Intrauterine	0.5	50	2	90.6	11.6	
Matthijs et al. 2003	Cervical	2.4	80	4	--	42.5±2.8	
	Cervical	2.4	20	4	--	31.7±1.0	
	Cervical	0.24	80	4	--	47.5±8.7	
Mezalira et al. 2005	Intrauterine	1.0	100	1	66.4±30.8	14.6±13.7	
	Intrauterine	0.5	100	1	63.9±39.8	12.6±12.3	
	Intrauterine	0.25	100	1	67.8±35.0	17.1±15.7	
Steverink et al. 1998	Cervical	6.0 3.0 1.0	80	2.5	70±3.4	25±1.3	
Willenburg et al. 2003	Cervical	0.5	80	8	89.75	54	
	Cervical	0.5	80	8	94.37	38	Estrogens
	Cervical	0.5	80	8	75.87	42	Oxytocin
	Cervical	0.5	80	8	87.62	34	PGF2α

Controlling the phagocytosis activity of PMNs towards the sperm and backflow following insemination could improve AI efficiency in this species. Moreover, new biotechnologies such as the use of frozen-thawed semen, new methodologies for insemination or sorted semen, will involve the use of a low number of sperm, so knowledge of sperm losses could improve their effectiveness.

2.2.5. Sperm selection in the uterus after insemination

The fact that only several thousand of spermatozoa reach the oviduct after the deposition of billions during insemination (Matthijs et al., 2003) suggests that, besides suffering backflow losses and phagocytosis by PMN, spermatozoa may be subjected to a rigid selection or unspecific clearance even before entering the oviduct (Taylor et al., 2008). There are different mechanisms along the female genital tract that allow the progressive selection of the most suitable spermatozoa for fertilizing, setting up different sperm subpopulations. These

subpopulations are partially or completely deficient in some of the aspects necessary to participate in the different steps of fertilization (Satake, 2006). Also, it has been demonstrated that each male produces his particular sperm subpopulations capable of reaching the oviduct (Holt, 2009).

Under normal circumstances, a low number of spermatozoa are sufficient for fertilization, and these establish themselves in the oviduct during the first hour after insemination (Hunter, 1981). Sperm population was studied in *ex vivo* conditions by the incubation of spermatozoa in different fractions of the uterus (Taylor et al., 2008). While the binding of viable sperm to the oviduct is thought to act as a SR, the retention of sperm cells in the uterus could serve to protect the viable spermatozoa from being removed with the backflow or to help sperm maturation (Taylor et al., 2008), so these findings could be interpreted as a pre-selection process.

These findings agree with reports in other species such as the ruminants where, the cervical crypts and grooves, aided by mucus, filter defective and immotile sperm, protect sperm from phagocytosis, act as safe storage areas and provide privileged paths for the transport of viable sperm (Mullins and Saacke, 1989). There are only a few reports about sperm selection in the female tract, so further studies should be performed to clarify how the sperm are selected along the uterus on their way to the oviduct.

2.2.6. AI methodologies

Oestrus is the period around ovulation in which sows show a standing response, allowing the boars to mate with the females. The duration of oestrus varies among sows from 24 h up to 96 h. The moment of ovulation after onset of oestrus also is highly variable (from 10 h to 85 h). A reliable prediction of ovulation time would be worthwhile, since fertilization results are highly dependent on the moment of insemination relative to the moment of ovulation. When the interval between insemination and ovulation is from 0 to 24 h, fertilization is optimal (Soede and Kemp, 1997). A prerequisite for optimal sow fertility is insemination with fresh extended semen during the 24 h period before ovulation. However, the large individual variation (both in gilts and sows) of the onset of estrus to ovulation interval limits the possibility to inseminate, in most of the cases, close to the optimal time (Steverink et al., 1999). Gilts show a shorter duration of oestrus than sows, therefore it is recommended that the time of gilt insemination, based on the onset of estrus, should differ from that for sows. Kaneko and Koketsu (2012)

showed that it was a good standard procedure to perform first insemination “immediately” after estrus detection and to perform second insemination “6 to 12 h” after first estrus detection. As mentioned, the optimal time of insemination in sows is 0 to 24 h before ovulation however, factors as duration of estrus, duration of first estrus after weaning or weaning-to-estrus interval must be evaluated to decide the number of inseminations per oestrus and at what point they should be done (Soede et al., 1995).

The main goal during mating or AI is that an adequate population of spermatozoa reach the site of fertilization during the peri-ovulatory period. In natural service, an enormous volume (~200-500 ml) and number of sperm (~20-70 billions) are deposited in the genital tract. Among other benefits (sanitary control, management, use of genetic superior males or control of semen quality) AI was introduced in pig production to optimize use of the male ejaculate. During natural mating only one male can serve one female. But with the use of the AI, approximately 20-25 females can be inseminated with one ejaculate if the sperm is deposited in the cervix.

In the past two decades, new strategies have been developed with the idea of depositing the semen close to the site of fertilization using a lower volume and number of cells than usual. These methods avoid the transit of spermatozoa through most of the female tract, ensuring that an optimal functional sperm population reaches the oviduct at the time of ovulation. So, what the pig AI industry aims to do is optimize boar ejaculation by decreasing the number of spermatozoa inseminated per dose, while maintaining the same efficiency in terms of pregnancy rate and litter size as afforded by traditional (cervical) insemination.

Cervical Insemination*:* Cervical AI (CAI) is the most widely used reproductive methodology in the porcine industry around the world. The technique is easy and simple to apply in field conditions, and basically consists of depositing the semen in the cervix (Figure 6) using a catheter. The concept behind it was logical and straightforward: to simulate *in vivo* conditions during mating. For this purpose, a catheter with the approximate length of the boar penis and finishing in a corkscrew shape (as the boar penis) was designed (See figure 7A-i). Actually, there are many different types of commercial cervical catheters available with differently shaped tips. However, little research has been conducted to compare them in the same study and so the use of one or another depends on the economics and personal preference on swine farms. Summarizing, the swine catheters can be grouped in to three types depending on the tip: spiral (Figure 7A-i), foam and multi-ring tip (Figure 7A-i and ii). It is important to take in to account the tip shape when insemination is carried out. Briefly, prior to insemination the vulva should be cleaned and the tip of the catheter coated with extender or non-spermicidal

lubricant. The catheter should be inserted into the vagina at a 45º angle to avoid its introduction into the urethra. Slide the catheter until feels a resistance, indicating that the catheter is at the entrance to the cervix. At this point, there are two manners of insert the catheter, depending on the tip. In the case of spiral type, turn it counter-clockwise until it locks into the cervix. With a foam or multi-ring tip just slide it (exerting some pressure) into the cervix until it locks. Once insemination has been made, the catheter is removed clockwise in the first case (spiral tip) and pulled softly outward in the others (foam and multi-ring tip).

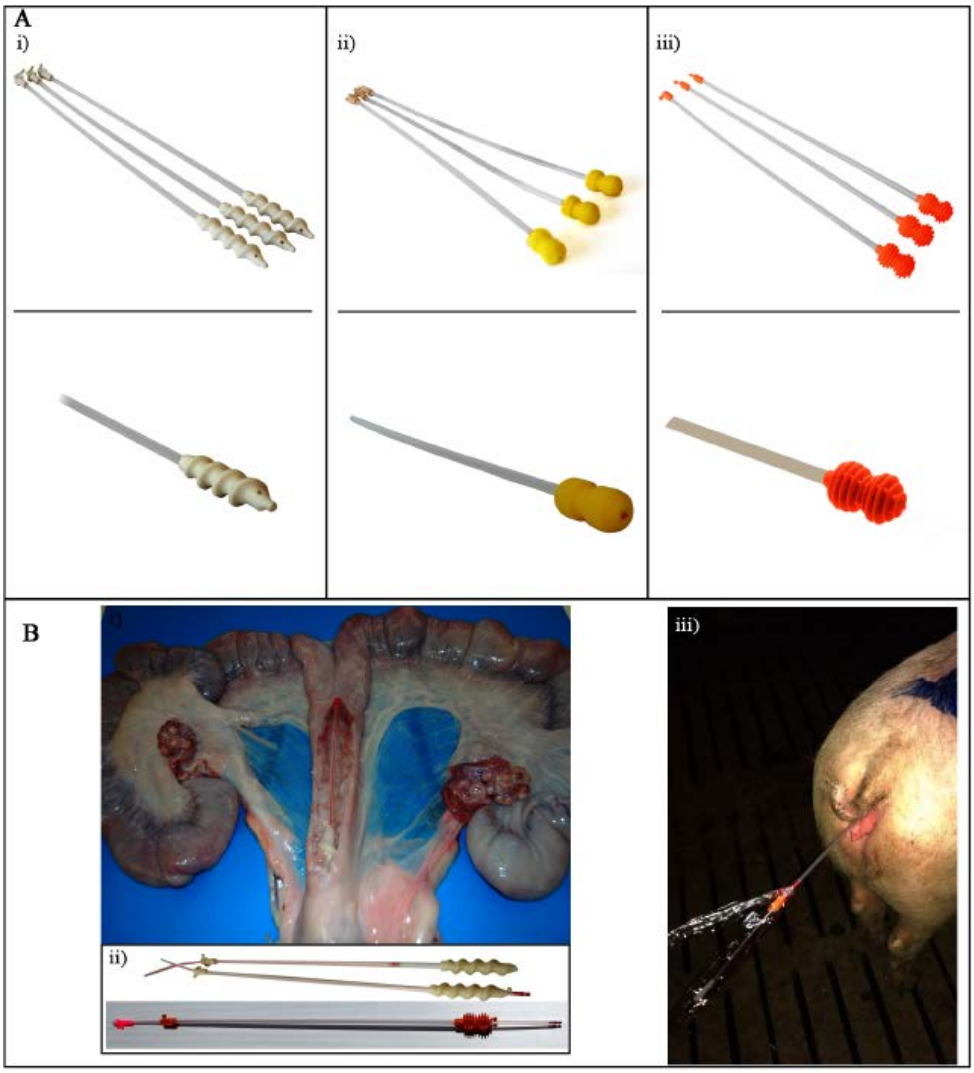

Figure 7. Catheters used in swine AI. (A) Different tips used in cervical catheter insemination: i) spiral; ii) foam and iii) multi-ring tip. (B) Post-cervical catheter: i) post-cervical catheter placed in *ex vivo* uterus; ii) flexible cannula of approximately 72 cm inserted into a conventional cervical catheter; and iii) swine insemination using post-cervical catheter. (Images provided by Import-Vet S.A. Spain).

Numerous investigations into the efficiency of CAI have been developed. In 1992, a study was conducted to examine the effects of mating by natural service and AI (Flowers and Alhusen, 1992). For this purpose the inseminations were carried out twice every oestrus for each female. In the case of AI the concentration dose was 7 billion sperm in 60 ml. When the AI was applied the reproductive parameters obtained were similar or even better than by natural service. Knowing the efficiency of AI, several studies have focused on analyzing the adequate number of sperm and volume for CAI. Watson and Behan (2002) compared three different sperm concentrations (1, 2 and 3 x 10^9 in 80 ml of extender). When $2x10^9$ sperm were inseminated the results were similar to those obtained with $3x10^9$ spermatozoa. Pregnancy and farrowing rates and litter size dropped drastically when the lower sperm concentration ($1x10^9$) was used. In addition to the number of sperm, the fluid of the inseminated dosage is an important factor to take into account for an adequate fertilization rate. Baker et al. (1968) inseminated gilts with a constant number of sperm ($5x10^9$) but in different dose volume (20, 100, and 200 ml). The authors concluded that using 100 ml during the insemination obtained a higher proportion of fertilized eggs and sperm attached than females inseminated with 20 and 200 ml. So, taking into account the results obtained in CAI, females are commonly inseminated using 2 to 3 billion sperm cells in an 80-100 ml volume.

Post-cervical insemination*:* As mentioned above, females are usually inseminated 2-3 times during oestrus with 2-4 billions sperm cells per dose, so that 4 to 12 billion sperm cells are used per female in each oestrus. These conditions limit the number of doses that can be prepared from one ejaculate. Various efforts to perform AI have been made by controlling the ovulation time, adding products to the dosage, etc. But, recently these efforts have been directed at reducing the number of sperm inseminated per dose and placing the sperm in different parts of the female reproductive tract rather than in the cervix. One of these techniques developed in the last decade is named post-cervical artificial insemination (post-CAI) (or intrauterine insemination) (Gil et al. 2000; Watson and Behan, 2002; Rozeboom et al., 2004; Mezalira et al., 2005; Roberts and Bilkei, 2005), which consists of depositing the sperm in the uterine body, after the cervix and just before the uterine bifurcation (Figure 6 and Figure 7B-i).

Several studies have been made to define the most suitable conditions (mainly number of sperm and dose volume) for this technique to reach at least similar results to CAI. First, let us look at some differences in the procedure between CAI and post-CAI methods. Post-CAI is performed with a combined catheter-cannula kit which consists of approximately a 72 cm long flexible cannula (15-20 cm longer than the common one) inserted into a conventional cervical catheter (Figure 7B). Unlike the CAI method, the sperm dose should be introduced quickly

(only a few seconds) to spread the dosage through the uterine horns, instead of several minutes used in the cervical method. The inner catheter is removed and then, with the cervical catheter still placed in the cervix and shaken in a rotational way, the neck of the womb is massaged for five seconds, after which the catheter is removed; this seems to stimulate ovulation.

Different authors (Watson and Behan, 2002; Mezalira et al. 2005; Araujo et al. 2009) have tested post-CAI in field conditions with reduced doses (1-2 x 10^9 sperm) obtaining similar results that when CAI was used. These data were confirmed when a similar number of sperm were found in the crypts and in the caudal isthmus region of the oviducts of sows inseminated by post-CAI ($1x10^9$ sperm) to those observed after conventional AI ($3x10^9$ sperm) (Sumransap et al., 2007; Tummaruk and Tienthai, 2010).

Other groups have attempted post-CAI using $0.5x10^9$ sperm per dose with controversial results. On the one hand, some authors (Gil et al., 2004; Mezalira et al., 2005; Araujo et al., 2009) found that using $0.5x10^9$ sperm with the post-cervical technique provided a similar results to CAI or post-CAI using a higher number of sperm. On the other hand, other authors (Rozeboom et al., 2004) reported a decrease in the farrowing rate and litter size when $0.5x10^9$ sperm were used in post-CAI in comparison with CAI group.

The application of post-CAI in field conditions implies several advantages. One of these is the use of a lower number of spermatozoa per dose, which increases the number of insemination doses produced per male. In current commercial conditions, one boar can produce up to 2000 doses per year with 3 billion sperm cells (Mezalira et al., 2005). By reducing the sperm number to 1000 million per dose, using the post-CAI method, the number of doses can be increased by up to 300%. In addition, the number of boars per farm could also be reduced, saving on the costs associated with buying and maintaining them. The use of the post-CAI method would ensure important savings.

Another point in favor the post-CAI technique is the time. CAI needs to be carried out more slowly than post-CAI mainly because of the lower volume used in post-CAI, where the dose influx can be very fast (few seconds) because the folds of the cervix are not a problem, and the sperm are released close to the fertilization site. In addition when the CAI method is used the catheter must remain in the uterus an additional few minutes after insemination to minimize backflow. Moreover, post-CAI insemination is straightforward and can be performed by the own farm technicians. Watson and Behan (2002) in their report concluded that the application of post-CAI in swine is simple, effective and safe.

Post-CAI has been applied in sows as well as in gilts (Dimitrov et al., 2007; Araujo et al., 2009). However, the use of this methodology in gilts is not as effective as in sows due to the physical impossibility which presents, in some cases, penetration of the post-cervical inner catheter in this type of female. However, new complementary methods can be used to enhance the use of post-CAI in gilts; for example, the application of Monzal® (Hidroclorhide of vetrobutin, Boehringer Ingelheim), a medicament routinely used to relax the uterine muscle during farrowing. The administration of this drug prior to post-CAI improves inner catheter penetration through the cervix in gilts (Hernández-Caravaca et al., 2013).

Deep intrauterine insemination*:* As mentioned several times through the present introduction, only a few of the total number of sperm deposited will reach the oviduct. Accordingly, some researchers have thought about the possibility of depositing only a few thousand of sperm in a place close to the fertilization place, the oviduct, in a technique denominated deep intrauterine insemination (DUI) (Figure 6). This insemination was used for the first time by Krueger et al. (1999). In this case, a surgical DUI was performed using a very low number of sperm (between 1 and 500 million) deposited close to the utero-tubal junction (UTJ) showing very encouraging results. The next step in DUI was the use of non-surgical insemination. For this purpose, Martínez et al. (2001) developed an optic fibre endoscope technique for non-surgical deep intrauterine insemination without sedation of the animal. But the problem with this technique was mainly the cost of the endoscope and impossibility of using it in field conditions. So, the same authors designed a new catheter constructed on the basis of the endoscope used previously (length 1.80 m, 4 mm outer diameter, and 1.80 mm diameter inner tubing) but less expensive. Briefly, deep uterine catheterization is performed after the insertion of a commercial AI spirette (to produce a cervical lock). The DUI catheter is then inserted through the spirette, moved through the cervical canal, and propelled forward along the uterine body and uterine horn (reviewed by Vázquez et al., 2008).

Although the sperm dose can be reduced to 150 x 10^6 (20-fold reduction) with the same pregnancy rate as in CAI, the litter size is reduced. This reduction in fertility represents a potential economic loss that must be considered in the total business model when using DUI in field conditions (Vázquez et al., 2008).

Other limitations of the technique for its application in field conditions are: 1) the high cost of the pipette for this procedure and the difficulty of executy the technique still represent impediments for its implantation on commercial farms (Da Costa et al., 2011); 2) the possibility

of uterine injury (Bathgate et al., 2008) due to the anatomical complexity of the sow's genital organs, 3) and the risk of infection (Carabin et al., 1996). At present the application of DUI is limited in field conditions but is very useful for using semen from superior boars or in new biotechnologies involving sex-sorted semen, frozen-thawed or genetically modified sperm (sperm mediated gene transfer) (García-Vázquez et al., 2011).

Recently, a new insemination device was developed and named double uterine deposition insemination (DUDI) (Mozo-Martín et al., 2012). This combines aspects of post-CAI and DUI, resulting in the post-cervical deposition of semen and approximately half-way along the uterine horn. When tested in field conditions this system provided similar fertility results to CAI when 750 million sperm in 30-50 ml was used (Mozo-Martín et al., 2012). Although the technique has provided encouraging results, further experiments are required, comparing DUDI with post-CAI and DUI methods.

Intraoviductal insemination*:* Another technique that permits a drastic reduction in sperm number during insemination is deposition directly into the oviduct (Figure 6) by laparotomy. This method is called intratubal artificial insemination (ITAI) and was first used during the 1970s by Polge et al. (1970), resulting in successful pregnancies.

Nowadays, this technique is undergoing resurgence through the application of new biotechnologies such as laparoscopy. The use of laparoscopy instead of laparotomy offers some advantages: 1) laparoscopy is considered a less invasive technique than laparatomy for introducing the semen into the uterus or in the uterine tuba (Vázquez et al., 2008); 2) it causes less stress and there is no problems of adherences in the postoperative period (Fantinati et al., 2005); 3) the procedure is relatively fast (approximately 15-20 min per animal) (Fantinati et al., 2005; Vázquez et al., 2008).

The laparoscopic ITAI method has permitted a reduction in sperm number deposited to $0.3\text{-}1x10^6$, while obtaining good oocyte penetration rates (Vázquez et al., 2008). However, this technique is still far from being commercially applied in swine. There are several difficulties involved in its application such as the need for trained personnel, equipment costs, risk of polyspermy (Hunter, 1973; Vázquez et al., 2008) and, in addition, the insemination should be realized in both uterine horns because the low concentration and the small volume used prevent sperm migration to the collateral horn (Fantinati et al., 2005).

2.3. FUTURE REMARKS

Although porcine biotechnology is improving day by day and new methods are a reality on the farm, such as post-CAI, there is still a long way to go. However, some techniques should be available to the porcine industry in the near future. For example, the use of sex-sorted sperm, whether the number of sperm sorted is improved, or the viability and fertility rate of frozen-thawed sperm is enhanced. Another aspect that should see progress is fresh sperm storage; for this purpose, investigations must focus on new extender formulations that permit long term storage (at least 7 days) at 15-20º C. This, in combination with other new strategies, such as post-CAI, would vastly increase boar effectiveness. The development of new *in vitro* technologies to predict male fertility is another theme that will be resolved sooner or later, permitting use of the best boars and at the most appropriate moment. Besides these, fresh knowledge on the physiology of reproduction is necessary before new strategies can be widely applied in swine-breeding practice. In this respect, knowledge of the exact mechanism by which sperm reach the oviduct, how sperm are selected during transit in their travel or, very ambitiously, what the special characteristics of the spermatozoa which fertilize the oocyte are, would provide to the reproductive industry new opportunities to enhance efficiency at farm level and provide economic savings.

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3. Objectives/Objetivos

The main aims of the present thesis are the following:

1. To evaluate the reproductive and economic benefits of using *post-cervical insemination* in farm conditions compared with traditional cervical insemination, and to evaluate the phenomenon of *backflow* **(Article 1)**.

2. To determine whether the *motility* of sperm doses can influence sperm volume and concentration and the percentage of motile spermatozoa collected in the backflow at different times post-insemination **(Article 2)**.

3. To study whether the *morphometry* of sperm cells is modified during and/or implicated in sperm selection after insemination, through a morphometric analysis of the sperm in the backflow (head and flagellum) and the utero-tubal junction (flagellum) **(Article 3)**.

4. To examine whether sperm *morphology* influences the selection of spermatozoa in the uterus by studying the sperm in the backflow and the utero-tubal junction, and to analyse the possible role of uterine fluid in morphological changes **(Article 4)**.

Los objetivos principales de la presente Tesis Doctoral son los siguientes:

1. Evaluar los rendimientos reproductivos y económicos del uso de la *inseminación post-cervical* en condiciones de granja comparados con la inseminación cervical tradicional, así como la evaluación de los *reflujos* **(Artículo1)**.

2. Determinar si la *motilidad* de las dosis espermáticas puede influir en el volumen, concentración espermática y el porcentaje de espermatozoides motiles recogidos en el reflujo a diferentes tiempos tras la inseminación **(Artículo 2)**.

3. Estudiar si la *morfometría* de las células espermáticas se modifica y/o está implicada en la selección espermática tras la inseminación, mediante el análisis morfométrico del esprmatozoide en el reflujo (cabeza y flagelo) y en la unión útero-tubárica (flagelo) **(Artículo 3)**.

4. Examinar si la *morfología* espermática influye en la selección de los espermatozoides en el útero, mediante el estudio de los espermatozoides en el reflujo y en la unión útero-tubárica; así como analizar el papel del fluido uterino sobre posibles cambios en la morfología **(Artículo 4)**.

4. Articles

Article 1

Reproductive performance and backflow study in cervical and post-cervical artificial insemination in sows

Animal Reproduction Science. 2012 Dec; 136 (1-2):14-22. doi: 10.1016/j.anireprosci.2012.10.007.

http://www.animalreproductionscience.com/article/S0378-4320%2812%2900310-7/abstract

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Reproductive performance and backflow study in cervical and post-cervical artificial insemination in sows

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ABSTRACT

The present study was developed to evaluate multiparous sow reproductive performance and backflow in post-cervical artificial insemination (post-CAI) using a reduced number of sperm than in cervical artificial insemination (CAI). The experimental groups were divided into sows inseminated by: 1) cervical artificial insemination (CAI): 3×10^9 spermatozoa/80 ml; 2) post-CAI: 1.5×10^9 spermatozoa/40 ml (post-CAI 1); 3) post-CAI using 1×10^9 spermatozoa/26 ml (post-CAI 2). Post-CAI 1 reproductive parameters were similar to those of post-CAI 2 (except for live born litter size which was greater in post-CAI 1) and better than for the CAI group ($p < 0.01$). In a second experiment the backflow volume, number of sperm, and sperm quality in the backflow were studied in the 3 experimental groups. The % of volume and spermatozoa in the backflow was higher in the CAI group ($p < 0.05$) than post-CAI groups (statistically similar between them). Moreover, the quality parameters (motility, progressive motility, viability, chromatin decondensation and morphology) in backflow semen were identical in all three experimental groups, but differed as regards the original insemination dose incubated inside a colostomy bag (sperm quality control group). The present study shows that the use of post-CAI (either post-CAI 1 or 2) in field conditions can be recommended because the efficiency is similar (in the case of post-CAI 2) or higher (post-CAI 1) than when using the traditional method (CAI), representing a reduction cost.

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Article 2

Boar sperm with defective motility are discriminated in the backflow moments after insemination

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Boar sperm with defective motility are discriminated in the backflow moments after insemination

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ABSTRACT

During insemination, a large number of spermatozoa are deposited in the female genital tract, but a very low percentage is able to colonize the site of fertilization. The influx of neutrophils into the uterine lumen and semen reflux (backflow) are known mechanisms that decrease the number of spermatozoa within the uterus. No report has attempted to ascertain whether the backflow is a random or selective process of the spermatozoa. In this work, sows were inseminated using two populations of spermatozoa in the same proportion: (1) unstained spermatozoa with high motility and (2) stained spermatozoa with low, medium, or high motility. Volume, number, and percentage of stained spermatozoa were evaluated in the backflow (collected at 0–15, 16–30, and 31–60 minutes after insemination). This article provides evidence that (1) the motility characteristics of the spermatozoa do not influence the percentage of sows with backflow, the volume and number of spermatozoa in the backflow; (2) the discarding of spermatozoa in the backflow is not specific during the first moments after insemination (0–15 minutes), whereas later (16–60 minutes), spermatozoa with defective motility (low and medium groups) are discarded in a higher proportion than high group in the backflow ([16–30 minutes: low, 85.13 ± 4.32%; medium, 72.99 ± 5.05%; and high, 54.91 ± 2.38%; $P < 0.0001$; 31–60 minutes: low, 87.16 ± 6.01%; medium, 87.02 ± 4.01%; and high, 59.35 ± 2.86%; $P = 0.001$]). Spermatozoa with poor motility are discarded in the backflow probably as a selective process, on the part of the female genital tract or as a result of the intrinsic low spermatozoa motility.

Article 3

Morphometry of boar sperm head and flagellum in semen backflow after insemination

Theriogenology. 2015. *In press*. doi:10.1016/j.theriogenology.2015.04.011.

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Morphometry of boar sperm head and flagellum in semen backflow after insemination

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ABSTRACT

Once deposited in the female reproductive system, sperm begin their competition and undergo a selection to reach the site of fertilization. Little is known about the special characteristics of sperm that reach the oviduct and are able to fertilize, with even less information on the role of sperm dimension and shape in transport and fertilization. Here, we examine whether sperm morphometry could be involved in their journey within the uterus. For this purpose, sperm head dimension (length, width, area, and perimeter) and shape (shape factor, ellipticity, elongation, and regularity), and flagellum length were analyzed in the backflow at different times after insemination (0–15, 16–30, and 31–60 minutes). Sperm morphometry in the backflow was also analyzed taking into account the site of semen deposition (cervical vs. intrauterine). Finally, flagellum length was measured at the uterotubal junction. Sperm analyzed in the backflow were small (head and flagellum) with different head shapes compared with sperm observed in the dose before insemination. The site of deposition influenced head morphometry and tail size both being smaller in the backflow after cervical insemination compared with intrauterine insemination. Mean tail length of sperm collected in the backflow was smaller than that in the insemination dose and at the uterotubal junction. Overall, our results suggest that sperm size may be involved in sperm transport either because of environment or through sperm selection and competence on their way to encounter the female gamete.

Article 4

Morphological study of boar sperm during their passage through the female genital tract

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Morphological study of boar sperm during their passage through the female genital tract

Running head: Sperm morphology in uterus environment

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Abstract

Once deposited in the female tract, sperm face a series of challenges that must be overcome to ensure the presence of an adequate normal sperm population close to the site of fertilization. Our aim was to evaluate the influence of the uterus milieu on boar sperm morphology. In experiment 1, sperm morphology was evaluated in the backflow (60 min after insemination) and within the utero-tubal junction (UTJ) (collected ~24h after insemination) following intrauterine sperm deposition (n=6) and compared with the morphology of the sperm in the insemination dose. In experiment 2, the influence of the uterine fluid (UF) on sperm morphological modifications was evaluated. For this purpose, ejaculated (n=4) and epididymal (n=4) sperm were *in vitro* incubated in the presence or not of UF for 2 and 24 h. In both experiments, sperm were classified as normal, having a cytoplasmic droplet (proximal or distal) or with tail defects. The results of experiment 1 pointed to an increase in morpho-abnormal sperm collected in the backflow (27.70%) and a reduction of the same in the UTJ (2.12%) compared with the insemination dose (17.75%) ($P<0.05$). In experiment 2, the incubation of ejaculated sperm with UF did not provoke any morphological modification; however, when epididymal sperm were incubated with UF, a pronounced increase in the percentage of normal sperm was evident after 24 h compared to the initial dose (from 25.77% to 53.58%, $P<0.05$), mainly due to distal cytoplasmatic droplet shedding (53.22 *vs.* 20.20%). In conclusion, almost all the sperm which colonize the UTJ had a normal morphology, part of the abnormal sperm having been discarded in the backflow and part selected/modified on their way to the oviduct. UF seems to influence cytoplasmic distal droplets removal as has been demonstrated previously in seminal plasma.

Keywords: backflow, cytoplasmic droplet, porcine, sperm morphology, uterine fluid.

22-May-2015

Dear Prof. García-Vázquez:

It is a pleasure to accept your manuscript entitled "Morphological study of boar sperm during their passage through the female genital tract" in its current form for publication in the Journal of Reproduction and Development. The comments of the reviewer(s) who reviewed your manuscript are included at the foot of this letter.

Thank you for your fine contribution. On behalf of the Editors of the Journal of Reproduction and Development, we look forward to your continued contributions to the Journal.

Sincerely,
Dr. Takashi Nagai
Co-Editor in Chief, Journal of Reproduction and Development
taku@fftc.org.tw

5. Conclusions/Conclusiones

Article 1:

1. The productive benefits of post-cervical insemination are comparable with or greater than those of cervical insemination, especially when sows with 2-3 parities and 6 or more.

2. Post-cervical insemination provides economic benefits compared with cervical insemination.

3. The site of sperm deposition influences the volume and number of spermatozoa collected in the backflow, both being greater in cervical insemination.

4. The spermatozoa collected in the backflow are of reduced quality compared with the spermatozoa of the insemination dose, which suggests that a selection process is operating in the sow uterus.

Article 2:

5. The percentage of motile spermatozoa in the insemination dose does not influence the number of sows with backflow.

6. The sperm of low motility are rejected in the backflow 15 minutes after insemination due to a selective process on the part of the female genital tract or as a result of the low motility of the spermatozoa.

Article 3:

7. After artificial insemination, spermatozoa of given dimensions (reduced head size and flagellum) and head shape (elliptic and elongated) are discarded in the backflow, suggesting a sperm selection process in the uterus based on their morphometric characteristics and/or a modification of the same in their transit towards the oviduct.

8. Sperm morphometrics are not influenced by the uterine fluid *in vitro*.

9. The insemination site influences the morphometric characteristics of the spermatozoa collected in the backflow – being larger (head and flagellum) when post-cervical insemination rather than cervical deposition is carried out.

10. Flagellum length is not involved in the capacity of spermatozoa to reach the utero-tubal junction.

Article 4:

11. Sperm selection in the female genital tract after artificial insemination is influenced by spermatozoa morphology – a greater proportion of morphoanomalous sperm being rejected in the backflow and during the transit towards the utero-tubal junction.

12. The uterine fluid does not influence morphological changes in ejaculated sperm *in vitro*, but does influence the reduction in distal cytoplasmatic droplets in epididymal spermatozoa.

Artículo 1:

1. Los rendimientos productivos de la inseminación post-cervical son comparables o superiores a los de la inseminación cervical, principalmente cuando se utiliza en cerdas con un número de partos de 2-3 e igual o superior a 6.

2. El uso de la inseminación post-cervical a nivel de granja supone un ahorro económico comparado con los rendimientos de la inseminación cervical.

3. El lugar de deposición espermática influye en el volumen y número de espermatozoides recogidos en el reflujo, siendo estos parámetros superiores cuando la deposición se produce a nivel cervical.

4. Los espermatozoides recogidos en el reflujo presentan una calidad espermática reducida en comparación con la dosis de inseminación, sugiriendo un proceso selectivo en el útero de la cerda.

Artículo 2:

5. El porcentaje de espermatozoides móviles presente en las dosis de inseminación no influye en el número de cerdas que presentan reflujo.

6. Los espermatozoides con baja motilidad son descartados a partir de los 15 minutos tras la inseminación en el reflujo debido a un proceso selectivo, ya sea por parte del tracto genital de la hembra o como resultado de la baja motilidad intrínseca de los espermatozoides.

Artículo 3:

7. Tras la inseminación artificial, los espermatozoides con unas determinadas dimensiones (tamaño de cabeza y flagelo reducido) y forma de la cabeza (más elípticos y elongados) son descartados en el reflujo, indicando una posible selección espermática en el útero de

la hembra basada en sus características morfométricas y/o una modificación de los mismos en su tránsito hacia el oviducto.

8. La morfometría espermática no se ve influida por el fluido uterino en condiciones *in vitro*.

9. El lugar de inseminación influye en las características morfométricas de los espermatozoides recogidos en el reflujo, siendo estos de mayores dimensiones (cabeza y flagelo) cuando se realiza la inseminación post-cervical comparada con una deposición en el cérvix de la hembra.

10. La longitud del flagelo no estar involucrado al menos en la capacidad de ascensión de los espermatozoides hacia la unión útero-tubárica.

Artículo 4:

11. La selección espermática en el tracto genital de la hembra tras la inseminación artificial se ve influenciada por la morfología del espermatozoide, descartando en el reflujo y en el transporte espermático hacia la unión útero-tubárica una mayor proporción de espermatozoides con morfoanomalías.

12. El fluido uterino no influye la modificación morfológica en espermatozoides eyaculados en condiciones *in vitro*, mientras sí provoca una reducción de gotas citoplasmáticas distales en espermatozoides epididimarios.

6. Annex: publications derived from the Thesis

The results obtained in the present Doctoral Thesis have been included in the following publications:

Articles in journals included in the Science Citation Index (SCI) of the Institute for Scientific Information (ISI):

- **Hernández-Caravaca I**, Izquierdo-Rico MJ, Matás C, Carvajal JA, Vieira L, Abril D, Soriano-Úbeda C, García–Vázquez FA. Reproductive performance and backflow study in cervical and post-cervical artificial insemination in sows. Animal Reproduction Science. 2012 Dec; 136(1-2):14-22. doi: 10.1016/j.anireprosci.2012.10.007.

- **Hernández-Caravaca I**, Soriano-Úbeda C, Matás C, Izquierdo-Rico MJ, García-Vázquez FA. Boar sperm with defective motility are discriminated in the backflow moments after insemination. Theriogenology. 2015. 83: 655-661. doi: 10.1016/j.theriogenology.2014.10.032.

- García-Vázquez FA, **Hernández-Caravaca I**, Yánez-Quintana W, Matás C, Soriano-úbeda C, Izquierdo-Rico MJ. Morphometry of boar sperm head and flagellum in semen backflow after insemination. Theriogenology. doi: 10.1016/j.theriogenology.2015.04.011.

- García-Vázquez FA, **Hernández-Caravaca I**, Matás C, Soriano-Úbeda C, Abril-Sánchez S, Izquierdo-Rico MJ. Morphological study of boar sperm during their passage through the female genital tract. Journal of Reproduction and Development. Accepted May 22, 2015. *In press*. Vol. 61, nº 5 (October, 2015).

Other articles:

- **Hernández-Caravaca I**, Izquierdo-Rico M.J, Matás C, García-Vázquez FA. Inseminación post-cervical: rentabilidad en la granja. Suis. Septiembre, 60. 26-33. 2009.

Abstracts in International Congress:

- **Hernández-Caravaca I**, Izquierdo-Rico M.J, García-Vázquez FA. Post-cervical insemination of sows with reduced sperm number in field conditions. Reprod Fertil Dev 21 (1): 105. Meeting abstract IETS. 2009.

- **Hernández-Caravaca I**, Izquierdo-Rico MJ, García-Vázquez FA. Inseminación artificial post-cervical en la especie porcina: efectos en los parámetros reproductivos. Comunicación-póster. VI congreso internacional de estudiantes de ciencias experimentales y de la salud, CEU. Moncada, Valencia, España. 2009.

- **Hernández-Caravaca I**, Izquierdo-Rico MJ, Carvajal JA, Abril D, Soriano-Úbeda C, Vieira L, Matás C, García-Vázquez FA**.** Semen backflow in sows after cervical and post-cervical artificial insemination. Comunicación-poster. The 2011 Allen D. Leman Swine Conference**.** St. Paul, Minnesota, EEUU. 2011.

- **Hernández-Caravaca I**, Soriano-Úbeda C, Izquierdo-Rico MJ, Matás C, García-Vázquez FA. Estudio del reflujo seminal tras la inseminación artificial cervical y post-cervical en porcino. Comunicación-oral. 9º congreso internacional de estudiantes de ciencias experimentales y de la salud, CEU. Moncada, Valencia, España. 2012. **2º PREMIO a la mejor comunicación**.

- **Hernández-Caravaca I**, Izquierdo-Rico MJ, Matás C, Soriano-Úbeda C, Abril-Sánchez S, García-Vázquez FA. Selection of morphologically normal sperm within the porcine uterus. VI International Congress of Histology and Tissue Engineering, Bilbao, Spain. 2015.

Abstracts in National Congress:

- **Hernández-Caravaca I**, Izquierdo-Rico MJ, Matás C, García-Vázquez FA. Inseminación post-cervical en cerdas con reducido número de espermatozoides. Comunicación oral. ANAVEPOR. Zaragoza, España. 2008. **1ER PREMIO a la mejor comunicación.**

- **Hernández-Caravaca I**, Soriano-Úbeda C, Izquierdo-Rico MJ, Matás C, García-Vázquez FA. Análisis del reflujo seminal en la especie porcina: inseminación artificial cervical *vs.* Postcervical. Comunicación-poster. III Congreso ANAVEPOR. Zaragoza, España. 2012.

- Soriano-Úbeda C, **Hernández-Caravaca I**, Izquierdo-Rico MJ, Matás C, García-Vázquez FA. La selección espermática en el útero de la cerda está influenciada por la calidad de los espermatozoides: análisis del reflujo. Comunicación-oral. XV Jornadas sobre Producción Animal (AIDA – Asociación Interprofesional para el Desarrollo Agrario). Zaragoza, España.. 2013.

- Yánez-Quintana W, **Hernández-Caravaca I,** Izquierdo-Rico MJ, Soriano-Úbeda C, Matás C, García-Roselló E, García–Vázquez FA. Boar sperm selection within uterus is influenced by flagellum length. Comunicación oral. 12º Congreso Internacional Asociación Española de Reproducción Animal (AERA 2014). Alicante 16 y 18 de Octubre de 2014. Reprod Dom Anim 49 (Suppl. 4), 97 (2014); doi: 10.1111/rda.12402. AERA. Alicante, España. 2014.

- Hernández-Caravaca I, Soriano-Úbeda C, Matás C, Izquierdo-Rico MJ, García-Vázquez FA. Backflow in sows inseminated with different sperm motility populations. Comunicación oral. I Jornadas Doctorales de la Universidad de Murcia. Murcia, España. 2015.