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Avances de la técnica de preselección del sexo en el ganado porcino mediante separación de espermatozoides X e Y por citometría de flujo

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1.- Introducción

La posibilidad de elegir el sexo de la descendencia, antes de la concepción, es uno de los objetivos más perseguidos dentro del campo de la tecnología de la reproducción, ya que supone poder controlar una serie de situaciones, algunas de gran importancia, que de otra manera dependen únicamente del azar. La mejora genética y la optimización de la producción en las explotaciones ganaderas, el control de enfermedades hereditarias ligadas al sexo en humana, o el manejo en cautividad y la recuperación de animales salvajes son algunos de los campos en los cuales la selección del sexo supone una herramienta muy importante.

En la especie humana, eludir las numerosas enfermedades existentes ligadas de forma específica al cromosoma X, así como el llamado desequilibrio familiar son las aplicaciones prioritarias de la elección del sexo de la descendencia. Alrededor de unas 6000 enfermedades de carácter hereditario pueden afectar al hombre, 370 de las cuales están ligadas al cromosoma X (McKusik, 1992). La hemofilia, la distrofia muscular de Duchenne o la hidrocefalia ligada al cromosoma X, son algunas de estas enfermedades que suelen ser de carácter recesivo, siendo expresadas por los hijos varones de madres portadoras (Johnson y Welch, 1997). En estos casos, poder determinar el sexo de la descendencia antes de la concepción resulta fundamental. En cuanto al desequilibrio familiar, algunos estudios realizados a este respecto han demostrado que la mayoría de parejas están a favor de la elección del sexo en la descendencia antes de su concepción, como solución a estos problemas, que se presentan cuando se tienen 2 ó 3 hijos del mismo sexo y se desea tener otro de sexo diferente (Rosenzweig y Aldeman, 1976; Markle y Nam, 1982; Fugger y cols., 1998). Sin embargo, cuestiones de tipo ético o

religioso (Schenker, 2002), así como cuestiones relacionadas con la seguridad del proceso, plantean ciertos reparos por parte de algunos sectores a la hora de aceptar la selección del sexo de los hijos sin obedecer a razones terapéuticas y como solución a un posible desequilibrio familiar (Seidel, 2003).

Predeterminar el sexo de la descendencia en animales destinados a deportes de élite, como perros, caballos e incluso camellos despierta también un gran interés en la actualidad, debido al alto valor económico que pueden alcanzar los individuos en función del sexo. La aplicación de la técnica en la producción de especies cinegéticas donde los individuos macho son mucho más valorados a la hora de la caza, así como la optimización de los esquemas reproductivos en los zoológicos, para evitar elevados niveles de consanguinidad y el rescate de especies en peligro de extinción cuando un escaso número de machos o hembras puede conducir a la desaparición total de la misma, entran también dentro del abanico de posibles utilidades del control del sexo de la descendencia (Seidel, 2003)

Por último, en diferentes áreas de investigación, como la transgénesis, la predeterminación del sexo supondría una reducción en los costes, ya que permitiría una reducción en el número de animales requeridos. Además, y debido al alto precio que alcanzan los animales pertenecientes a líneas transgénicas, la aplicación de esta técnica para planificar la producción supondría una gran ventaja (Seidel, 2003). Por otro lado, las continuas investigaciones que se realizan en el campo del sexaje de espermatozoides pueden dar lugar a la obtención de información básica para otras disciplinas, como ocurre por ejemplo en el caso de la inmunología (Blecher y cols., 1999).

Es sin embargo en la producción animal donde la predeterminación del sexo de supone una herramienta extremadamente útil desde un punto de vista productivo. Una aceleración en los programas de mejora genética, un incremento en la eficiencia biológica y económica de la producción y una mayor flexibilidad en los sistemas de manejo son algunas de las ventajas que esta técnica podría aportar a las explotaciones ganaderas (Schenk y cols., 1999; Maxwell y cols., 2004). El ejemplo más claro lo tenemos en el ganado bovino, donde la obtención de hembras resulta fundamental en

las explotaciones lecheras, mientras que en las dedicadas a la producción cárnica será más rentable obtener el mayor número de terneros posibles (Seidel y Johnson, 1999). En este último caso, se ha descrito una mejora de alrededor del 5% en la eficiencia de la producción gracias a la obtención de animales de sexo deseado (Doyle, 2000). En el caso concreto del porcino, la producción de machos y hembras de líneas híbridas selectas vería aumentada su eficiencia y rentabilidad mediante la preselección del sexo de la descendencia (Johnson, 2000). La aplicación de esta técnica en los núcleos de multiplicación porcina, sería también ventajosa ya que permitiría producir machos o hembras de acuerdo a las necesidades de producción. De igual manera la preselección de sexo en la progenie supondría un gran impulso para los programas de mejora genética en esta especie. La posibilidad de obtener sólo hembras como producto final en la cadena de producción haría posible eliminar la castración como práctica sistemática de manejo en estos núcleos productivos para conseguir una óptima calidad de la canal, ya que aunque actualmente está permitida en España, es muy probable que sea clasificada como práctica poco adecuada cuestionándose su utilización en la implementación de las nuevas normativas de bienestar animal propuestas por la Unión Europea.

1.1.- Estructura de la tesis y objetivos

El empleo de la separación espermática mediante citometría de flujo, como método para predeterminar el sexo de la progenie, ha dado lugar a la obtención de descendencia viva en diferentes especies de mamíferos domésticos, incluido el ganado porcino. Sin embargo, existen algunos aspectos, tanto técnicos, como relacionados con los efectos del proceso de separación sobre los espermatozoides de verraco separados, que deben ser estudiados más detalladamente con el fin de maximizar los rendimientos del procedimiento y hacer posible la utilización de esta tecnología de forma generalizada en ganado porcino.

El presente trabajo ha sido realizado utilizando un citómetro modelo Epics ALTRA (Coulter Corporation, Miami, FL. USA) preparado para trabajar a alta

velocidad y modificado específicamente y por primera vez para separación espermática. Las modificaciones consistieron en la incorporación de un fototubo frontalmente al impacto del láser dotado de un ajuste de foco, en la sustitución de la cámara de flujo estándar de separación celular por una aguja de inyección de muestra con el extremo biselado, y por ultimo en la reubicación de la cámara de video necesaria para el control del proceso de separación. Estas modificaciones hacen que el proceso de ajuste o alineamiento del citómetro, básico para conseguir la máxima eficiencia del procedimiento, sea más complejo que en otros equipos.

En los citómetros estándar de separación celular este alineamiento se realiza con esferas regulares fluorescentes creadas específicamente para ello. Sin embargo y debido a las particularidades que presenta la célula espermática, este procedimiento debe realizarse con partículas los más parecidas posibles a estas (Johnson y Welch, 1999). En general se tiende a utilizar espermatozoides pertenecientes a especies en las cuales la diferencia de ADN entre los espermatozoides X e Y es grande, además de presentar una morfología de la cabeza adecuada para una fácil identificación y separación de las poblaciones.

Aunque hasta este momento no se habían separado espermatozoides de macho cabrío, la elección de estos espermatozoides para la realización de la primera experiencia vino determinada en primer lugar porque desde un punto de vista filogenético era de esperar que presentaran unas diferencias en el contenido de ADN próximas a las del ganado ovino (4'2 %) y superiores a las existentes en porcino (3'6%). En segundo lugar, la morfología de la cabeza de los espermatozoides de caprino tiene también un papel relevante, ya que su forma aplanada permite distinguir muy bien entre el perfil y la cara plana de la misma, facilitando la identificación de los espermatozoides orientados, y confirmando de forma definitiva la utilidad y el correcto funcionamiento del procedimiento bajo nuestras condiciones de trabajo. Finalmente la importancia de esta especie en el sector ganadero regional determinó su elección como elemento de estudio, ya que nuestros resultados podrían ser de utilidad "per se" en este sector. Los resultados obtenidos en esta experiencia demostraron la utilidad de los espermatozoides de macho cabrio como herramienta para el ajuste del

equipo previamente a la realización de los experimentos de separación de espermatozoides de verraco.

Una vez comprobado que el sistema está ajustado y es capaz de identificar los espermatozoides X e Y de forma adecuada, la determinación de la eficiencia del proceso de separación, definida como el grado de pureza de las poblaciones obtenidas, antes de su aplicación mediante diferentes técnicas de reproducción asistida se convierte en una necesidad prioritaria (Welch y Johnson, 1999). Con este objetivo se realizó la experiencia 2, en la cual se diseñó y utilizó la hibridación in situ fluorescente directa (FISH: fluorescente in situ hybridization) como método para la determinación del porcentaje de espermatozoides portadores del cromosoma X e Y presentes en muestras de espermatozoides separadas.

El elemento más importante en una reacción de FISH es la sonda de ADN, la cual puesta en contacto con el material nuclear del espermatozoide dará lugar a una marca específica de un determinado cromosoma fácilmente identificable mediante microscopía de fluorescencia. Debido a que no existen, de forma comercial, sondas de ADN para reacciones de FISH en porcino, se planteó la necesidad de construirlas mediante amplificación por PCR de fragmentos específicos pertenecientes a los cromosomas 1 e Y porcinos, y posterior marcaje fluorescente de los mismos utilizando la técnica de desplazamiento de cortes (nick translation). También se comprobó la utilidad del método de marcaje y de las sondas de ADN construidas, directas y específicas para cromosomas 1 e Y porcinos. Con el fin de evaluar los rendimientos del equipo en cuanto a la pureza de las poblaciones obtenidas y una vez comprobada la utilidad de las sondas, se analizaron muestras de espermatozoides separados siguiendo criterios de separación más o menos estrictos (altas y bajas purezas, respectivamente), directamente relacionados con diferentes tamaños en las ventanas de selección de la población.

La obtención de descendencia del sexo deseado tras inseminación con espermatozoides seleccionados mediante citometría de flujo es un hecho demostrado en diferentes especies de mamíferos domésticos. Sin embargo, una reducción en las tasas de gestación así como en los tamaños de camadas, ha sido descrita como

resultado de la inseminación con espermatozoides separados en algunas de estas especies. Actualmente, la causa principal de esta reducción en la fertilidad de los espermatozoides separados todavía debe ser determinada. Daños en el ADN, aumento de la perdida de embriones en estadios tempranos de la gestación, y otros factores relacionados con la dosis de inseminación, momento de realización de la misma en relación con el momento de la ovulación, o la reducida viabilidad de los espermatozoides separados en el tracto genital femenino son algunos de los factores a estudiar para conseguir unos niveles de viabilidad y fertilidad post-separación adecuados (Maxwell y cols., 2004). En este sentido se diseñaron dos experiencias destinadas a evaluar la viabilidad y capacidad fecundante *in vitro* e *in vivo* de los espermatozoides de verraco sometidos al proceso de separación.

En primer lugar (experiencia 3) se evaluó como la conservación en medio líquido de los espermatozoides separados de verraco afectó a la motilidad, viabilidad y capacidad fecundante *in vitro* de los mismos. Este sistema de conservación resulta de vital importancia para los espermatozoides separados, ya que habitualmente el laboratorio donde se realiza la separación se encuentra alejado del lugar de inseminación. Por tanto, es necesario conocer cuanto tiempo son capaces los espermatozoides separados de mantener su viabilidad y capacidad fecundante tras la separación, porque esto determinara el tiempo dentro del cual debe realizarse la inseminación. Además y derivado del bajo rendimiento del proceso de separación conseguir un número suficiente de espermatozoides separados para su inseminación requiere en ocasiones tiempos de separación prolongados, por lo que la población espermática resultante estará constituida por espermatozoides almacenados durante diferentes tiempos tras la separación determinando una gran heterogeneidad de la misma. Por tanto, y como se ha señalado anteriormente, evaluar el tiempo durante el cual el espermatozoide separado mantiene la capacidad fecundante, resulta de trascendente importancia. El principal problema que aparece asociado a la perdida de esta capacidad de penetración es la capacitación prematura, estadio que aparece de forma característica en espermatozoides de verraco que han sufrido el proceso de separación por citometría de flujo (Maxwell y Johnson, 1999), al igual que lo hace en

aquellos que son sometidos a procesos de congelación y descongelación (Pursel y Johnson, 1975). La relación entre el estado de precapacitación y la capacidad fecundante de los espermatozoides separados, así como la influencia de las condiciones de almacenamiento, fueron también analizadas en esta experiencia.

En segundo lugar, y tras comprobar la capacidad fecundante *in vitro* de los espermatozoides de porcino sometidos a procesos de separación por citometría de flujo, se procedió a evaluar la fertilidad *in vivo* de los mismos. En la realización de esta cuarta experiencia, se usaron espermatozoides procesados reproduciendo las condiciones del proceso de separación, pero recogiendo la totalidad de la población sin seleccionar ninguna en concreto con el fin de conseguir un mayor número de espermatozoides. Las inseminaciones se realizaron mediante el procedimiento de inseminación intrauterina profunda (Martínez y cols., 2001 a y b), tanto en cerdas con ovulación inducida como en cerdas con ovulación espontánea, con el fin de determinar las condiciones mas adecuadas para la obtención de los mejores resultados de fertilidad tras la inseminación de estos espermatozoides.

La obtención de descendencia viva de siete especies de mamíferos diferentes (incluyendo el hombre) tras la inseminación con espermatozoides separados mediante citometría de flujo (Fugger y cols., 1998; Amann, 1999; Seidel y Johnson, 1999; Garner, 2001; Seidel y Garner, 2002) sin aparición, en ningún caso, de alteraciones fenotípicas, indica que el procedimiento es seguro a nivel genético. Sin embargo esta seguridad es solo una hipótesis aceptada como valida en ausencia de evidencias fenotípicas que demuestren lo contrario. Teniendo en cuenta que la tinción del ADN de los espermatozoides con el fluorocromo Hoechst 33342, y que el impacto del láser ultravioleta sobre estas células son dos etapas, que podrían alterar la integridad del ADN (Ashwood-Smith, 1994), fundamentales en la separación espermática mediante citometría de flujo, la demostración objetiva de la seguridad genética de la técnica, fue otro de los fines contemplados en este trabajo. Por tanto, y para finalizar el estudio se evaluó si realmente el fluorocromo por sí sólo, o en combinación con el impacto del láser, podría inducir un daño a nivel del ADN espermático produciendo un efecto mutagénico en los espermatozoides, que se manifestaría con un incremento en la

presencia de alteraciones genotípicas o fenotípicas en la descendencia. En la experiencia 5 se analizaron, en primer lugar, los parámetros reproductivos (porcentaje de partos y tamaño de camada) derivados de las inseminaciones realizadas con espermatozoides teñidos y con espermatozoides teñidos y sometidos al proceso de separación. En segundo lugar, se realizó un análisis fenotípico y genotípico de los de los lechones obtenidos. Con el fin de determinar si existe algún tipo de alteración en los parámetros analizados, que puede ser debida al efecto del Hoechst 33342 o la combinación del mismo con el láser, se compararon los datos con los obtenidos en un grupo control formado por inseminaciones realizadas con semen no teñido, ni procesado y por los lechones nacidos de estas inseminaciones.

Por todo lo anteriormente expuesto los objetivos fundamentales del presente trabajo fueron:

1.- Comprobar la utilidad de los espermatozoides de macho cabrío como herramienta en el alineamiento del citómetro de flujo previo al posterior análisis y separación de espermatozoides de verraco, así como determinar la diferencia en el contenido de ADN entre los espermatozoides X e Y de esta especie.

2.- Diseñar un procedimiento sencillo de hibridación in situ fluorescente directa en espermatozoides de verraco, mediante la fabricación de sondas de ADN especificas para cromosomas porcinos (1 e Y) marcadas mediante el método de desplazamiento de cortes (nick translation) y evaluar la eficiencia de este método de marcaje así como la utilidad de la hibridación in situ fluorescente directa como herramienta para la determinación de la eficiencia del proceso de separación.

3.- Conocer la influencia del tiempo de conservación en medio líquido sobre la motilidad, viabilidad, estado acrosomal y capacidad de penetración de ovocitos madurados *in vitro* de los espermatozoides de verraco sometidos al proceso de separación mediante citometría de flujo.

4.- Determinar la capacidad fecundante *in vivo* de los espermatozoides de verraco sometidos al paso por el citómetro de flujo y depositados en el tracto genital de la cerda mediante inseminación intrauterina profunda, así como definir las condiciones más adecuadas para la obtención de las mejores tasas de fertilidad tras la aplicación de los espermatozoides separados con este sistema de inseminación.

5.- Evaluar el posible efecto genotóxico del proceso de separación espermática por citometría de flujo sobre los espermatozoides separados de verraco mediante la valoración de la normalidad fenotípica y citogenética de los lechones nacidos tras las inseminaciones realizadas con espermatozoides separados.

2.-Revisión Bibliográfica

Poder determinar el sexo de la descendencia antes de la concepción, tanto en animales domésticos como en la especie humana, es una idea que ha ocupado la mente del hombre desde hace miles de años. Los primeros intentos realizados en este sentido se describen ya en la época de los antiguos filósofos griegos y estaban basados, como norma general, en la mitología así como en supersticiones y creencias populares. Beber vino y sangre de león en tiempo de luna llena o incluir carne roja y comidas amargas en la dieta de las madres que desearan tener un hijo varón, además de oraciones, danzas y una gran variedad de remedios secretos, eran algunos de estos métodos. Aún a finales del siglo XVIII, todavía existía la firme creencia de que la situación anatómica (lado izquierdo o derecho) de los testículos o los ovarios daba origen a esta diferencia de sexos (Gledhill, 1988). Estas técnicas iban destinadas fundamentalmente al hombre, pero dieron lugar a otras muchas que fueron aplicadas en las especies de interés ganadero. La falta de base científica y el fracaso sistemático eran el denominador común de todas ellas.

Las primeras evidencias científicas convincentes sobre la determinación sexual del individuo llegan en el siglo XX con el descubrimiento de los cromosomas sexuales y su papel principal en dicha determinación (Guyer, 1910). A partir de este momento los mayores esfuerzos para controlar el sexo de la descendencia se han dirigido siempre a la manipulación *in vitro* del espermatozoide antes de la fecundación.

2.1.- Métodos para la determinación del sexo de la descendencia

Debido al interés que despierta, las técnicas desarrolladas para la identificación y selección del sexo de la descendencia han sido numerosas. Según el momento en el que se realicen, estas técnicas pueden clasificarse en postimplantacionales (tras la implantación del embrión) y preimplantacionales (antes de la implantación embrionaria), las cuales a su vez pueden realizarse tras la fecundación o previamente a la misma. En este último caso se habla de predeterminación o preselección del sexo de la descendencia.

La identificación del sexo del feto mediante ultrasonografia es el método postimplantacional mas utilizado en gran variedad de especies. Sin embargo, presenta ciertas limitaciones ya que permite la identificación del sexo pero no la selección del mismo, a menos que se utilice el aborto selectivo. Además, es una técnica que aunque resulta de gran utilidad en especies monotocas (Fricke, 2002) presenta un interés nulo en especies politocas como es el caso del porcino. El cariotipado de células metafásicas del feto, obtenidas por amniocentesis o tras toma de muestras de las vellosidades coriónicas, es otra técnica que puede ser utilizada para la determinación del sexo tras la implantación, pero en este caso su principal aplicación es el diagnóstico de anomalías cromosómicas, fundamentalmente la trisomía del 21, en la especie humana (Larrabee y cols., 2004).

Otra alternativa para conseguir seleccionar el sexo de la descendencia es el análisis de los embriones antes de la implantación, cuyo principal inconveniente es la necesidad de la manipulación extracorpórea de los mismos para la toma de muestras mediante biopsia embrionaria (Handyside y cols., 1990). Este sexaje preimplantacional durante estadios tempranos del desarrollo embrionario es fundamental en humanos para el diagnóstico de enfermedades ligadas al sexo y para la detección de alteraciones a nivel cromosómico, permitiendo la selección y posterior transferencia únicamente de los embriones sanos (Kuliev y Verlinsky, 2002; 2004). Métodos inmunológicos, basados en la detección de antígenos específicos de individuos machos como es la

identificación del antígeno H-Y (Betteridge y cols., 1981; van Vliet y cols., 1989; Wachtel, 1984), cuantificación de enzimas íntimamente ligadas al cromosoma X (van Vliet y cols., 1989), análisis citogenéticos (Betteridge y cols., 1981; King, 1984; van Vliet y cols., 1989) y detección de secuencias específicas de cromosoma Y mediante PCR (Handyside y cols., 1989,1990; Liu y cols., 1994; Schroder y cols., 1990; Fajfar-Whetstone y cols., 1993) o hibridación in situ (West y cols., 1987; Leonard y cols., 1987) son algunas de las técnicas que se pueden utilizar para definir el sexo de los embriones en mamíferos, incluido el hombre. El análisis citogenético, así como la PCR o la hibridación in situ, son más precisas y además pueden realizarse sobre una muestra biopsiada del embrión, mientras este sigue su desarrollo *in vitro* o es criopreservado (Jericho y cols., 2003).

Realizar la predeterminación del sexo antes de la concepción (preselección del sexo) es el método más eficaz para la obtención de descendencia del sexo deseado (Johnson, 2000). Teniendo en cuenta que en la dotación cromosómica del ovocito está siempre presente el cromosoma X, resulta evidente que la determinación sexual del individuo depende del cromosoma X o Y que posea el espermatozoide que fecunde ese ovocito. El método más exacto, eficiente y potencialmente más rentable para predeterminar sexo de la descendencia pasa por la separación de los espermatozoides en función de su dotación cromosómica (presencia del cromosoma X o del Y) y su posterior utilización mediante diferentes técnicas de reproducción asistida (Johnson, 2000). Aunque a simple vista podría parecer que esta separación puede llevarse a cabo mediante estrategias sencillas, el hecho de que las diferencias fenotípicas entre los espermatozoides X y los Y sean mínimas (Seidel, 1999) dificulta sobremanera su identificación y selección.

2.2.- Preselección del sexo mediante separación espermática

La predeterminación del sexo de la descendencia mediante separación espermática agrupa a todos aquellos métodos que basándose en diferencias existentes

entre los espermatozoides X y los Y son capaces de modificar la proporción de individuos machos y hembras impuesta por la naturaleza. Según Jafar y Flint (1996), cualquier técnica de separación espermática debe cumplir tres premisas fundamentales: producir una desviación evidente en el ratio de espermatozoides X/Y de una población espermática, no interferir en la capacidad fecundante *in vivo* o *in vitro* de los espermatozoides separados y, por último, dar lugar a la obtención de descendencia (embriones o camadas) viva que confirme la desviación eficaz de la proporción de espermatozoides X e Y hacia uno u otro lado. El cumplimiento de las dos últimas premisas implica la necesidad de combinar estos procedimientos de separación espermática con técnicas como la inseminación artificial, la fecundación *in vitro* y/o la transferencia de embriones (Johnson y cols., 2005).

En los últimos 70 años han sido propuestos numerosos métodos para la separación de espermatozoides X e Y (Tabla 1), basados tanto en aspectos físicos de los espermatozoides (diferencias de peso, tamaño, motilidad, densidad e incluso de carga superficial existentes entre ellos), como en aspectos inmunológicos de los mismos (presencia del antígeno H-Y en la membrana plasmática del espermatozoide o la existencia de una proteína específica del sexo (sex specific protein SSPs)).Sin embargo, las experiencias realizadas por Johnson (1988) para comprobar la efectividad de estas técnicas demostraron que ni los métodos basados en las diferencias físicas entre los espermatozoides portadores del cromosoma X y los portadores del cromosoma Y, ni aquellos que se apoyaban en las diferencias existentes en la superficie espermática presentaban una efectividad aceptable.

Tabla 1: Diferencias existentes entre espermatozoides X e Y, utilizadas por diferentes autores como base para la separación espermática (modificado de Johnson, 1995)

Parámetro	Diferencia entre X e Y	Referencia
DNA	Mayor contenido de DNA en el espermatozoide X	Pinkel y cols.,1982; Moruzzi,1979 Johnson y cols., 1989; Johnson, 1991; Cran y cols., 1995
Tamaño	Mayor tamaño del espermatozoide X	Cui and Mathew, 1993; van Munster y cols., 1999a; van Munster y cols., 1999b van Munster, 2002
Motilidad	Mayor rapidez del espermatozoide Y	Ericcson y cols., 1973; Ericsson y Ericsson, 1999; Ollero y cols., 2000
Carga superficial	Espermatozoides portadores del cromosoma X migran al cátodo	Kiddy y Hafs, 1971; Kaneko y cols., 1984; Mohri y cols., 1987
Superficie del espermatozoide	Existencia del antígeno H-Y en espermatozoides portadores del cromosoma Y Presencia de una proteína específica del sexo (sex specific protein, SSPs)	Hendriksen y cols., 1993; Hoppe y Koo, 1984; Howes y cols., 1997. Hendriksen y cols., 1996; Blechery cols., 1999
F-body	Brazo largo del cromosoma Y	Barlow and Vosa (1970)

Aunque la existencia de los cromosomas sexuales era conocida desde principios del siglo XX (Guyer, 1910), la posibilidad de diferenciar los espermatozoides en función del cromosoma sexual que presentaran no fue vislumbrada hasta que Moruzzi (1979) demostró, mediante la observación de los cariotipos, la existencia de numerosas especies de mamíferos que poseían diferencias evidentes en la cantidad de ADN entre los espermatozoides portadores del cromosoma X y los portadores del cromosoma Y. Esta diferencia se debe al mayor tamaño que presenta el cromosoma X en relación al Y, y es la única diferencia cuantificable y válida desde un punto de vista científico como base para la separación efectiva de los espermatozoides X e Y (Johnson, 1998).

Por otro lado, si el objetivo perseguido no es predeterminar de forma exacta el sexo de la progenie sino provocar una ligera desviación en las proporciones de machos y hembras presentes en la descendencia, pueden utilizarse otros sistemas. En este sentido, se han realizado algunos estudios para intentar demostrar que adecuando el momento de inseminación al momento de ovulación, se podrían regular los porcentajes de machos y hembras obtenidos (Ballinger, 1970; Foote, 1977) y aunque en un primer momento esta técnica demostró no ser fiable, debido a que los resultados obtenidos no eran repetitivos, estudios más recientes (Rorie, 1999) utilizando sistemas más precisos para el control del ciclo reproductivo de las hembras han demostrado que el ratio de machos y hembras nacidos puede ser modificados en algunas especies mediante el ajuste de la inseminación en relación al momento de la ovulación, así como mediante tratamientos de sincronización de celo y de ovulación. La aparente capacidad que tienen muchas especies de regular el sexo de su descendencia según las necesidades del grupo, parece estar íntimamente ligada a esta relación entre la deposición de los espermatozoides y el momento de la ovulación (Rorie, 1999). Sin embargo, hasta el momento todas las observaciones realizadas han sido de naturaleza empírica careciendo en todo momento del necesario fundamento científico (Seidel y Johnson, 1999).

2.3.- Separación espermática mediante citometría de flujo en función del contenido en ADN

Predeterminar el sexo de la descendencia, utilizando espermatozoides separados en base a su contenido en ADN, es hoy en día una realidad en mamíferos, incluido el hombre, gracias a la citometría de flujo (Johnson, 2000).

Esta técnica se basa en la diferente fluorescencia que emiten los espermatozoides tras la tinción de su ADN debida, a su vez, a la diferente cantidad de ADN que existe entre el cromosoma X y el cromosoma Y (Pinkel et al., 1982; Seidel y Johnson, 1999; Vázquez y cols., 2001b).

Las diferencias entre los espermatozoides X e Y no son iguales en todas las especies, siendo de un 7'5% en el caso de las chinchillas y de, solamente, un 2'8% en el caso de los espermatozoides humanos. En el caso de las especies de interés productivo estas diferencias se encuentran entre estos límites siendo de un 3'6% en verraco, 3'7% en caballos, 3'8% en toros y 4'2 % en moruecos (Welch y Johnson, 1999). A medida que esta diferencia es mayor, la separación de los espermatozoides X e Y se ejecuta con mayor facilidad.

Los espermatozoides de las diferentes especies de interés en producción ganadera (porcino, bovino, ovino y equino) han sido separados mediante citometría de flujo en poblaciones X e Y, con purezas de alrededor de un 90 a 95%, para ser después utilizados en combinación con diferentes técnicas de reproducción asistida (Johnson, y cols., 1989; Johnson, 1991; Cran y cols., 1993, 1997; Catt y cols., 1996, Buchanan, 2000). La obtención de animales del sexo deseado de forma repetitiva entre diferentes laboratorios confirma la utilidad de la citometría de flujo como técnica para la separación y selección de espermatozoides X e Y en base a la diferencia en el contenido de ADN (Johnson y cols., 2005).

2.3.1.- Metodología de separación de espermatozoides X e Y mediante citometría de flujo.

A diferencia de otros procesos en los que están implicadas las técnicas de citometría de flujo, cuando se trabaja en separación de espermatozoides, este procedimiento complejo tiene siempre como doble objetivo el obtener espermatozoides separados en poblaciones X e Y manteniendo al mismo tiempo la viabilidad de los mismos para asegurar la fecundación in vitro o, preferentemente, in vivo.

El procedimiento de separación de espermatozoides X e Y mediante citometría de flujo puede dividirse en tres diferentes fases (Figura 1)

1.- Preparación y tinción de la muestra
2.- Análisis de la muestra y discriminación de las poblaciones de espermatozoides X e Y.
3.- Separación de los espermatozoides X e Y.

2.3.1.1 Preparación y tinción de la muestra.

Tras la obtención de los eyaculados, los espermatozoides deben ser diluidos en un medio que les asegure la viabilidad hasta el momento de la separación. Estos espermatozoides diluidos deben ser incubados durante 1 hora a 35°C con una solución del fluorocromo bis-benzimide Hoechst 33342 (H-42) el cual atraviesa las membranas espermáticas uniéndose, sin intercalarse, a la doble hélice de ADN de modo que emite más o menos fluorescencia en función de la cantidad de ADN que presenten los espermatozoides (Johnson y Schulman, 1994). El proceso de tinción es uno de los pasos críticos en el proceso de separación espermática por citometría de flujo, ya que cuando es realizado de manera correcta los espermatozoides presentan una tinción uniforme ayudando a la obtención de pequeños coeficientes de variación y a una

óptima diferenciación de las poblaciones de espermatozoides X e Y presentes en el eyaculado, incrementándose la eficiencia del procedimiento (Johnson, 2000). Después de la incubación es importante verificar al microscopio que la tinción es correcta así como que los espermatozoides presentan una motilidad similar a la que tenían antes de la incubación (Vázquez y cols., 2002).

2.3.1.2.- Análisis de la muestra y discriminación de las poblaciones de espermatozoides X e Y.

Tras la tinción de los espermatozoides, se procede al análisis para intentar discriminar dos poblaciones en base a la cantidad de ADN que presenten. Este procedimiento no fue viable hasta que en 1982, Pinkel y cols., demostraron que esta diferenciación era posible en núcleos de espermatozoides de ratón utilizando un equipo denominado citómetro de flujo. Posteriormente, Garner y cols. (1983) realizaron este mismo experimento utilizando espermatozoides de especies domésticas. En 1989, Johnson y cols., obtuvieron los primeros nacimientos en conejos utilizando esta tecnología.

El citómetro de flujo es un equipo altamente sofisticado, cuya función es la de medir células, las cuales pueden ser analizadas en base a sus características físicas (tamaño, complejidad celular) o en base a la fluorescencia que emiten tras ser marcadas con un fluorocromo.

Los componentes básicos de un citómetro son el circuito de admisión de la muestra, el haz de luz láser que excitará el fluorocromo, la cámara de flujo o lugar de encuentro entre la célula y el láser, y el bloque óptico cuyo objetivo es el de recoger la fluorescencia emitida por la célula y de este modo poder cuantificar esa emisión y analizar la célula en función de la misma. Los resultados de fluorescencia obtenidos son analizados mediante diferentes programas informáticos y son representados en

forma de poblaciones obedeciendo a sus características de fluorescencia (Shapiro, 2003).

El mayor obstáculo que se presenta en la separación de espermatozoides, además de la pequeña diferencia en la cantidad de ADN que existe entre el cromosoma X y el Y, es la morfología espermática (Gledhill y cols., 1976; Van Dilla y cols., 1977; Pinkel y cols., 1979). Los espermatozoides son células planas que van a emitir una fluorescencia diferente en función de la posición en la que reciban el impacto del láser. Esto hace que la diferente intensidad de fluorescencia que el espermatozoide emite según la posición en la que interseccione con el haz de luz ultravioleta sea mayor que la diferencia de fluorescencia emitida por el mismo en función de que transporte un cromosoma X o un cromosoma Y, resultando imposible la identificación de forma correcta de las poblaciones de espermatozoides X e Y presentes en los eyaculados (Gledhill y cols., 1976). Por tanto, es necesario hacer un primer análisis de posición del espermatozoide para, simultáneamente y sólo en aquellos espermatozoides que pasan en una posición determinada a través de la cámara de flujo, analizar la cantidad de ADN de su interior y de este modo poder diferenciar espermatozoides X e Y. Este doble análisis obliga a modificar los citómetros convencionales, siendo necesaria la instalación de un fotomultiplicador frontal (0° respecto al impacto del láser) capaz de recoger fluorescencia (Johnson y Pinkel, 1986), en sustitución del detector de luz estándar que incorporan los citómetros de flujo de forma habitual en esta posición. Situado lateralmente (90° respecto al láser) se ubica otro fotomultiplicador presente en todos los citómetros cuya función es la de discriminar entre espermatozoides orientados/no-orientados mientras que el frontal servirá para discriminar entre poblaciones de espermatozoides X e Y. Aunque de este modo era posible diferenciar, dentro de los espermatozoides orientados, las poblaciones de espermatozoides X e Y en los primeros equipos utilizados, el porcentaje de células espermáticas orientadas de forma adecuada era extremadamente bajo (Pinkel y cols., 1982). Por este motivo, se hace necesario incorporar sistemas que permitan incrementar el porcentaje de espermatozoides orientados que serán analizados.

El primer dispositivo diseñado con este fin consistía en la inyección de las células espermáticas a través de una aguja biselada que le daba al chorro de muestra una forma plana (Figura 2a) y permitía obtener porcentajes de orientación alrededor de un 25-30% (Johnson y Pinkel, 1986). La incorporación del fotomultiplicador frontal y de la aguja biselada junto con el protocolo de tinción con Hoechst 33342 dieron lugar al desarrollo de una técnica de separación espermática por citometría de flujo en base al contenido de ADN ("Beltsville Sperm Sexing Technology) que hizo posible la obtención de camadas del sexo deseado tras la aplicación de estos espermatozoides en inseminación artificial (Johnson y cols., 1989).

Posteriormente y en la misma línea, Rens y cols. (1998) desarrollaron un sistema pensado exclusivamente para la separación espermática y destinado a mejorar el número de espermatozoides orientados con el objetivo final de aumentar la eficiencia del proceso de separación. En este caso, se modificó una cámara de flujo de las llamadas de chorro al aire ("jet in air") dándole una forma elíptica a los extremos (de entrada y de salida) de la misma, lo que determina la aparición de unas fuerzas en el fluido que transporta la muestra provocando la orientación correcta de los espermatozoides (Figura 2b) y pudiendo llegar a incrementar el porcentaje de espermatozoides orientados hasta el 70% (Rens y cols., 1998). Este incremento trajo consigo un aumento significativo en el rendimiento de los citómetros convencionales adaptados a la separación espermática, ya que permitía la obtención de 800.000 espermatozoides separados por hora en lugar de los 350.000 obtenidos en las primeras aplicaciones de la técnica (Rens y cols., 1999). Sin embargo, el paso definitivo hacia la optimización de los rendimientos de los equipos de separación espermática llegó con la adaptación del dispositivo orientador diseñado por Rens y cols. (1998) a los citómetros de alta velocidad (Peters y col., 1985; van den Neg y Stokdijk, 1989). El elevado porcentaje de espermatozoides orientados en combinación con la gran capacidad de separación de los sistemas de alta velocidad y ciertas mejoras introducidas en los dispositivos electrónicos de los equipos han permitido aumentar los rendimientos de la técnica, hasta alcanzar 9 millones de espermatozoides separados por

hora para cada una de las poblaciones (X e Y) manteniéndose las purezas alrededor del 90% en ambos casos (Seidel, 2003).

El análisis de los espermatozoides se produce en el momento de la intersección de estos con el láser. Sólo los resultados de los análisis correspondientes a los espermatozoides orientados quedan reflejados en un histograma donde aparece, a la derecha, la población más fluorescente, es decir, aquella que corresponde a espermatozoides X y, a la izquierda, una población menos fluorescente que corresponde a espermatozoides Y quedando entre ambas una hendidura que las separa. En función de que esta hendidura sea más o menos pronunciada se podrá seleccionar, para su separación, una población más o menos grande. Los análisis que dan lugar a histogramas con hendiduras pequeñas, como es el caso del porcino, obligan a seleccionar una población pequeña ya que gran parte de la misma quedará ubicada en la denominada "zona de confusión", un área común compartida entre las poblaciones de espermatozoides X e Y (Figura 3).

2.3.1.3.- Separación de los espermatozoides X e Y. Eficiencia y rendimientos.

Las células que pasan por el citómetro de flujo se encuentran suspendidas en el chorro de muestra y son conducidas hasta el lugar de impacto con el láser (lugar de análisis) envueltas por un medio salino compuesto comúnmente por PBS conteniendo EDTA y un 1% de Albúmina sérica bovina (Johnson y cols., 1989). Una vez realizado el análisis, y debido a la vibración a alta velocidad de la cámara de flujo, se produce la división del chorro de muestra en numerosas gotas pequeñas y uniformes (microgotas) que transportan las partículas analizadas de forma individualizada. En este punto y conforme las gotas van cayendo, se activan los circuitos de carga y cada una de las gotas es cargada eléctricamente según el espermatozoide que transporte. Tanto las gotas cargadas como las no cargadas pasan a través de un campo electrostático (alrededor de 2000V) existente entre las placas de alto voltaje (placas deflectoras) con las que va equipado el citómetro. Las gotas que transportan un espermatozoide X serán cargadas positivamente y desviadas hacia el polo negativo de estas placas,

mientras que las gotas portadoras de un espermatozoide Y serán cargadas negativamente y desviadas hacia el polo positivo. Aquellas gotas atraídas hacia uno u otro polo y por tanto portadoras de uno u otro tipo de espermatozoide serán recogidas en tubos mientras que aquellas gotas que no transportan ninguna partícula, o que por otro lado envuelven una partícula que no cumple las condiciones definidas para la población seleccionada, no recibirán carga de ningún tipo y serán descartadas (Johnson, 1992. Ver figura 1)

Los tubos en los que se recogen los espermatozoides separados incluyen un medio llamado "TEST-yolk" (Johnson y cols., 1989), que estabiliza las membranas espermáticas y amortigua el impacto de los espermatozoides contra el fondo del tubo, lo cual es necesario ya que las microgotas impactan sobre la superficie del tubo a más de 100 Km./h. Este medio está compuesto por una solución de Test- Tris-Glucosa a la que se le añade un porcentaje variable de yema de huevo que oscila entre el 2 % y el 20 % del volumen final y plasma seminal (desde el 1% al 10%) o fracciones proteicas del plasma seminal. Sobre estos tubos se suele recoger 9 veces el volumen del medio de recogida depositado.

La velocidad de separación depende, entre otras cosas, de la presión a la que se haga pasar la muestra a través del citómetro. Aunque la presión normal de trabajo es de 12 p.s.i., los nuevos sistemas de separación van equipados con los módulos "hypersort" que permiten incrementar esta presión hasta 100 p.s.i. Hasta el momento, se ha comprobado que presiones de trabajo de hasta 62 p.s.i. no afectan a la viabilidad de la célula espermática (Parrilla et al. 2001).

Por último, es necesario señalar que la eficiencia del proceso de separación espermática mediante citometría de flujo debe ser evaluada teniendo en cuenta dos factores muy relacionados entre sí, como son el número de espermatozoides separados por unidad de tiempo (rendimiento) y la pureza de la población separada. Ambos factores dependen casi exclusivamente del porcentaje de espermatozoides que presenten una orientación correcta (Rens y cols., 1999). Algunos estudios recientes (Seidel y Garner, 2002; Maxwell y cols., 2004) aseguran que dependiendo de factores como el macho donante de espermatozoides, el eyaculado utilizado, incluso de la

habilidad del técnico que maneja el equipo, sólo aproximadamente un 30 % de los espermatozoides presentes en una muestra son separados, mientras que el resto se pierden antes, durante y después de la separación. En la actualidad se pueden obtener unos rendimientos de 10-15 millones de espermatozoides separados por hora, con purezas del 90%(Johnson y Welch, 1999).

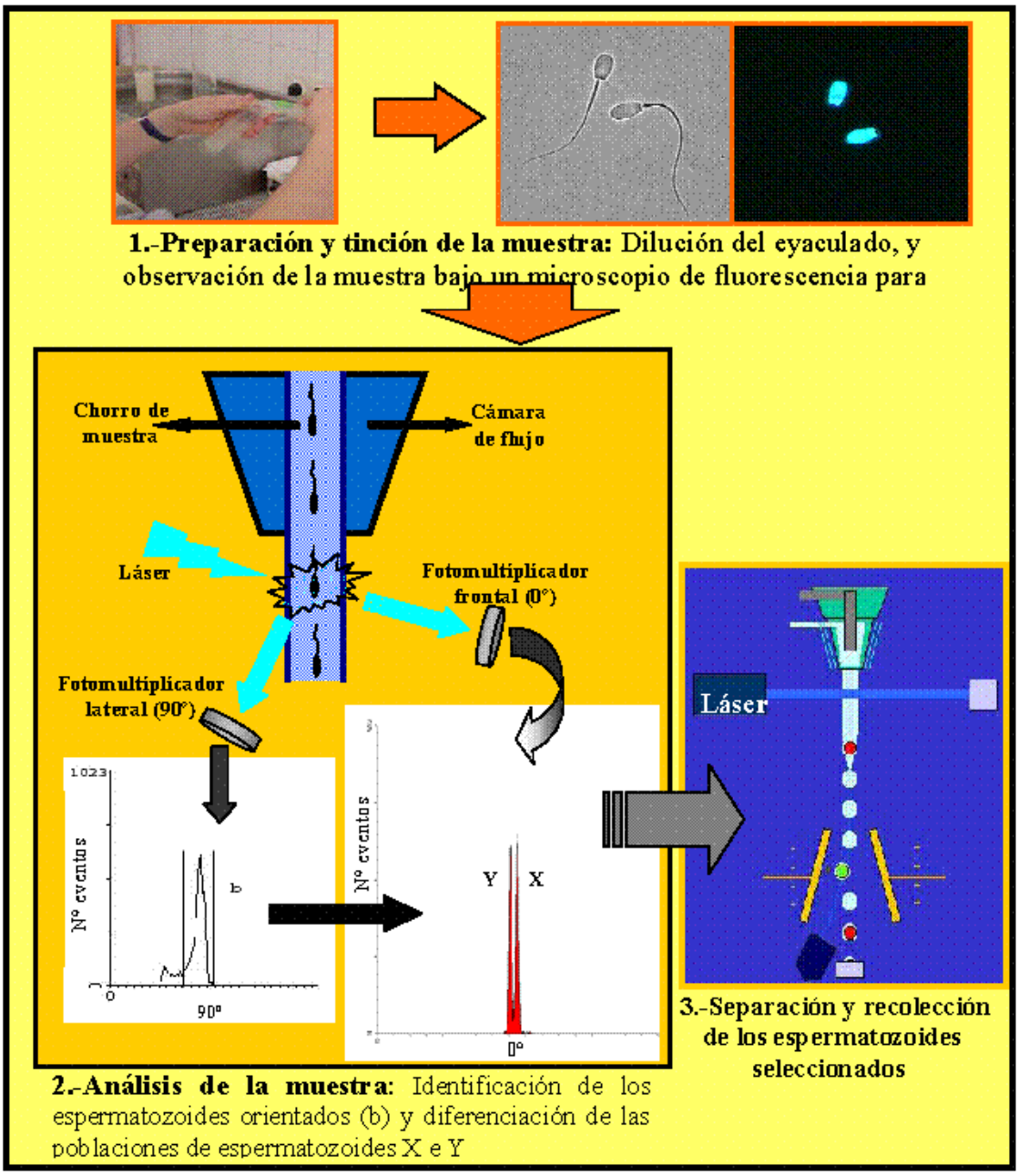

Figura 1: Esquema general del proceso de separación

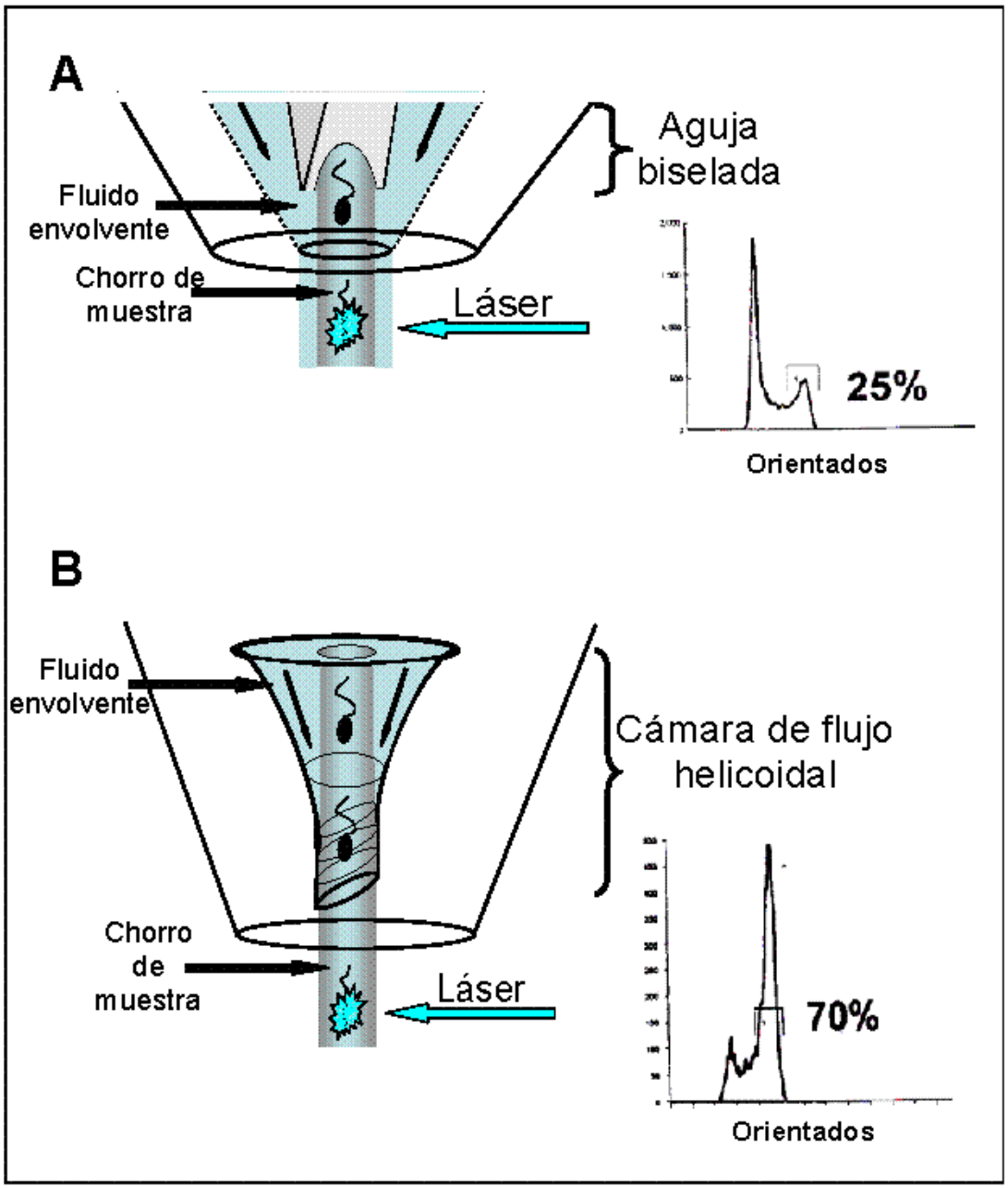

Figura 2: Diagrama de la aguja biselada (A) y de la cámara de flujo helicoidal (B) mostrando la posición de un espermatozoide correctamente orientado respecto al láser. Así como el porcentaje de espermatozoides orientados en cada uno de los casos.

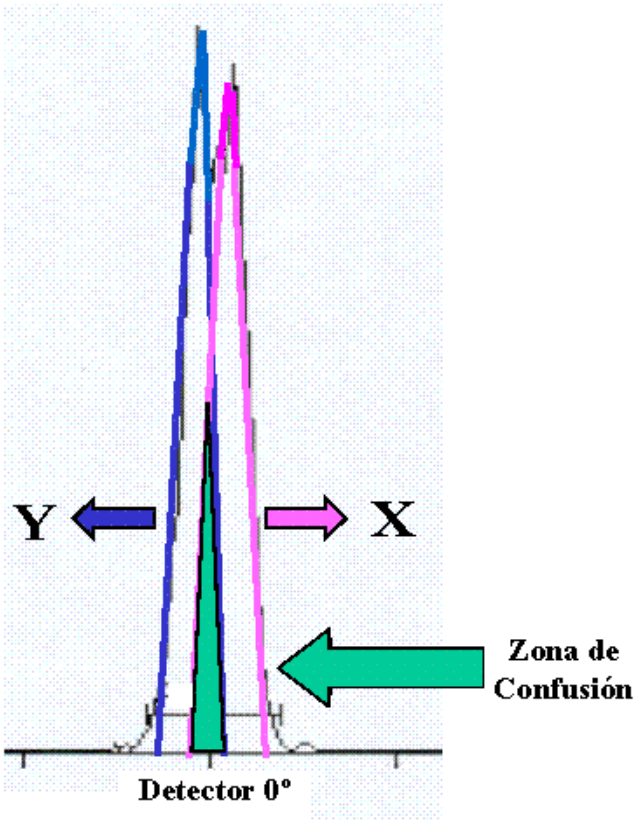

Figura 3: Histograma correspondiente a las poblaciones X e Y, mostrando la zona de incertidumbre o confusión.

2.4.- Situación actual de la técnica

La separación espermática por citometría de flujo, en base a la diferencia en el contenido de ADN existente entre los espermatozoides portadores del cromosoma X y los portadores del cromosoma Y, es actualmente el único método fiable y eficaz para la obtención de descendencia del sexo deseado (Maxwell y col., 2004).

Desde las primeras aplicaciones de esta técnica, se han realizado continuas mejoras e innovaciones tanto en los protocolos como en el equipamiento de los citómetros utilizados, resultando en la obtención de animales de sexo deseado en diferentes especies. Aún así, y como corresponde a toda técnica nueva y en vías de desarrollo, todavía existen una serie de aspectos limitantes.

A pesar de que con las mejora en el procedimiento de separación así como en lo medios de recogida, los porcentajes de espermatozoides viables separados se han incrementado notablemente, la utilización de diferentes técnicas para evaluación de la viabilidad, motilidad o estado a acrosomal de los espermatozoides separados (Maxwell y Johnson, 1997; Centurión y cols., 2000), ha demostrado que éstos, en las condiciones y con los medios actuales, son células inestables, en un estado de capacitación o precapacitación debido al estrés del procedimiento y, sobre todo, a la alta dilución a la que se ven sometidos. Una de las mayores limitaciones de este método es la reducida vida útil que presentan los espermatozoides tras el proceso de separación, determinando que su viabilidad y capacidad fecundante en el tracto genital femenino se vea también disminuida. El diagnóstico del momento de la ovulación mediante ecografía transrectal y la realización de las inseminaciones lo más próximas posibles al mismo, para así disminuir el tiempo que los espermatozoides permanecen almacenados en el tracto genital femenino, se hacen necesarios como pasos fundamentales en los protocolos de inseminación con semen sexado (Maxwell y cols., 2004). Otra de las grandes necesidades del momento actual es el desarrollo de nuevos sistemas para la evaluación del efecto que el proceso de separación tiene sobre el espermatozoide. Un estudio detallado de estos efectos implicaría el estudio de cambios espermáticos a nivel molecular, incluyendo la detección de cambios en la arquitectura de la membrana plasmática (Gillan y cols., 2004).

Los rendimientos de los citómetros actuales son mucho mayores que los obtenidos en un principio, pero en el caso concreto de la especie porcina siguen siendo insuficientes para la utilización de los espermatozoides separados en inseminación artificial, haciendo necesaria la combinación de esta tecnología de separación, con técnicas, como la fecundación *in vitro* (FIV) y la transferencia de embriones (ET)

(Rath y cols., 1997,1999). Aunque un estudio reciente describe la obtención de lechones tras inyección intracitoplasmática (ICSI) de espermatozoides separados y posterior transferencia de embriones (Probst y Rath, 2003), los protocolos de fecundación *in vitro* y transferencia embrionaria en combinación con la separación espermática todavía necesitan ser mejorados en la especie porcina para resultar útiles desde un punto de vista comercial (Maxwell y cols., 2004). Sólo un procedimiento no invasivo de inseminación uterina profunda (DUI: deep uterine insemination) o de transferencia de embriones sería aceptable actualmente, tanto desde un punto de vista económico como de bienestar animal para la aplicación de espermatozoides sexados en esta especie.

Resultados altamente satisfactorios han sido descritos por numerosos autores tras inseminación uterina profunda en diferentes especies (Bovino: Seidel y cols., 1997, 1999a y b; Ovino: Cran y cols., 1997; Hollinshead y cols., 2002; Equino: Lindsey y cols., 2002a,b; Morris y cols., 2002), a pesar de lo cual la producción *in vitro* de embriones a partir de ovocitos de hembras jóvenes o adultas se perfila en la actualidad como el método más prometedor para generar descendencia con semen sexado en la mayoría de estas especies (Maxwell y cols., 2004).

La criopreservación de espermatozoides separados ha sido y está siendo también utilizada con éxito en diferentes especies de mamíferos domésticos obteniéndose descendencia tras la descongelación e inseminación de los mismos (Bovino: Schenk y cols., 1999; Seidel y cols., 1999b; Porcino: Johnson y cols., 2000a; Ovino: Hollinshead y cols., 2001; Equino: Lindsey y cols., 2002a). En este sentido recientemente (Hollinshead y cols., 2003; O' Brien y cols., 2004) se ha descrito la obtención, *in vitro*, de embriones viables en ganado ovino a partir de espermatozoides previamente congelados, los cuales fueron descongelados sometidos al proceso de separación y congelados de nuevo para ser posteriormente descongelados y utilizados en FIV. La posibilidad de poder separar espermatozoides congelados-descongelados y volverlos a congelar hasta el momento de su uso, facilitaría la aplicación de la tecnología de separación espermática en aquellos casos en los que esta podría verse limitada por la distancia entre los equipos y los animales (Maxwell y cols., 2004), ya

que permiten un transporte adecuado de los mismos, antes y después de la separación, manteniendo su viabilidad y capacidad fecundante.

Si bien en el caso del vacuno, la experiencia en el proceso de congelación, la congelabilidad del espermatozoide, y la disposición anatómica de los cuernos uterinos de la vaca han permitido el nacimiento de terneros tras inseminación artificial con espermatozoides separados y congelados, en el caso del porcino sólo se han conseguido nacimientos tras la inseminación quirúrgica de las cerdas con espermatozoides de verraco separados y congelados (Johnson y cols., 2000). Sin embargo, los resultados obtenidos actualmente en congelación de espermatozoides de verraco (Roca y cols., 2004; Carvajal y cols., 2004; Eriksson y cols., 2001) y la utilización con éxito de sondas de inseminación intrauterina profunda en la cerda (Martínez y cols., 2001a y b, Martínez et al., 2002) determinan que la combinación de las tecnologías de separación y congelación espermática, junto con la aplicación de los espermatozoides resultantes mediante procedimientos no quirúrgicos representen una herramienta fundamental para incrementar la eficiencia de esta técnica en producción porcina.

Sin duda y gracias a los continuos avances en la instrumentación utilizada, así como en la manipulación de los espermatozoides separados, la eficacia del proceso se verá incrementada. Este aumento de la eficacia junto a las mejoras en los sistemas de aplicación de los espermatozoides separados, podría suponer un gran paso en la aplicación práctica de esta tecnología de manera generalizada en las diferentes especies.

Experiencia 1

Flow cytometry identification of X- and Y-chromosome-bearing Goat Spermatozoa

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1.1 Abstract

Flow cytometric sorting technology was used to measure the difference in DNA content between X-and Y- chromosome bearing spermatozoa in bucks. Spermatozoa were analysed by flow cytometry to characterise X- and Y-chromosome bearing sperm populations and to quantify the DNA difference between them. Two symmetrical, overlapping and clearly separated peaks, corresponding to X- and Y-bearing spermatozoa, were detected. The difference in fluorescence intensity between peaks was 4.4 ± 0.03% without significant variations inter- or intra-animal detected. Therefore, the identification and selection of high purity samples of sperm populations for sex-sorting is easier in bucks compared to other domestic species.

1.2 Introduction

Sexing mammalian spermatozoa for the production of offspring with the desired sex is one of the most important new biotechnologies available for livestock producers. Such strategy allows various options when seeking the improvement of efficiency in production (Johnson 2000). For instance, predetermination of sex for breeding would reduce costs by decreasing the number of animals required (Seidel, 2003). To date, the only reliable procedure to separate X- and Y- chromosome bearing spermatozoa –as validated in numerous laboratories- is flow cytometry/cell sorting for DNA sperm content (Johnson 2000).

The DNA difference between X and Y spermatozoa varies among species being, for farm animals, in the range of 3.5% to 4.5% (Johnson 1995). A clear identification of the two sperm populations to sort depends on this DNA difference which conditions the size and correct setting of sorting gates and thus, the final output of the process (Vazquez et al 2001).

Although sperm sexing technology has demonstrated its effectiveness for many species (Seidel and Garner 2002), no data is yet available about DNA content in goat spermatozoa. Taking into account that the isolation of recombinant proteins from transgenic milk goats has attracted interest in the last few years (Balsadarre et al. 2003), sexing technology could be of great interest for this kind of biotechnological research.

The aim of this paper was to determine the difference in DNA content between X- and Y-chromosome bearing goat spermatozoa.

1.3 Material and Methods

Semen samples were weekly collected via artificial vagina from 6 fertile Murciano-Granadina bucks (between 18 and 36 months old) and, following immediate evaluation of normality (motility >80%; viability>85%; total spermatozoa per ejaculate>$3x10^9$; acrosomal abnormalities<10%; abnormal spermatozoa morphology<15%), extended in Beltsville Thawing Solution to a final concentration of $150x10^6$ spermatozoa/ml. The extended semen was then processed for sperm sorting following the general procedure described by Johnson et al. (1989). Briefly, one ml of extended spermatozoa were stained with Hoechst-33342 fluorophore (22.4 μM, Sigma Chemical Co., Alcobendas, Madrid, Spain) and incubated for at least 2 h at 35°C. The stained spermatozoa were passed through a modified EPICS Altra flow cytometer/cell sorter (Coulter Corporation, Miami, FL. USA) operating at 42 p.s.i. The fluorophore loaded spermatozoa were excited with 120 mW of ultraviolet (UV; 351, 364 nm) lines of a 6-W 90-6 Innova Laser (Coherent Lasers, Inc., Palo Alto, CA). Two UV blocking filters (460 and 450 nm band passes for 0° and 90° detectors, respectively) were used. The fluorescence emitted by the spermatozoa was collected from both the 0 and the 90 degree detectors and stored as frequency distributions (histograms). A minimum of 100,000 spermatozoa per sample was recorded in duplicate. The histograms were fitted

to a pair of Gaussian distributions, whose means and relative areas were adjusted to give the best least squares fittings to the data.

The relative areas of the two peaks obtained were assumed to give the proportion of X- and Y- spermatozoa in a sample, with the separation of the fluorescence intensity indicating differences in the relative content of DNA of the X- and Y-chromosome bearing sperm population. The experiments were individually performed with spermatozoa collected from 6 bucks during 8 consecutive weeks. Each ejaculate sample was run in duplicate. The percentage of separation (%) of the two peaks was calculated by the formula: $\%= 100\ (x-y)/0.5\ (x+y)$, where **x** and **y** were the respective means for the two peaks. The data corresponded to the mean ± SEM of the percentage of separation. Analyses of variance (ANOVA) were carried out using the general linear model procedure implemented in the SPSS 11.5/PC statistical package (SPSS, Inc., Chicago, IL, USA).

1.4. Results

A biparametric histogram representing the fluorescence detected by the 90° detector and the fluorescence directed to the 0° detector (Figure 1) was used to identify the properly stained sperm population. Only those spermatozoa falling within gate A (Figure 1), which represented about 90% of the total spermatozoa, were analysed using the signal gathered by the 90° detector (Figure 2). This figure shows the best orientated population on the right of the curve (between vertical lines). This well-orientated population represented between 60 and 70% of the total of spermatozoa in all samples analysed. These clearly orientated spermatozoa were used for the accurate measurement of DNA content by the 0° detector (Figure 3). Here, two distinct minimally overlapping peaks were visible, corresponding to the X (right peak) and Y (left peak) -chromosome bearing sperm populations. The analyses of the means of the intensity of fluorescence of these X-Y peaks revealed they differed by 4.4 ± 0.034% of DNA content. No significant variation ($p>0.05$) were seen within male (among

ejaculates) or among males. The proportions of X- and Y-chromosome bearing spermatozoa were close to the expected 50:50 in all analysed samples.

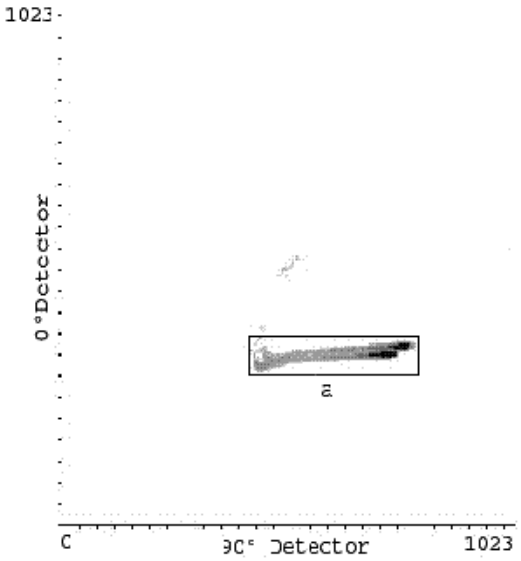

Figure 1. Density plot of 90° vs. forward fluorescence detector (0°) for goat male spermatozoa stained with Hoechst 33342. Only the spermatozoa falling within the gate **a** (about 90%) were considered properly orientated.

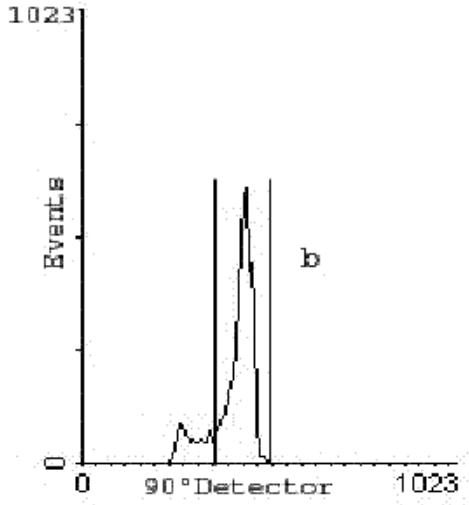

Figure 2. Histogram pattern of signals obtained by the 90° detector of the gated region a in Figure 1. Selected oriented population represents approximately 70% of the total analysed spermatozoa.

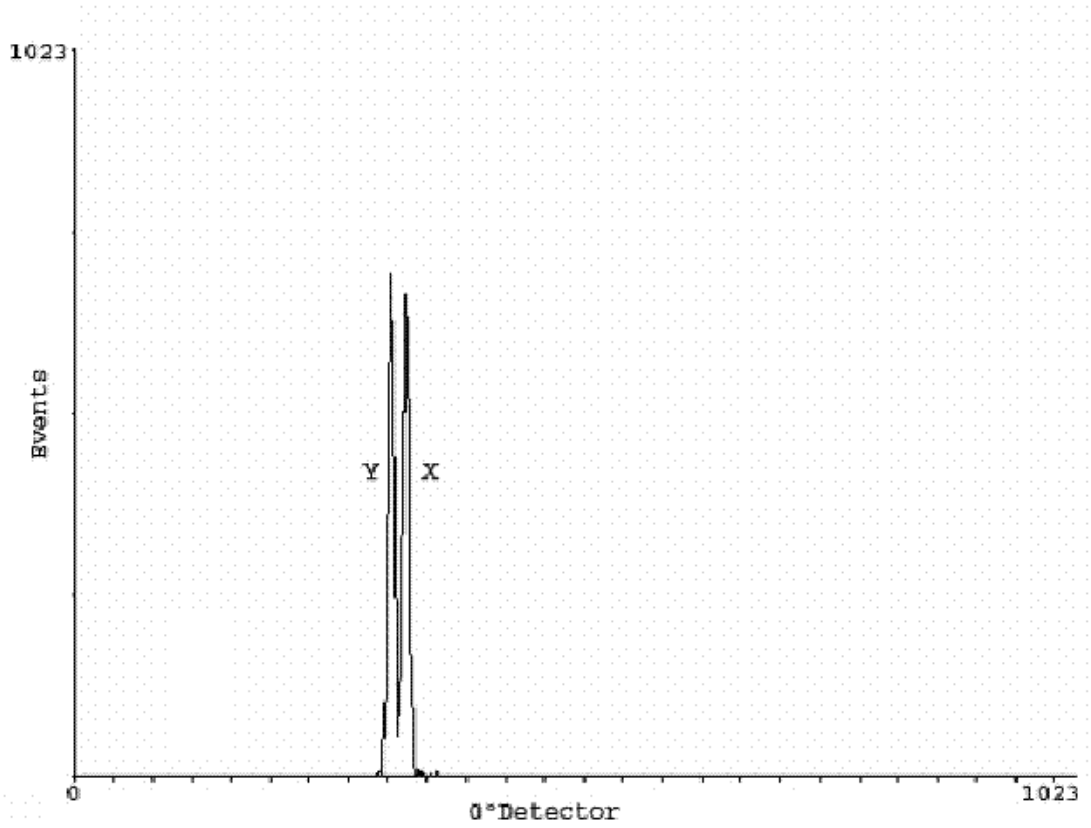

Figure 3: Histogram pattern of signals obtained by the forward fluorescence (0°) detector of the gated region b in Figure 2. The presumptive X and Y populations are clearly visible.

1.5. Discussion

The present analyses established for the first time the difference in DNA content between X- and Y-chromosome bearing buck spermatozoa. The difference in DNA content detected (4.4%) is close to the values established for ram spermatozoa (4.2%, Johnson and Pinkel 1986). Such a difference provides histograms with peaks

separated by a deep split, clearly resolving both X- and Y- bearing sperm populations. The depth of the split between the X and Y peaks gives an indication of how easy is to sort highly pure sperm X and Y populations (Johnson 2000). Consequently, high sorting rates with good accuracy of sorting, both parameters of utmost importance for the optimality of the efficiency of sorting, can be expected for goat spermatozoa. Moreover, the difference in the content of DNA between X- and Y- buck sperm populations was not significantly different ($p>0.05$) between males of the same breed, as it occurs among bulls (Garner et al. 1983), albeit only a breed was evaluated in the present experiments. Further experiments are needed to disclose eventual differences among breeds.

The practical application of sexing spermatozoa, synergistically with other reproductive techniques, could improve the efficiency of goat production both in biological and economic terms. Milk from transgenic goats produced by somatic cell nuclear transfer (SCNT) technologies appears to be a very attractive source of pharmaceuticals ("gene-pharming") mostly owing to the low frequency of failure compared to other species (Baguisi et al., 1999). For this reason, the use of X-chromosome bearing spermatozoa populations should be a useful tool in the application of the transgenic technology (Seidel, Jr., 2003). Finally, the wide difference in DNA-content shown between X and Y spermatozoa makes possible the use of these spermatozoa for checking the correct alignment of any flow cytometer as a first step before starting any sorting procedure even when spermatozoa from other species are to be sorted or analysed.

In conclusion, the rather large difference in DNA content present between X- and Y- chromosome bearing buck spermatozoa allows a clear identification of these two sperm populations, doing possible an accurate and with optimal output rates spermatozoa sorting, thus making the technology attractive for goat breeders as well as for transgenesis research.

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Experiencia 2

Fluorescence *in situ* hybridization in diluted and flow cytometrically sorted boar spermatozoa using specific DNA direct probes labelled by nick translation

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2.1 Abstract

Successful evaluation of X- and Y- chromosome bearing sperm separation technology using flow cytometry-cell sorter is of great importance. Fluorescence *in situ* hybridization (FISH), which allows for the detection of specific nucleic acid sequences on morphologically preserved spermatozoa, is an ideal method for quantitatively and qualitatively assessing the purity of sorted sperm samples. In this study specific porcine DNA direct probes for small regions of chromosomes 1 and Y were used. Chromosome 1 was labelled in green and used as internal control to detect a lack of hybridization, whereas chromosome Y was labelled in red. Nick translation was used as the labelling method for the preparation of these probes. Spermatozoa, unsorted and sorted for high and low Y-chromosome purity from ejaculates of five boars were fixed on slides and two-colour direct FISH was performed for chromosome 1 and Y. About 500 non-sorted and 200 sorted spermatozoa per sample were scored. The proportion of Y-chromosome-bearing spermatozoa was determined by the presence of red fluorescent signal on the sperm head and the proportion of X-chromosome-bearing spermatozoa was determined by subtraction. The efficiency of the hybridization procedure was established as near 98 % on sorted and unsorted samples. The results of this study confirm that direct FISH using specific pig DNA probes labelled by nick translation provides a useful tool for laboratory validation of sperm separation by flow sorting technology. Moreover, the ease of nick translation and the quality of the fluorescent signal obtained using this method makes this procedure the most appropriate method for labelling pig DNA probes to be used for direct FISH on pig spermatozoa.

2.2 Introduction

The sex of offspring can be predetermined with variable precision using a flow cytometric sperm sorting technique. This method is based on the differential amount of DNA present in X- and Y-chromosome bearing spermatozoa (Johnson and Pinkel, 1986). This difference can be measured by the use of a modified flow cytometer-cell sorter which is able to sort into populations enriched for X- or Y-chromosome-bearing spermatozoa with a high percentage of purity (Johnson et al., 1989; Johnson, 1991; Johnson, 1997; Seidel and Garner, 2002). The current production rate using this technology is 10-15 x 10^6 X- and Y-chromosome-sorted spermatozoa per hour (Johnson and Welch, 1999).

Determination of the resulting sex ratio before inseminating the sorted spermatozoa is essential to make this reproductive technique economically feasible. The sex of the offspring depends on the purity of the sorted spermatozoa in terms of X: Y-chromosome sperm ratio and, in turn, the purity of the sorted spermatozoa depends on the width of the sorting area setting (Johnson, 1997; Seidel and Garner, 2002). Unfortunately, the production rate also depends on the sorting windows; increasing the size of the sort windows by 15%, allows about a 20% increase in the rate of sorting spermatozoa to be achieved; however, the purity of the sorted population defined as the percentage of spermatozoa bearing the selected chromosome (X or Y) decreases by 10% (Johnson, 1997). Thus, the purity of the sorted spermatozoa decreases as production rate increases during the sorting. Although a high purity with a low production rate can be selected when sorted spermatozoa are used for IVF or intracytoplasmic sperm injection, a high production rate is needed for deep intrauterine insemination with sorted spermatozoa in sows (Vazquez et al., 2003). Therefore, the use of a wide sorting window is necessary during the sorting with consequent detrimental effect on the purity of the sperm population.

Since the introduction of sperm-sexing technology, several laboratory validation methods have been developed for verifying the purity of sorted X- and Y-chromosome bearing spermatozoa. The sort re-analysis method described by Johnson et al. (1987; 1989) involves the re-analysis of spermatozoa that have already been sorted flow cytometrically. As this method relies on the same instrumentation that produced the original sperm separation, it is not truly independent. Besides, when the difference in DNA content between X- and Y- chromosomes is small, as in human spermatozoa, sort re-analysis cannot be performed with the required accuracy (Johnson et al., 1993; Fugger et al., 1998).

Molecular biology offers various techniques for assessing the effectiveness of X- and Y-chromosome separation. PCR DNA amplification of single cells has been used to assess the purity of the sorted spermatozoa (Welch et al., 1995). However the labour required to conduct PCR of single cells likely prohibits its use for the routine monitoring of sorted sperm.

Fluorescence *in situ* hybridization (FISH) has also been used to analyse sorted spermatozoa. Kawarasaki *et al.* (1998) showed that two-colour FISH using a combination of DNA probes specific for chromosomes Y and 1 was useful for estimating the purity of X and Y sperm cells sorted by flow cytometry. This method offers the opportunity to use an independent technique for validating sorted sperm samples quantitative and qualitatively (Welch and Johnson, 1999).

The most important reagents on a FISH procedure are the fluorescent DNA probes. After or during DNA amplification steps by PCR, specific DNA must be labelled by incorporating fluorescent nucleotides. It is important to use a labelling method that allows the production of probes with good specificity that are easy to visualise to produce optimal hybridization results. The type of probe and labelling method should be chosen according to the sensitivity requirements of the situation.

The FISH technique has been performed on bull (Hassane et al., 1999; Piumi et al., 2001) and boar spermatozoa (Kawarasaki et al., 1995; 1996), and boar and cattle sperm flow sorting has also been evaluated by indirect FISH probes (Kawarasaki et al.,

1998; Rens et al., 2001). However, no results have been described using direct probes for evaluating the purity of X- and Y- chromosome sorted spermatozoa.

Nick translation reaction is an enzymatic labelling method (Rigby et al., 1977) used for incorporating nucleotide analogues (Langer et al., 1981). This method is frequently used for labelling DNA probes on human cytogenetical analysis. Piumi et al. (2001) used this technique to prepare an indirect fluorescent probe for X chromosome detection on bovine sperm. In this procedure one strand of the double-stranded DNA is nicked with deoxyribonuclease (DNase I). Subsequently, a 5'-3' exonuclease, DNA polymerase I, extends the nicks to gaps and the polymerase replaces the excised nucleotides with fluorescent-labelled substitutes on the basis of the complementary strand.

The aim of the present study was to determine an easy procedure for direct FISH on boar spermatozoa using nick translation-labelled pig DNA direct probes specific for small regions of chromosomes 1 and Y. The efficiency of the nick translation reaction was evaluated by performing a FISH assay on pig spermatozoa that were unsorted and sorted for high and low purity. The advantages and disadvantages of this procedure were analysed.

2.3. Material and Methods

All reagents used in this study were provided by Sigma-Aldrich Co. (Alcobendas. Madrid. Spain), unless otherwise stated.

2.3.1. Preparation of DNA direct probes labelled by nick translation

2.3.1.1. PCR amplification of 1- and Y-chromosome specific fragments

Pig chromosomes 1- and Y -specific primers were designed according to oligonucleotide sequences described by Rubeš et al. (1999). PCR amplification resulted in products of 377 bp and 244 bp for the Y- and 1- chromosome specific fragments, respectively. The sequence for 1 chromosome (X51555) is 313 nucleotides in length with a copy number of 2n=3000-6000 (Jantsch et al., 1989) whereas the sequence for Y chromosome (X12696) is 3832 nucleotides in length with no marked internal repetitions (McGraw et al., 1988).

Oligonucleotide sequences were as follows:

Chromosome 1
- *Forward*: 5'- GTT GCA CTT TCA CGG ACGCAG C-3'
- Reverse: 5'- CTA GCC CAT TGC TCG CCA TAG C-3'

Chromosome Y
- Forward: 5'- AAT CCA CCA TAC CTC ATG GAC C-3'
- Reverse: 5'- TTT CTC CTG TAT CCT CCT GC-3'

All reagents were thawed in advance and kept on ice before use in the PCR reaction. The master mix was prepared by mixing 0.4 µl of a mixture of 4 dNTPs (dTTP; dATP; dGTP and dCTP each at 2 mM) , 2.5 µl (from a 10pmol/µl stock solution) of primers for chromosomes 1 and Y, 5 µl of pig genomic DNA containing 10-100 ng of DNA; 5 µl of 10 x PCR buffer (100 mM Tris-HCl, pH 8.3 at 25°C; 500 mM KCl; 15 mM $MgCl_2$; 0.01% gelatine) and 0.5 µl of 5 U/µl *Taq* DNA polymerase. The volume reaction was made up to 50 µl with water. Amplification cycles were performed in a thermocycler (Personal Master Cycler. Eppendorf) and consisted of a first denaturation step before the first cycle at 95°C for 5 min, followed by 35 cycles of the following programme: denaturation at 95° C for 15 s, annealing at 60°C for 1

min and extension at 72°C for 15 s. Finally, an elongation step for 7 min at 72°C was performed after the last cycle.

Ten microlitre samples of each PCR product were subjected to electrophoresis in 1% (w/v) agarose gels. Amplified products were stained with ethidium bromide and visualized, and then photographed under UV light. The remainder of the PCR product was precipitated with ethanol and sodium acetate before determining the final concentration of amplified DNA.

2.3.1.2.- Nick Translation reaction

The specific DNA sequences of 1 and Y chromosomes amplified by PCR were used as DNA templates and were labelled using the modified deoxyuridine triphosphates (dUTP), Cy3-dUTP (Amersham Pharmacia Biotech Europe GmbH. Barcelona) which provides a red fluorescent signal for chromosome Y, and Alexa Fluor 488-5-dUTP (Molecular Probes Europe BV. The Netherlands) which provides green fluorescence signal for chromosome 1.

All chemicals used in this reaction were purchased from Amersham Pharmacia Biotech Europe GmbH (Barcelona). The nick translation reaction (Nick Translation kit; N 5500®. Amersham Pharmacia biotech) was performed by adding a 15 µl of PCR product containing 1µg of template DNA to a microcentrifuge tube containing 20 µl of nucleotide buffer (14 µM for dATP, dGTP, and dTTp and 4µM for dCTP), 1µl of fluorescent labelled nucleotide (1 mM), 10 µl of enzyme mix (0.5 units/µl DNA polymerase 1 and 10 pg/µl DNase I in Tris-HCl pH 7.5, $MgCl_2$, glycerol and bovine serum albumin), 1 µl of freshly diluted DNase I (0.002333 units of enzyme); the final volume was adjusted to 50 µl with water. An incubation step of 4 hours and 15 min was needed to complete the reaction. After this incubation time, 5 µl of 0.2 M EDTA pH 8.0 was added to stop the reaction.

2.3.2. Flow cytometric sperm sorting

Semen samples were collected from five fertile boars and diluted on Beltsville hawing solution (BTS) (205mM Glucose, 20.39 mM NaCl, 5.4mM KCl, 15.01 mM $NaHCO_3$ and 3.35 mM EDTA) (Johnson et al., 1988) to a final concentration of $3x10^7$ spermatozoa/ml.

Sorted spermatozoa were obtained following the general procedure described by Johnson et al. (1989). Briefly, 50 ml of diluted spermatozoa was stained with Hoechst-33342 (9 μM) and incubated for 1 h at 35°C. After 15 min at room temperature (25°C), stained sperm were sorted with a modified EPICS Altra Flow Sorter (Coulter Corporation, Miami, FL. USA) operating at 42 p.s.i and with a power laser of 160 mW. Spermatozoa were sorted for Y-chromosome-bearing spermatozoa according to the criteria of high purity or low purity by adjusting the size of the sort window. A wide sort window allows a high production rate with a low purity, whereas a narrow sort window allows a low production rate but with a high purity. Control samples consisted of unsorted spermatozoa from the same boars.

2.3.3. Fluorescence in situ hybridization

Direct FISH was performed on sorted and non-sorted boar spermatozoa. The specificity of the probes was evaluated using metaphase spreads from pig lymphocytes.

2.3.3.1. Preparation of spermatozoa

Five millilitres of unsorted (3×10^7 spermatozoa/ml) or 10-15 ml of sorted spermatozoa (0.5×10^6 spermatozoa/ml) were centrifuged for 5 min at 1200 x g and resuspended in a KCl hypotonic solution (75 mM), providing a final volume of 6 ml. This step was repeated at least twice. Subsequently, the supernatant was discarded, and the pellet was resuspended in fresh, cold fixative (methanol-glacial acetic acid 3:1),

bringing the sperm suspension to a volume of 4-6 ml (depending on the sperm concentration). The fixed sperm suspension was spread on a clean glass slide and air-dried.

Before hybridization, the slides were washed in 2 x saline-sodium citrate buffer (SSC) to remove excess fixative, dehydrated by passing through a series of ethanol (70%;85%;100%) and air-dried. Subsequently, the slides were incubated for 15 min at 37°C in a 5 mM dithiothreitol (DTT) solution (pH 7.4) to reduce the protamine disulphide bonds and, thus to produce the decondensation of sperm nuclei. For boar D, it was necessary to repeat this sperm decondensation step for further 10 min. The slides were washed again in 2 x SSC, dehydrated by passing through a series of ethanol and air dried. Denaturation was carried out in 70% (v/v) formamide/2x SSC solution at 75°C for 5 min. Finally, the slides with sorted and unsorted spermatozoa were dehydrated again by passing through a new series of ethanol and dried at room temperature.

2.3.3.2.- Preparation of metaphases

Peripheral blood was aseptically collected from the same five boars that were used to obtain sperm samples. Metaphase chromosome spreads obtained from pig lymphocyte cultures were used as FISH controls. Cell suspensions were cultured using the protocol described for goat metaphases by Di Berardino et al. (1996) with minor modifications. Briefly, aliquots of 0.5 ml whole blood containing 6 x 10^6 lymphocytes were added to each of five culture flasks containing 8 ml of RPMI 1640 medium without L-glutamine (Gibco. Life Technologies. Barcelona. Spain), including 1 ml of fetal bovine serum, 0.1 ml of L-glutamine, 50 µl of antibiotic-antimycotic solution, and 0.1 ml of pokeweed mitogen (lectin from Phytolacca Americana) to stimulate mitogenetic activity . The cultures were allowed to grow for 68 h at 38.5 °C, and colcemid (Gibco) was added at a final concentration of 0.1 µg/ml at 15 min before harvesting. Harvested cells were treated with hypotonic solution (75 mM KCl) and

fixed in methanol: acetic acid (3:1). After fixation, the metaphases were dropped on to clean microscope slides and air-dried.

Denaturation processing of these slides was as follows: the slides were first placed in a Coplin jar with 2 X SSC for 1 h. Subsequently, 100 µl of RNase solution (100 µg/ml in 2 X SSC) was deposited on to the slide and covered with at 24 mm x 36 mm cover glass and incubated at 37°C for 1 h. The slides were then washed in 2 x SSC at room temperature for 5 min, and pepsinized (5 µl of 0.01% w/v of pepsin in 50 µl of 10 mM HCl) for 5 min. Treatment after fixation was as follows: 5 min in PBS containing 50 mM of $MgCl_2$; 10 min in 1 % formaldehyde solution in PBS-$MgCl_2$ 50 mM and finally 5 min in PBS. After a dehydration step the slides were air-dried and observed by phase-contrast microscopy (x 400). Denaturation solution (70 % (v/v) deionized formamide, 2 X SSC, adjusted to pH 7.0) was freshly prepared and heated just before use. Slides were denatured for 5 min at 75°C, dehydrated in a new ethanol series and air-dried.

2.3.3.3.-Hybridization and detection

Three microlitres of Y-chromosome labelled probe and 3 µl of 1-chromosome labelled probe were precipitated by cold ethanol and sodium acetate, for 3 hours at -80°C or overnight at -20°C. After precipitation, probes were washed with 70% (v/v) ethanol, and centrifuged at 23000 x g for 30 min. DNA pellets were resuspended in 10 µl of Hybridization Solution ®. This hybridization mixture was denatured at 75°C for 5 min, and then dropped on to the previously denatured slides and finally covered with a coverslip. The slides were sealed with rubber cement and placed in a dark moist chamber at 37°C for 72 h. After hybridization, slides were washed in 0.4 x SSC solution at 75°C for 2 min, followed by a further wash in 2 x SSC/0.1% (v/v) Tween 20 at room temperature for 2 min, dehydrated by a series of ethanol (70%;85%;100%) and air-dried. The slides were finally counterstained with 8 µl of 4', 6-diamino-2-phenylindole (DAPI) antifade solution

2.3.4. Analysis of the samples

The slides were examined with a fluorescence microscope (Leica DMRB Fluo. equipped with a DAPI, FITC and Texas Red filters. Heerbrugg. Switzerland). A total of 2500 non-sorted spermatozoa, 2000 sorted spermatozoa (for low and high sorted purity) and 50 metaphase plates were analysed. Control slides were observed and the correct localization of the fluorescent signal on the metaphase spreads was evaluated. Spermatozoa were scored only if they were intact, non-overlapped, had a clearly defined border, and had not decondensed to more than twice the size of a non-decondensed sperm head, which could produce large and sometimes fragmented FISH signals.

A χ^2 test was used to investigate the deviation from the expected ratio 50:50 (X: Y) of the percentages of putative Y-chromosome-bearing spermatozoa for each unsorted and sorted population and to compare the percentages of spermatozoa showing signal on chromosome 1.

2.4. Results

Specific pig DNA probes for direct FISH were labelled with fluorescent nucleotides using nick translation. The time invested in performing this labelling reaction and the ease of the procedure were evaluated. The technical manipulation of the reagents was minimal and, thus, the protocol for preparing specific DNA fluorescent probes by NT was very easy to perform.

Control slides prepared with metaphase chromosome spreads from boar lymphocytes exhibited signals at the appropriate regions of the 1- and Y-chromosomes illustrating the high specificity of the probes for their respective complementary sequence (Fig.1). Signal for chromosome 1 was observed on the centromeric region whereas the fluorescent mark for Y chromosome was visible on the long arm of the chromosome.

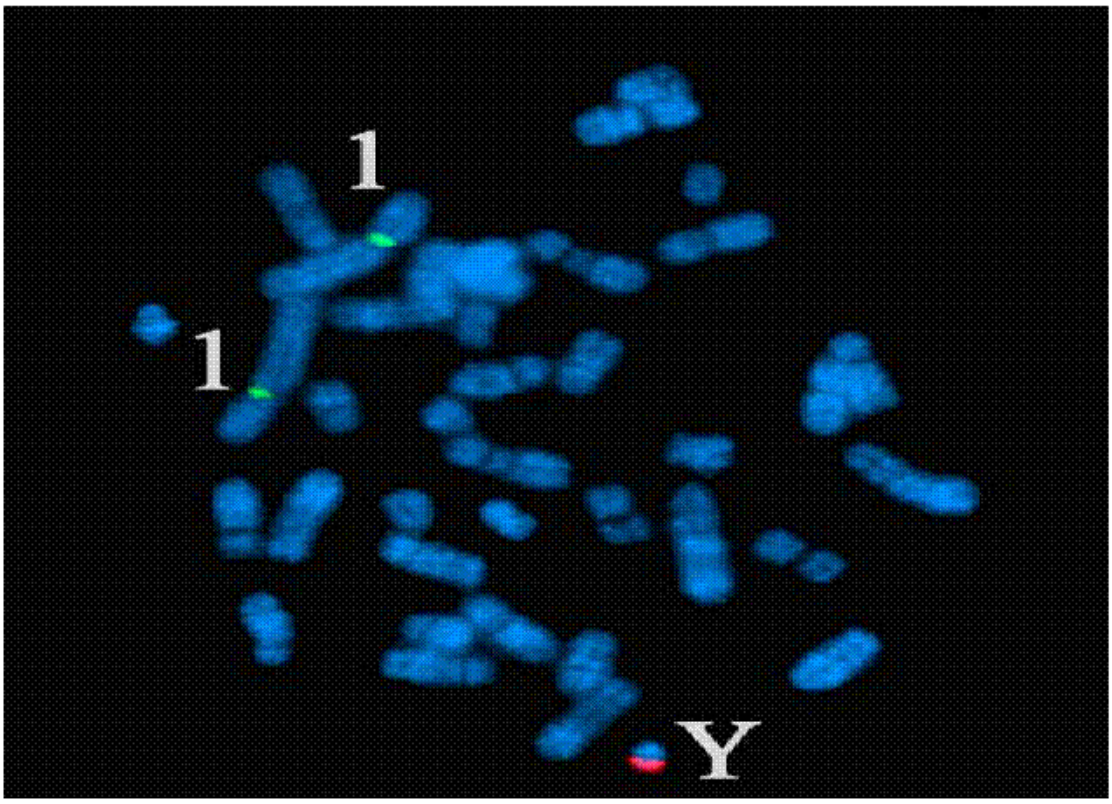

Figure 1. Normal boar metaphase chromosomes demonstrating hybridization signal on the 1 - and Y- chromosomes (green and red respectively) obtained with NT labelled probes.

One of the crucial steps for FISH experiments is to achieve proper nuclear decondensation, so as to obtain efficient hybridization results. In boar D, the additional 10 min decondensation treatment was necessary to obtain adequate decondensation.

The results obtained after the FISH procedure on sorted and unsorted spermatozoa are shown (Figs 2 and 3, respectively). The green signal on chromosome 1 is clearly identifiable on the decondensed sperm heads independent of the sperm sample used. The red signal produced with the Y probe obtained by nick translation was stronger than the signal produced by the chromosome 1 probe and comprised a larger area of the sperm head, making identification of the Y chromosome easier.

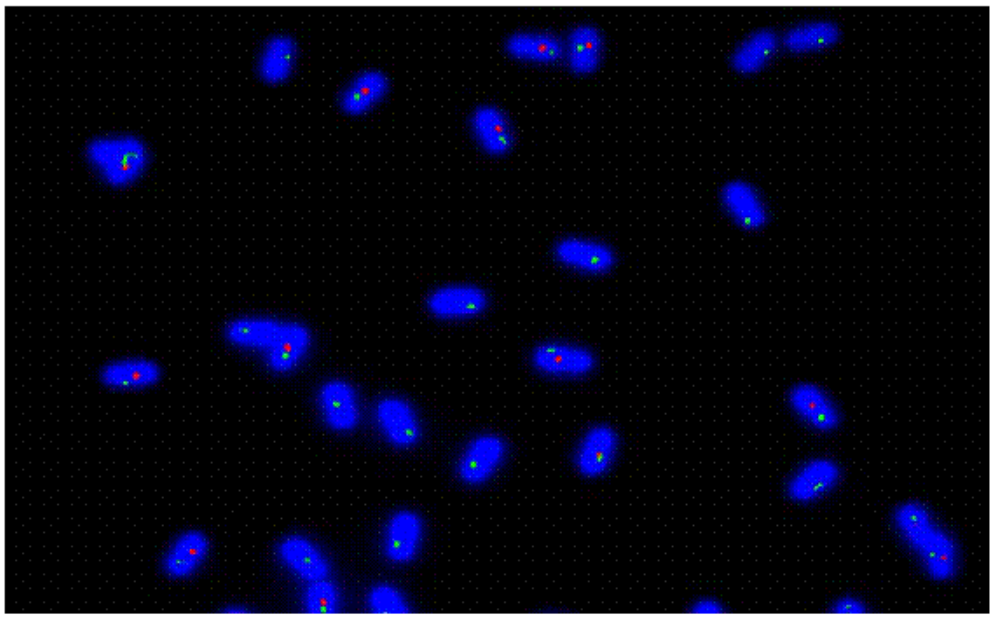

Figure 2. Unsorted spermatozoa 1 and Y – chromosomes hybridized with NT labelled probes. Only sperm heads with green signal were considered as X-bearing sperm.

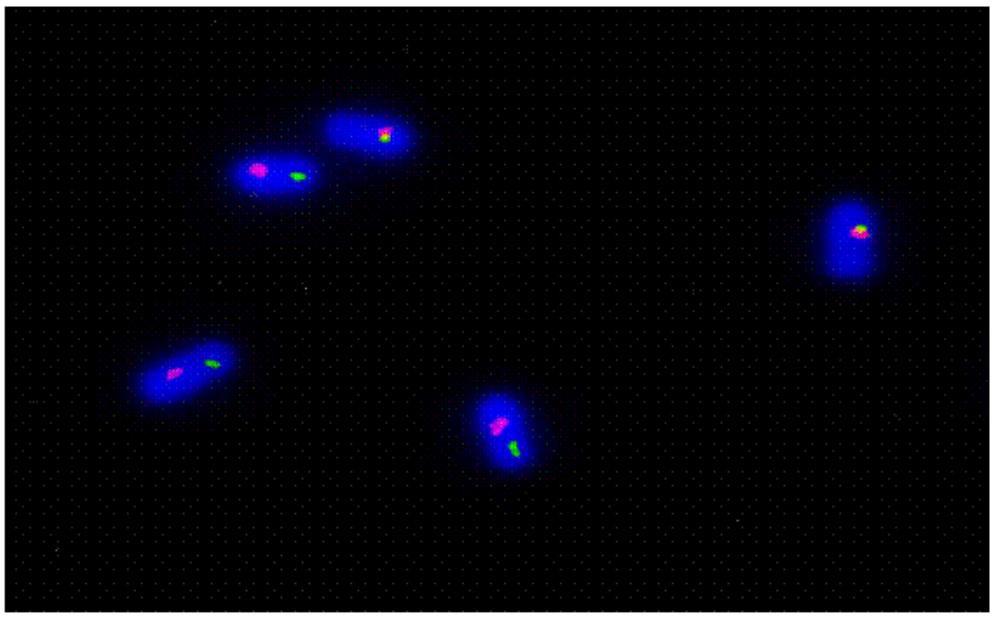

Figure 3. 1 (green signal) and Y (red signal) NT probes hybridized to a Y-sorted sample.

However, subjective analysis of the quality of the fluorescence presented by spermatozoa showed that nick translation probes produce a very clear hybridization signal for both chromosomes. Hybridization efficiency was not affected by the sperm

treatment and the percentages of sperm with chromosome-1 fluorescent signal were similar for sorted and non-sorted spermatozoa. In both cases this efficiency (number of spermatozoa with the chromosome-1 signal divided by the number of spermatozoa counted) was near to 98% (Tables 1 and 2). Spermatozoa showing signal on chromosome-Y but not on chromosome-1 were not observed. Furthermore, the average frequency of aneuploid, probably diploids, spermatozoa was below 0.1% (spermatozoa showing two red and green signals or two green signals separated by at least one and a half diameter of the respective fluorescence mark within that sperm cell).

Table 1. Percentage X- and Y-chromosome unsorted boar spermatozoa by fluorescence *in situ* hybridization (FISH)

Boars	Number counted	Y (Y and 1 signals*)	X (Only 1 signal†)	Without signals	Significance (deviation from 50:50)
A	500	48	50.4	1.6	NS
B	500	51	46.6	2.4	P<0.05
C	500	50.6	47.6	1.8	NS
D‡	500	48.2	49.2	2.6	NS
E	500	52.8	46	1.2	P<0.05

* Spermatozoa labelled with the green and red fluorescent signals (chromosomes 1 and Y, respectively).
† spermatozoa labelled only with the green fluorescent signal (chromosome 1).
‡ In this boar additional 10 min decondensation treatment was necessary
NS: not significant (P>0.05)

Percentages of Y-chromosome bearing boar spermatozoa were first determined on unsorted samples (Table 1). The Y probe gave a single red signal on approximately half of the spermatozoa, which is consistent with half of the spermatozoa carrying a Y chromosome on unsorted samples (ratio 1:1). Spermatozoa with fluorescent mark for

chromosome 1 (green signal) but without red signal for Y-chromosome were considered as putative X-chromosome bearing spermatozoa. On flow sorted sperm samples, the red signal was observed on approximately 75 and 93 % of the spermatozoa scored for low and high Y purity sorting, respectively (Table 2).

There were no differences at the $P< 0.1$ level between the theoretical ratio (50:50) and the observed ratio before sorting, but for boars B and E (Table 1) there was a significant excess of Y-chromosome-bearing sperm at the conventional $P<0.05$ level. However, the percentages of Y-bearing spermatozoa after sorting were significantly different ($p<0.0001$) from the 50:50 ratio for both high and low Y purity sorting.

Table 2. Percentage X- and Y- sorted spermatozoa by flow cytometry for low and high Y purity sorting verified by FISH.

BOAR	Sorted spermatozoa for Y chromosome with low purity (%)					Sorted spermatozoa for Y chromosome with high purity (%)				
	Number counted	Y (Y and 1 signals)	X (Only 1 signal)	Without signals	Significance (deviation from 50:50)	Number counted	Y (Y and 1 signals)	X (Only 1 signal)	Without signals	Significance (deviation from 50:50)
A	200	77.5	20.5	2	p<0.0001	200	92.5	6.5	1	p<0.0001
B	200	74.5	27	2.5	p<0.0001	200	90	7.5	2.5	p<0.0001
C	200	75	22.5	2.5	p<0.0001	200	89.5	9	1.5	p<0.0001
D*	200	74	24	2	p<0.0001	200	93	5	2	p<0.0001
E	200	71	27.5	1.5	p<0.0001	200	92	6.5	1.5	p<0.0001

* In this boar additional 10 min decondensation treatment was necessary

2.5. Discussion

Evaluating the efficacy of a sorting procedure for the separation of X- and Y-chromosome-bearing spermatozoa is an important step before using these spermatozoa in reproductive techniques.

FISH has been defined as the most effective method for evaluating sorted spermatozoa as it can be used to examine sperm cells by viewing sperm chromosomes directly (Kawarasaki et al., 1998). The highly qualitative and quantitative independent evaluation is the main advantage of FISH over the use of flow cytometry reanalysis or single cell PCR evaluation.

One of the major problems when FISH is performed on spermatozoa is the extreme degree of condensation of the DNA, making access to specific hybridization sites very difficult. As the time course of sperm decondensation varies considerably among species (Perreault et al., 1988), different decondensation protocols have been established for different species. Nuclei must be decondensed to allow the DNA probes access to the sperm chromatin, but if the nuclei are swollen to more than twice their original size the signal from one chromosome may split and appear as two or more signals, causing the spermatozoa to be falsely scored as disomic (Wyrobèk et al., 1993). Many laboratories have accepted that, for two signals to indicate the labelling of two separate chromosomes, the signals should be separated by at least one-half the size of the signal to ensure they arise from two separate chromosomes and not from a split signal on single chromosome. Decondensation protocols that have been used successfully on humans do not work well on bovine sperm (Hassanane et al., 1999). A human sperm DNA decondensation protocol (Rousseaux et al., 1995) adapted for boar spermatozoa resulted in spermatozoa with intact morphology of both the head and the tail and allowed efficient double direct FISH to be performed. This protocol is based on treating spermatozoa with dithiothreitol (DTT) to reduce nuclear protamine disulphide bonds and, thereby, accelerating sperm nuclear decondensation. A similar

DTT decondensation protocol has been applied by Kawarasaky (1996) on flow-sorted and unsorted boar sperm. Variation among boars was detected in this study with respect to the optimal time for sperm head decondensation. This difference in sperm susceptibility to the decondensation process is dependent on the content in chromatin disulphide bridges of the sperm nuclei (Rodriguez et al., 1985), which is variable between species and also between individuals of the same species, as has been demonstrated.

Rens et al. (2001) performed a FISH experiment on bull spermatozoa sorted by the established Beltsville Sperm Sexing Technology. The result of this study showed that flow sorted cattle spermatozoa were more sensitive than diluted spermatozoa to the decondensation protocol used and that the reagent concentration had to be adjusted to obtain optimal decondensation on the sorted sperm samples. However, our results showed that this procedure for flow cytometric sorting of boar spermatozoa, does not affect nuclear decondensation. Moreover, the accessibility of the probe to the target DNA sequence is not affected by the Hoechst 33342 staining, although this fluorochrome binds tightly to DNA in the minor groove (Johnson and Schulman, 1994). Previous studies in which indirect FISH has been performed on flow- sorted boar spermatozoa do not describe any interaction between Hoechst 33342 and specific fluorescent DNA probes (Kawarasaki et al., 1998).

For FISH procedure to be carried out, a direct or indirect fluorescent DNA probe complementary to a specific DNA target sequence is needed. FISH experiments with indirect DNA probes have been performed previously on different species of domestic mammals to identify sex chromosomes in spermatozoa (Kawarasaki et al., 1995, 1996; Hassanane et al., 1999; Piumi et al., 2001; Rens et al. 2001). Although indirect probes are useful for detecting specific DNA sequences, they would perhaps not be the most useful methods for detecting a DNA sequence because their use requires at least one, and usually three detection steps before visualization under a microscope. Therefore direct detection is faster and simpler. However it should be

noted that direct FISH works only with extremely long targets or with targets consisting of highly repeated short DNA sequences.

The present study used direct DNA probes prepared by nick translation. This method is defined as a homogeneous labelling, owing to the random incorporation of labelled nucleotides throughout the length of the probe. If the reaction is optimally controlled, nick-translated probes can give the highest sensitivity compared with probes labelled by other methods.

The frequency of spermatozoa showing a signal for chromosome 1 was about 98%, demonstrating the high efficiency of the hybridization. Moreover, the results obtained from the high and low purity samples and the unsorted samples showed the efficacy of the probes for determining percentage of Y-chromosome-bearing spermatozoa.

As expected, the highest purity was obtained by narrowing the selection window. The width of the selected sort window is a key factor for obtaining sorted populations of high purity (Johnson, 1997) although at low rates. However, using a wide window it is not possible to produce enough sperm for artificial insemination in pigs economically using this method. Additional information about the percentage of aneuploidies for chromosome 1 and Y was obtained. This percentage was below 0.1% and is in accordance with the results reported by Rubes et al. (1999).

It was noted in the present study that the nick translation method does not involve extensive manipulation of the samples and, therefore, the risk of contamination is lower than in labelling procedures such as PCR labelling, which is often used to label DNA probes. However, in the FISH protocol, the labelled DNA fragments are of adequate size to combine with its complementary sequence on sperm heads and, thus, the nick translated DNA fragments yield a clear signal and a clean hybridization. By contrast, with PCR labelling the labelled fragments of DNA may be up 4-5 kb. Because of their size, the fragments do not penetrate the structures surrounding the

DNA target and may result only in background hybridization. Additional steps with partial DNase digestion are then necessary to shorten the labelled DNA.

In conclusion, two-colour direct FISH with pig chromosome-specific DNA probes prepared by nick translation provides a useful tool for determining purity on flow cytometry-sorted spermatozoa with a good specificity. The ease of the labelling procedure and the quality of the fluorescent signal obtained demonstrate that nick translation is an ideal labelling method for the preparation of fluorescent pig DNA probes to be used for evaluating flow sorting process on boar spermatozoa. These probes could also be a useful tool in the determination of percentages of chromosomal aneuploidy or other chromosome alterations on pig spermatozoa. Evaluation of the sex of the embryos and the determination of the appropriate development of the embryos are also possible.

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Experiencia 3

Influence of storage time on functional capacity of flow cytometrically sex sorted boar spermatozoa

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3.1 Abstract

Sex sorting of boar spermatozoa is an emerging biotechnology still considered suboptimal owing to the slowness of the process, which requires long sorting periods to obtain an adequate number of spermatozoa to perform a non-surgical insemination. This period involves storage of sorted cells that could impair their functional capacity. Here we have studied how the storage of sex sorted boar spermatozoa affects their functional capacity. Sorted spermatozoa were assessed at various times (0, 2, 5 or 10 hours) during storage after sorting and compared with diluted and unsorted spermatozoa for sperm motility patterns, plasma membrane and acrosomal integrity and their ability to penetrate homologous IVM oocytes. Sex-sorted sperm motility and membrane integrity only decreased significantly ($P<0.05$) by the end of the storage period (10h) compared to unsorted spermatozoa. Sperm velocity, ALH and Dance increased significantly ($P<0.05$) immediately post-sorting, returning to unsorted sperm values during storage. Acrosome integrity was not seriously affected by the sorting process, but decreased ($P<0.05$) during storage after sorting. Sorted spermatozoa stored 2 h after sorting did not differ from unsorted in penetration rates and numbers of spermatozoa per oocyte, reaching the highest ($P<0.05$) penetration rates and sperm numbers per oocyte when co-cultured for 6 or more hours. Non-storage or storage for 5 h or 10 h negatively ($P<0.05$) affected sperm penetration ability. In conclusion, although flow cytometrically sex sorted spermatozoa are able to maintain motility, viability and acrosomal integrity at optimal levels until 10 h of storage after sorting, fertilizing ability is maintained only over shorter storage times (<5h).

Key words: Flow cytometry, sex-sorting, seminal plasma, sperm quality, oocyte penetration, *in vitro*, pig.

3.2 Introduction

The development of semen sexing is widely accepted as a major advance in reproductive technology. The pre-selection of the sex accelerates genetic progress as well as enhancing benefit the management and efficiency of pig production. The effectiveness of this technology for producing offspring of the pre-selected sex in swine has been proven in numerous trials where the semen was sorted and surgically inseminated [1] or used for in vitro fertilization [2,3,4] or intracytoplasmic sperm injection [5] and subsequent embryo transfer. Recently, offspring have been produced after non-surgical deep intrauterine insemination [6] of either fresh sorted [7, 8] or sorted frozen-thawed (Maxwell WMC, personal communication) spermatozoa. This technology allows farrows to be obtained with doses as low as 50 million spermatozoa per insemination although the success of these inseminations has been suboptimal.

Although the current throughput of sperm sexing instruments is fast (5000-6000 sperm/s) [9], this yield is relatively slow and long sorting times are required to obtain the adequate number of sorted boar spermatozoa. Therefore, the final sperm population will be characterized by its heterogeneity, since it consists of sperm cells ranging from spermatozoa subjected to relatively long holding times after sorting to spermatozoa that have only just been sorted.

It is known that the sperm membrane is partially compromised during the flow cytometry and sorting process, which affects viability, storage capability and fertilization ability of spermatozoa [10]. Both, the physical effect of the sorting procedure and the high dilution rate by sheath fluid cause in the spermatozoa a short lifespan after sorting [11]. To improve post-sorting sperm survival, the impact that each sorting steps has on the sperm population should be evaluated, and the interval during which the sperm cells preserve their fertilizing capability after sorting needs to be defined. This would help to establish the potential fertilizing capability of the sex-sorted sperm population. Although the effect of the Hoechst 33342 staining [12] and

the sorting procedure [11, 13, 14] has been studied on boar spermatozoa, there are no reports monitoring the effects of liquid storage of flow cytometrically sorted boar spermatozoa up 10 hours on sperm functionality; this is a critical time for both the sorting and the transport of the spermatozoa from the cell sorter laboratory to the farm for performing a non-surgical insemination.

In the present study we have monitored the changes in viability, motility, acrosomal exocytosis and ability to penetrate IVM oocytes of flow sorted boar spermatozoa stored for 0, 2, 5 and 10 h. The main goal of this study was to know the variability in the sperm functionality of sex-sorted spermatozoa when a long holding time is required.

3.3 Material and Methods

All chemicals used in this study were provided by Sigma-Aldrich Co. (Alcobendas, Madrid, Spain), unless otherwise stated.

3.3.1. Semen collection and flow cytometric sorting

Experiments were carried out with semen from five mature Pietrain boars that had previously sired offspring. The boars were housed in individual pens in temperature-controlled environments (23 ± 2°C). Ejaculates (sperm-rich fraction) were obtained weekly using the gloved-hand method and extended in Beltsville Thawing Solution (BTS) [15] to 150 x 10^6 spermatozoa/mL. Shortly after collection, the semen samples were evaluated and used if they had met the minimum criteria: motility >80%, viability >85%, hypo-osmotic swelling response >70%, total sperm count per ejaculate >20 x 10^9, acrosomal abnormalities <10%, and abnormal sperm morphology <1 %

[16]. An aliquot of diluted semen was stored at 20°C until the analysis and was used as reference value of sperm characteristics before sorting while the rest of the diluted semen was processed for sex sorting.

Samples of semen were sorted for chromosomal sex by flow cytometric sorting using the Beltsville Sperm Sexing Technology protocol [17] as adapted for high speed sorting [18]. These spermatozoa are hereby named as sex-sorted spermatozoa (SS spermatozoa). Briefly, spermatozoa were loaded with Hoechst 33342 (0.3 μM per $1x10^6$ spermatozoa), followed by incubation in darkness for 1h at 35°C. After incubation, samples were filtered though a 30 μm nylon mesh filter to remove debris or clumped spermatozoa. The stained spermatozoa were sorted using an EPICS Altra high speed flow sorter (Coulter Corporation, Miami, FL, USA), operating at 3,655 kg/cm^2 and modified as described previously [19] with a Model 90C-6, 6-watt argon laser operated in the ultraviolet wave-length (351,364 nm) at 175 mW (Coherent Lasers, Inc., Santa Clara, CA).

The SS spermatozoa were collected in 10 mL BSA-coated plastic tubes containing 1 mL of TEST-yolk (2%) extender containing 10% of seminal plasma at 20°C. Spermatozoa were kept at 20°C during 0, 2, 5 and 10 hours after sorting.

3.3.2. Assessment of sperm characteristics

Sperm motility patterns, plasma membrane integrity and acrosomal exocytosis as well as the ability to penetrate homologous *in vitro* matured oocytes were assessed in unsorted and sorted spermatozoa immediately after sorting (0h), 2, 5 and 10 hours after sorting.

3.3.2.1. Assessment of motility

For sperm motility patterns analysis, aliquots of 4 μL from each group were placed in a warm (38°C) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38°C) of a Nikon Labophot light microscope (Tokyo, Japan), equipped with phase contrast optics. The microscope was equipped with a monochrome video camera (Hitachi CCD model, Chiba, Japan), connected to a personal computer. The sperm analysis was performed using the software Sperm Class Analyser (SCA, Barcelona, Spain). The programme settings were as follows: Frame rate= 25Hz, Search radius= 11.5 μm, Minimum track points= 7 frames, Threshold straightness= 75%. The motion variables measured were: curvilinear velocity (VCL, time-average velocity of the sperm head along its actual trajectory), straight-line velocity (VSL, time-average velocity of the sperm head along a straight line from its first position to its last position), amplitude of lateral head displacement (ALH, the average value of the extreme side to side movement of the sperm head in each beat cycle), dance (curvilinear velocity multiplied by the amplitude of lateral head displacement), beat cross frequency (BCF, the frequency with which the actual track crosses the smoothed track) [20].

3.3.2.2..Assessment of plasma membrane integrity

Sperm viability was assessed using the LIVE/DEAD Sperm Viability Kit (Molecular Probes Europe, Molecular Probes Europe, Leiden, The Netherlands). Briefly, 0.5 mL of sperm suspension containing 5×10^5 sex sorted spermatozoa was stained with 25 nM SYBR-14 solution and 12 μM Propidium Iodide (PI) solution. Samples were incubated at room temperature in darkness for 10 min before cytometric analysis. All analyses were carried out by analytical flow cytometry using an EPICS XL (Coulter Corporation Inc., Miami, FL, USA) equipped with standard optics, an argon ion laser (Cyonics, Coherent, Santa Clara, CA, USA) with 15 mW laser power at

488 nm and the software EXPO 2000 (Coulter Corporation Inc., Miami, FL, USA). Forward and sideways light scatter were recorded for a total of 15,000–25,000 events per sample and only sperm-specific events, which appeared in a typically L-shape scatter profile, were positively gated for the analysis. SYBR-14 was measured through a 525 nm band pass filter while PI was collected through a 635 nm band pass filter. Viable spermatozoa were defined as those stained with SYBR-14 and not stained with PI.

3.3.2.3. Assessment of acrosomal exocytosis

Acrosome exocytosis was evaluated on the sex sorted spermatozoa using simultaneously PI to stain dead cells and the lectin FITC-PNA to evaluate disrupted acrosome. Briefly, 0.5 mL of sperm suspension containing $5x10^5$ of sex-sorted spermatozoa was stained with 12 μM of PI and 5 μL of FITC-PNA stock solution (1mg/mL in bi-distilled water). Spermatozoa were incubated for 5 min in the dark and analysed immediately on the flow cytometer (see above). FITC-PNA was measured through a 525 nm band pass filter while PI was measured through a 635 nm band pass filter. Spermatozoa were identified in one of the three following populations: PI positive, non-viable cells; PI negative and FITC-PNA negative, live spermatozoa with intact acrosome and PI negative and FITC-PNA positive corresponding to live spermatozoa with exocytosed acrosome.

3.3.2.4. Assessment of IVM oocytes penetration

In order to assess the ability of spermatozoa to penetrate homologous oocytes matured in vitro, ovaries were obtained from pre-pubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl containing 70 μg/mL kanamycin, maintained at 34-37°C. Cumulus-oocyte-complexes (COC´s) were

aspirated from medium sized follicles (3-6 mm in diameter) with an 18-gauge needle fixed to a 10 mL disposable syringe. Only COC´s having a compact cumulus mass, an intact ZP and an oocyte with an evenly granulated cytoplasm were selected. For IVM, the COC´s were washed three times in BSA-free North Carolina State University (NCSU) 23 medium [21] supplemented with 10% (v:v) porcine follicular fluid, 0.1 mg/mL cysteine and 10 ng/mL epidermal growth factor (EGF). This medium is named "maturation medium" henceforth. Thereafter, COC´s were transferred to a Nunc 4-well multidish plate (50-100 COC´s/well) submerged in 500 µL of pre-equilibrated maturation medium (previously covered with warm mineral oil), supplemented with 10 IU/mL eCG (Intervet International BV, Boxmeer, The Netherlands) and 10 IU/mL hCG (Intervet International BV, Boxmeer, The Netherlands), and cultured at 39°C in 5% CO_2 in air for 22 h. The medium was then changed for maturation medium without hormone supplementation, and incubated at 39°C in 5% CO_2 in air for another 22 h. After IVM, the expanded cumulus cell cloud was removed with 0.1% hyaluronidase in maturation medium and washed three times with pre-equilibrated IVFmedium [22]. This medium consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM $CaCl2{\cdot}2H_2O$, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 1mM caffeine and 0.2% BSA (fraction V; A 7888, initial fractionation by cold alcohol precipitation). After washing, batches of 50 oocytes were placed in 50 µL drops of the same medium covered with warm mineral oil in a 35 x 10 mm petri dish. The dishes were kept in the incubator for about 30 min before spermatozoa were added for fertilization assays. Sorted and stored spermatozoa as well as unsorted spermatozoa were centrifuged at 500 x g for 3 min and re-suspended to a final concentration of 2 x 10^6 spermatozoa/mL in IVF medium and 50 µL of this sperm suspension containing 10^5 spermatozoa/mL were added to oocytes in IVF medium so that the relation oocyte:spermatozoa was 1:2,000. The oocytes were co-incubated with the spermatozoa at 39°C in an atmosphere of 5% CO_2 in air for 2, 3, 6 or 18h. After co-incubation, oocytes were mounted on slides, fixed in 25% (v/v) acetic acid in ethanol at room temperature for 48-72 h, stained with 1% lacmoid in 45% (v/v) acetic acid, and examined under a phase contrast microscope at

magnifications of x 400. Oocytes were considered penetrated when they had one or more swollen sperm heads and/or male pronuclei and their corresponding sperm tails.

3.3.3 Statistical Analyses

All data editing and statistical analyses were performed in SPSS, version 11.5 (SPSS Inc., Chicago, IL). Data were analyzed by ANOVA using the MIXED-procedure according to a statistical model including the fixed effect of post sorting storage time and the random effect of replicate. In the in vitro fertilization experiment, the time of coincubation was included as a fixed effect. To analyze data of sperm viability, motility and acrosome status, percentages were subjected to arcsine transformation before analysis. Data of percentage of penetration were modeled according to the binomial model of parameters [23] before analysis. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be significant when $p<0.05$. Experiments were replicated five times.

3.4. Results

3.4.1. Analysis of motility parameters

The results are summarized in Figure 1. The percentage of motile SS spermatozoa was maintained around 80% during storage up 5 hours to decrease significantly ($p<0.05$) with respect to the unsorted spermatozoa only by the end of the storage time (10h) (Figure 1A). Among the other variables explored by the CASA instrument, sperm velocity (VCL or VSL) and angularity parameters (ALH and Dance) increased significantly ($p<0.05$) immediately after sorting (Sorted 0h) to either return to unsorted sperm values by 2 h of storage (Fig 1 B-C and D-E, respectively). BCF variable was not affected by the storage time.

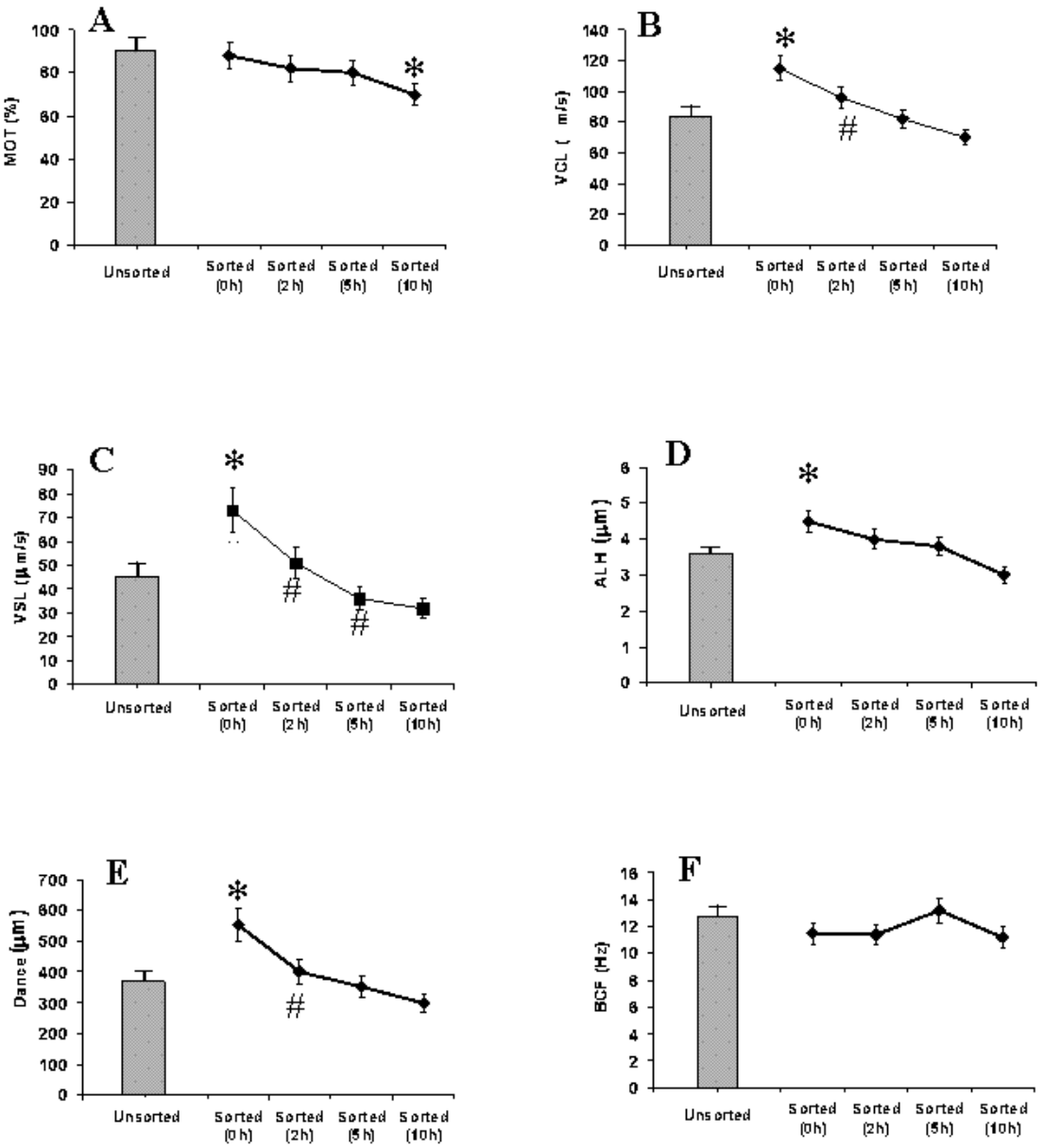

Figure 1. Changes in motion parameters in unsorted and flow cytometrically sorted spermatozoa analyzed by computer-assisted sperm motion analysis (CASA) after 0h, 2h, 5h, and 10h of storage after sorting. (A) Percentage of motile sperm (MOT); (B) curvilinear velocity (VCL); (C) straight-line velocity (VSL); (D) amplitude of lateral head displacement (ALH); (E) curvilinear velocity multiplied by amplitude of lateral head displacement (Dance); and (F) beat cross frequency (BCF). Each point represent the mean ± SEM. * $P<0.05$ compared to the diluted sample (control), # $p<0.05$ compared to the previous point.

3.4.2. Analysis of plasma membrane integrity

The results are presented in Table 1. Sperm sex sorting procedure had no significantly negative influence on sperm viability when spermatozoa were stored up 5 hours after sorting. However, sperm viability then decreased significantly ($p<0.05$) for SS spermatozoa stored between 5 and 10 hours (70.3±2.8) when compared with the unsorted spermatozoa (85.9±0.63).

Table 1. Percentage (mean ± SEM) of viable sperm populations identified with SYBR-14 (SY) and Propidium Iodide (PI) after cytometric analyses of flow cytometrically sex sorted spermatozoa stored for 0, 2, 5 and 10 h.

Spermatozoa (%)	Unsorted	Sorted and Stored			
		0 h	2 h	5 h	10 h
Live[1]	85.9±0.63^{a}	78.5±1.92^{a,b}	75.3±2.42^{a,b}	74.1±3.18^{ab}	70.3±2.8^{b}
Dead[2]	13.4±0.82^{a}	19.6±1.15^{a,b}	22.4±0.96^{a,b}	23.2±1.19^{ab}	26.2±1.01^{b}
Moribund[3]	0.67±0.13^{a}	1.9±0.13^{b}	2.3±0.11^{b}	2.7±0.09^{b,c}	3.5±0.12^{c}

[1] live spermatozoa, SY+/PI-; [2] dead spermatozoa, SY-/PI+; [3] moribund spermatozoa, SY+/PI+.
$^{a-c}$: Values with different superscripts within the same sperm population stained (same row) differ significantly.

3.4.3. Analysis of acrosomal exocytosis

The results are presented in Table 2. Although sex sorting significantly decreased ($p<0.05$) the percentages of live spermatozoa with intact acrosomes (PNA-/PI-) compared with the unsorted spermatozoa, high levels of intact spermatozoa (around 65-70%) were obtained after sorting and maintained during all storage periods.

Significant differences ($p<0.05$) in percentage of live cells with acrosomal exocytosis were found between spermatozoa before and after sorting. However, this percentage was maintained during the storage time ($p>0.05$) ranging from 3.6±0.92 just after sorting to 5.6±0.92 when spermatozoa were stored 10 h after sorting.

Table 2. Percentage (mean ± SEM) of sperm population with acrosomal exocytosis identified with FITC-PNA lectin (PNA) and Propidium Iodide (PI) after cytometry analyses of flow cytometrically sex sorted spermatozoa stored during 0, 2, 5 and 10 h.

Spermatozoa (%)	Unsorted	Sorted and stored			
		0 h	2 h	5 h	10 h
Acrosome Intact[1]	83.3±1.44[a]	74.1±2.11[b]	69.0±1.87[b]	68.8±2.55[b]	65.6±3.02[b]
Acrosome Reacting[2]	1.4±0.12[a]	3.6±0.92[b]	4.4±1.19[b]	3.9±0.82[b]	5.6±0.94[b]
Dead[3]	15.3±0.54[a]	22.3±2.57[b]	26.6±2.90[b]	27.3±3.54[b]	28.8±4.05[b]

[1] Acrosome Intact, live spermatozoa with an intact acrosome (PNA-/PI-) [2] Acrosome Reacting, live spermatozoa with an acrosome reacting (PNA+/PI-), [3] Dead, dead spermatozoa (PI+).
[a-b]: **Values with different superscripts within the same sperm population stained (same row) differ significantly.**

3.4.4 Analysis of IVM pig oocytes penetration ability

To evaluate the penetration rates following co-culture of SS boar spermatozoa with IVM oocytes for 2h, 3h, 6h or 18h, a total of 5,060 IVM oocytes were inseminated in five replicates. The results are summarized in Figure 2. At 2h after co-incubation, around 20% of oocytes were penetrated when either SS spermatozoa non stored or stored for 2 hours were inseminated. No differences with unsorted spermatozoa were found ($p>0.05$). Penetrations of spermatozoa stored more than 2 h remained lower ($p<0.05$). A sperm-oocyte co-incubation period of 3 h increased the penetration rate, reaching 55% for SS spermatozoa stored for 2 h as well as in unsorted cells. Sperm penetration levels around 80% were not reached unless the co-incubation time was of 6 h or above. Spermatozoa stored for 2 h after sorting were able to penetrate IVM oocytes to similar rates as the unsorted, irrespective of the time of co-incubation with the oocytes. Spermatozoa stored for 5 or 10 h showed, consistently, significantly lower ($p<0.05$) penetration rates than the others groups, irrespective of the co-incubation time. The mean number of spermatozoa that penetrated an oocyte following co-culture of SS and stored boar spermatozoa with IVM oocytes for 2h, 3h, 6h or 18h is presented in Figure 2B. Spermatozoa stored for 2 h after sorting showed the highest number of spermatozoa per oocyte and no differences with the unsorted cells were found, irrespective of the time of co-incubation with the oocytes. Co-incubation just after sorting or storage for 5 or 10 hours negatively ($p<0.05$) affected the number of spermatozoa per oocyte.

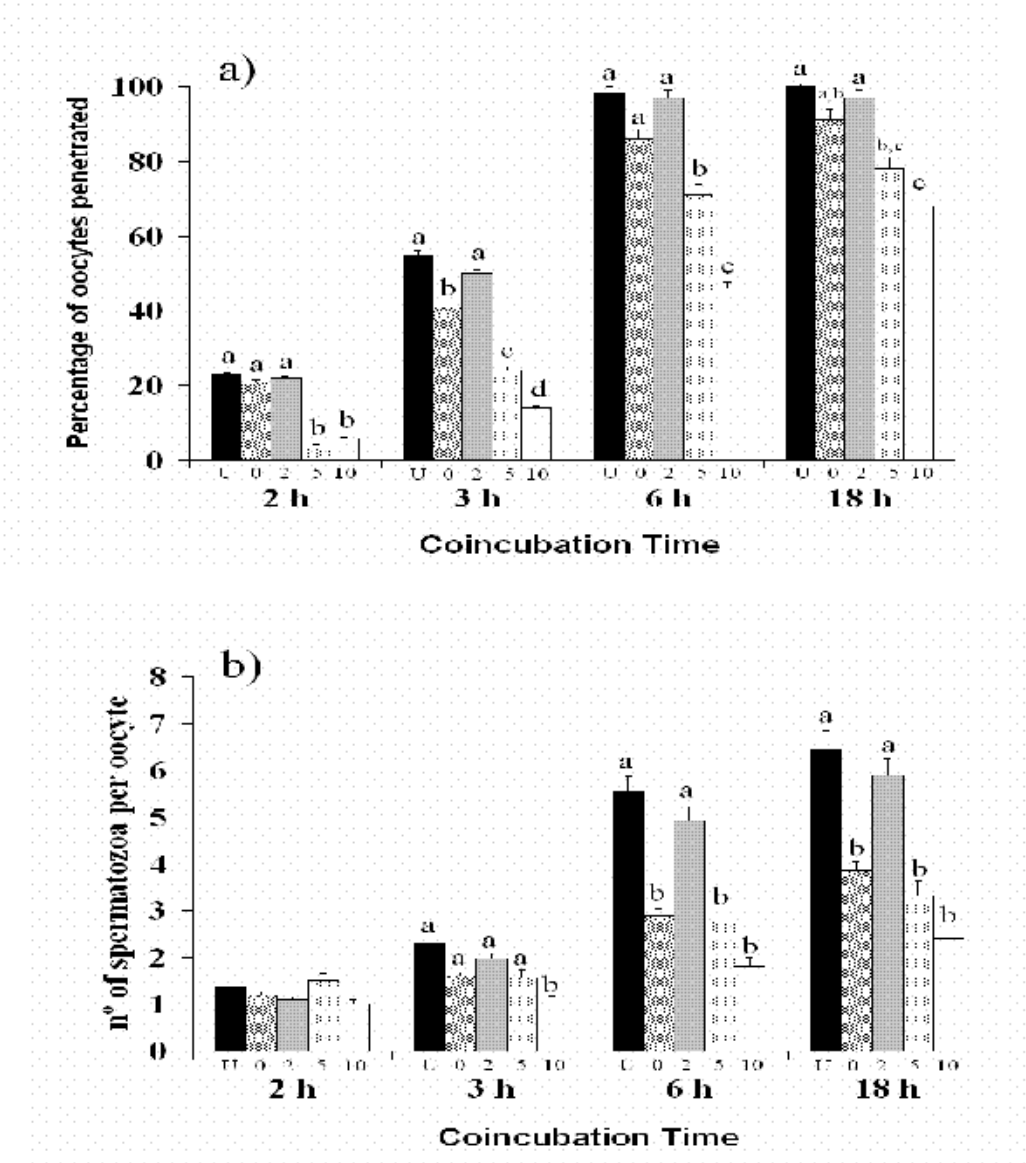

Figure 2: Effect of storage time of unsorted and flow cytometrically sorted spermatozoa on time-course (2, 3, 6 and 18 hours) penetration rates (A) and number of spermatozoa per oocytes (B). U, unsorted spermatozoa; 0, spermatozoa sorted and coincubated just after sorting; 2, spermatozoa sorted and stored 2h; 5, spermatozoa sorted and stored 5h; 10, spermatozoa sorted and stored 10h.

[a-c]: Histogram bars with different lettering differ significantly, $p<0.05$.

3.5. Discussion

Storage of SS spermatozoa in a liquid medium is necessary since the current throughput of sperm sexing instrument is low for the requirements of a practical deep insemination procedure. Because the outcome of the fertilization with SS spermatozoa is suboptimal when compared with unsorted spermatozoa [7], determining how storage time affects these spermatozoa is essential for knowing the sperm fertilizing ability expected after insemination. Thus, and taking account that the final population for insemination will be composed of spermatozoa ranging from cells just sorted to cells sorted and stored for a time longer as 10 hours [7], this study describes the viability, motility, acrosome exocytosis and penetrating ability in matured pig oocytes of SS boar spermatozoa stored after sorting for 0, 2, 5 and 10 hours in TEST-yolk 2% + 10% seminal plasma. This medium has been widely used as collection medium of sorted boar spermatozoa. The protective effect of this medium has been shown on highly diluted boar spermatozoa (unsorted and sorted) when spermatozoa are stored until 4 h [11]. Moreover, this medium preserves the viability, motility and fertilizing ability in unsorted boar spermatozoa stored during 10 h (I. Parrilla, unpublished observations).

Since during the sorting procedure a food dye is added to gate out the dead cells from the sperm population, a highly motile population is obtained after sorting [18]. In our study, viability and the percentage of motile cells were unaffected by the sorting procedure during the first 5 h after sorting despite the fact that in this experiment we have not used, for obvious reason, the food dye to eliminate non-viable spermatozoa.

Although the percentage of motile spermatozoa was unaffected, the motility patterns were affected. An increase in the motility parameters Dance, ALH, VCL and VSL just after sorting (0h of storage time) was observed. Taking account that during the flow sorting procedure the spermatozoa are diluted and processed only using

isotonic media containing balanced salts without presence of membrane-protective components, a cleansing of the sperm plasma membrane appears. Capacitation-like changes have been observed after sorting of both ram and boar spermatozoa [10, 11, 13, 14, 24]. An increase in sperm motility characteristics is related to the onset of the capacitation process [25] and thus, the changes in motility observed in flow sorted spermatozoa might indicate the beginning of the capacitation process in these cells. The motility results of the present study accord with this hypothesis. However, our results also show that this hyperactive motility could be reversed after 2h of storage to reach values similar to those obtained for non-sorted spermatozoa. These findings are probably related to the presence of seminal plasma (10%) in the collection medium, and seem to be related to its ability to stabilize the sperm plasma membrane and to reverse the capacitation status with which the boar spermatozoa emerge from the flow cytometer [14]. Addition of seminal plasma to the collection medium may attenuate the removal of many beneficial seminal plasma components extracted during the high dilution steps. A similar result has been found adding the heterodimer PSP-I/PSP-II obtained from the boar seminal plasma [26]. Although capacitation status was not directly measured in the present study, we evaluated the percentage of cells with exocytosis as a consequence of the capacitation of the spermatozoa. Moreover, the lectin PNA used in the study usually detects the earliest signs of the initiation of acrosome exocytosis, as soon as the fusion pores between the plasma and acrosomal membranes appear [27]. A significant increase in percentages of spermatozoa with acrosomal exocytosis was found in SS spermatozoa with respect to spermatozoa from the control group.

Sorted spermatozoa maintained their motility and viability until 10 hours of holding time after sorting. At this time, motility and viability decreased when compared with the control group. In this way, a slight decrease in the boar sperm viability has been reported when spermatozoa were held for up to 4 hours after sorting [13], but to the best of our knowledge, no data about longer storage times after sorting are available. Although the addition of membrane-protective component to the

collection medium (seminal plasma and/or egg yolk) can help to stabilize the sperm cell, slight changes on the cells related to dilution, pressure and temperature changes cannot be clearly visible just after sorting. Therefore, it could be expected, as occurred in our experiment, that with long holding times (10 h), the percentage of damaged cells increases, reducing the lifespan of sorted boar spermatozoa and compromising their fertilizing ability. However, it could be noted that this reduction is not dramatic. The percentage of sorted spermatozoa with motility and an intact plasma membrane at 10 h of storage after sorting was found, in our case, to be higher than those reported previously [13,14] for sorted boar spermatozoa even when analysed immediately after sorting. This finding could be explained by the development of the current high speed flow sorting technology [18] that allows the sexed sperm concentration to be increased, decreasing the possible sperm damage induced by the sorting procedure [10] and therefore resulting in optimal levels of flow sorted boar spermatozoa functionality.

Although a recent study on ram spermatozoa [28] demonstrated that sorted ram spermatozoa progressed more rapidly to an acrosome reacted state than non sorted spermatozoa over a 6 h of incubation period, we have not observed an increase in the percentages of live spermatozoa with an acrosomal exocytosis related with storing time. However, it should be noted that boar spermatozoa should be less susceptible than ram spermatozoa to the sex sorting procedure as boar spermatozoa are less susceptible than ram spermatozoa to damage for high dilution [29].

Motility, viability and acrosomal exocytosis values are useful characteristics for analysing the impact of the sex sorting procedure on the spermatozoa. However, the power of these assays to predict sperm fertility is limited. Homologous in vitro fertilization using both immature [30] and in vitro matured [31] pig oocytes is, at this moment, the best assay for the evaluation of sperm fertility since a number of physiological and biochemical events must occur in the spermatozoa (e.g. capacitation; acrosome reaction; recognition, attachment and penetration of the zona pellucida; and binding to the plasma membrane of the oocyte). Taking account that the fertilizing ability of the sorted spermatozoa may decrease during storage, the time course of in

vitro penetration of spermatozoa sexed and stored during 0, 2, 5 and 10 hours was examined.

The overall *in vitro* fertilization rates after insemination with sorted and non sorted spermatozoa were high in this study. The concentrations likely used in our study were higher than optimal because polyspermy rates were relatively high (≈ 85%). It should be noted that we did not attempt to optimise sperm concentration for IVF, and this was not an objective of our study. Moreover, the number of spermatozoa per oocyte and consequently, the incidence and degree of polyspermy, is an indication of the number of spermatozoa with ability to fertilize the oocytes [32], one of the objectives pursues in this paper. The sperm penetrating ability was generally lower in sorted spermatozoa (except for spermatozoa sorted and stored during 2 hours) compared with unsorted spermatozoa. This result was not expected since previous reports showed that to achieve a percentage of monospermic oocytes using sorted spermatozoa requires a half reduction of the normal (unsorted) sperm concentration [4]. It should also be noted that, in our experiment, sperm were collected in a medium containing seminal plasma and it has been shown that the presence of seminal plasma in the collection medium substantially reduces the penetration rates of oocytes in vitro [14].

The first evidence for penetration of the in vitro matured oocytes was observed 2 hours after co-culture. At this time, between 5% and 25% of oocytes were penetrated when the oocytes were co-incubated with spermatozoa that had been sorted and stored for 10 h or 2 h, respectively. Maximum penetration was attained at 18 hours in all groups studied. However, there was no significant increase in the penetration rate since 6 hours of co-culture except for spermatozoa stored 10 hours after sorting which reached the maximum and significant penetration rate at 18 h after co-culture. This initial penetration time was in accordance with studies reported previously in vivo [32]. However, our results are disagreeing with others previously reported by our laboratory where the initial penetration time was established in 3 hours. It should be

noted that, in our previous experiment, immature oocytes and freshly ejaculated spermatozoa were used [33].

Surprisingly, sorted spermatozoa stored for 2 hours had higher penetration rates and number of spermatozoa per oocyte than spermatozoa stored 0, 5 or 10 hours. While the weakness of spermatozoa sorted and stored 5 or 10 hours might have been responsible for reducing the number of penetrating spermatozoa, this hypothesis is not valid for spermatozoa inseminated just after sorting. One explanation for this difference in the penetration rates may be based in that the flow sorting procedure induces a scrambling in the plasma membrane components, affecting glycoproteins related with the oocyte recognition and penetration. The 2 hour incubation of the spermatozoa in a medium containing membrane-protective components might reorganize the plasma membrane, restore the fertilizing capability, increasing the percentage of penetrating spermatozoa. However, further research is required to clarify these results. If this explanation turns out to be correct, application of a storage of sorted samples for 2 hours may be needed before insemination with these SS spermatozoa.

In summary and from a practical point of view, the present study shows the necessity of depositing the spermatozoa as near as possible to the ovulation time and site of fertilisation before 5 hours post-sorting because the fertilizing ability of SS spermatozoa is beginning to decrease at this time of storage. However, in the light of our results, it could be noted that storage for 2 hours enhances the fertilizing ability of SS spermatozoa. Consequently, this short storage time could be beneficial when spermatozoa are directly inseminated close to the oocytes in vitro (IVF) or in vivo (e.g. oviductal insemination). In vivo insemination trials are in progress to evaluate whether this in vitro beneficial effect also results in an increase in the in vivo fertility rates. On the other hand, if a high number of spermatozoa are required for insemination (e.g. deep intrauterine insemination), it is necessary to take account the high heterogeneity composition of the sorted sperm population which may be responsible for the outcome of the insemination. Further research is necessary to reduce the heterogeneity of the

sperm population to a minimum, increasing the fertilizing ability of spermatozoa stored from 5 h forward.

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Experiencia 4

Birth of piglets after deep intrauterine insemination with flow cytometrically sorted boar spermatozoa

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4.1 Abstract

The present study was carried out to determine the pregnancy rates, farrowing rates and litter size in sows with either induced or spontaneous ovulation inseminated with flow cytometric sorted spermatozoa using deep intrauterine insemination technology. Spermatozoa were stained with Hoechst 33342 and sorted by flow cytometry/cell sorting but not separated into separate X and Y populations. In Experiment 1, sows (n=200) were weaned and treated for estrus/ovulation induction with eCG/hCG. Inseminations with either sorted (70 or 140 million) or non-sorted (70 or 140 million) spermatozoa were done using a specially designed flexible catheter. Farrowing rates were 39.1 and 78.7% for 70 million of sorted and non-sorted, respectively, and 46.6 and 85.7% for 140 million of sorted and non-sorted, respectively ($P<0.05$). The litter size in sows inseminated with sorted spermatozoa showed a tendency to be lower than when non-sorted spermatozoa were inseminated. In Experiment 2, sows (n=140) were inseminated as in Experiment 1 except that natural estrus was used. The ovaries of these sows were evaluated by transrectal ultrasonography. Farrowing rates were 25 and 77.2% for 70 million of sorted and non-sorted, respectively, and 32 and 80.9% for 140 million of sorted and non-sorted, respectively ($P<0.05$). These results show that the Deep Intrauterine Insemination technology can be successfully used to produce piglets from sorted spermatozoa when sows are hormonally treated to induce synchronous post weaning oestrus and ovulation.

Keywords: Pig; Flow cytometry; Deep insemination

4.2 Introduction

Pre-selecting the sex of the offspring will have a significant impact on pig production. The application of sex pre-selection to genetic programs in pig production will accelerate genetic progress. The only effective sex pre-selection methodology [1, 2] has been validated in numerous laboratories and is based on measuring the differential amount of DNA present in X- and Y-chromosome bearing spermatozoa. This difference is measured in a modified flow cytometer/cell sorter and the spermatozoa sorted into populations enriched for X- or Y-bearing spermatozoa with a high percentage of purity. The current production rate using the Beltsville sperm sexing technology (BSST) is 10 to 15 million X and Y sorted populations per h. Purities of the X and Y range from 85 to 95% [3]. The effectiveness of this technology for producing offspring in swine of the preselected sex has been proven in numerous trials [2,4,5,6,7] were the semen was sorted and surgically inseminated or used for in vitro fertilisation or intracytoplasmatic sperm injection and subsequent embryo transfer.

Any methodology applied to sexing swine spermatozoa must search for the appropriate application in the industry. The most economical means of using sexed boar spermatozoa in practical settings would be through non-surgical low dose artificial insemination of sows. Although the efficiency of the sorting procedure has increased considerably during the last five years with the development of improved nozzle designs [8] and adaptation to high speed cell sorting [3], its usefulness remains limited in the pig due to the large number of sperm required for routine cervical insemination (2×10^9 to 3×10^9 spermatozoa per insemination dose) under industry conditions.

It is known that the spermatozoal membrane is partially compromised during the flow cytometry/sorting process which affects viability, storage capability and fertilisation. This is due to the different stages that the spermatozoa must pass during

the sorting process. These stages involve staining and incubation of the spermatozoa with Hoechst 33342, sperm dilution, exposure to high pressure and to the laser beam; the rapid projection into the collection tubes and lastly the centrifugation process necessary to concentrate the sorted spermatozoa. After sorting, the spermatozoa have a short lifespan. Thus, there is an acute necessity to deposit the sexed spermatozoa close to the site of fertilisation to avoid premature death that would likely occur during the movement of the spermatozoa from the cervix to the oviduct [9]. Previous studies [2, 4] have sought to overcome this difficulty by applying surgical intratubal insemination to deposit the spermatozoa directly into the oviduct. Such a technique is useful for research purposes but is unlikely to be applied effectively in practical pig production.

Recently, a new procedure for non-surgical deep intrauterine insemination (DUI) in non-sedated sows has been described [10]. This procedure is based on a previous system using a fiberoptic endoscope [11] and it consists of a specially designed flexible catheter that allows passage through the cervix and deposition of the sperm in the anterior region of the uterine horn. Using this DUI procedure it has been demonstrated that only 50 million spermatozoa are required to achieve pregnancy. Based on these result, we have hypothesised that 50 to 70 million sorted spermatozoa would be sufficient to attain pregnancy in sows with our DUI technique without a decrease in farrowing rates. However, even with this low number of sperm inseminated, sorting sufficient numbers of sperm to achieve pregnancies with either X- or Y- sorted sperm to ascertain the effectiveness of the technique with sexed sperm is difficult at the present time. In other species, the use of sexed sperm in conjunction with deep uterine insemination [12, 13] has been found to be effective for producing sexed offspring.

Larsson [14] observed that insemination close to ovulation led to the highest fertility results when hCG-treated sows were inseminated with frozen semen. Since the spermatozoal membranes may be compromised by flow cytometric sorting (FCS), we determined it advantageous to induce ovulation in conjunction with the use of DUI. Ovulation time can also be determined by frequently repeating ultrasound examination

[15]. The use of the ultrasound to evaluate the follicular diameter as an approach to pin-point ovulation time could help to avoid the necessity for inducing ovulation with hormones.

The aim of this study was to ascertain the effectiveness of the DIU when flow cytometrically sorted boar spermatozoa (mimicking the conditions that sperm are exposed to during the BSST) are inseminated in sows after induction of ovulation or in which spontaneous ovulation was detected by ultrasongraphy.

4.3. Material and Methods

4.3.1. Semen processing

Experiments were carried out with semen from two mature boars which had previously sired offspring. The boars were housed in individual pens in controlled environment (20 ± 2°C). Sperm-rich ejaculate fractions were obtained using the gloved-hand method and diluted in Beltsville thawing solution (BTS) [16] to 30 x 10^6 spermatozoa/ml. Shortly after collection, the semen samples were evaluated and used if they met the minimum criteria: percentage of motile spermatozoa >80%, viability using propidium iodide > 85%, hypo-osmotic swelling response > 70%, total sperm per ejaculate >20 x 10^9, acrosomal abnormalities < 10%, abnormal sperm morphology <15% [17]. Diluted spermatozoa were splited. An aliquot of 500 ml was used for FCS while the rest was kept at 17°C until the insemination of the sows used as controls.

Samples of semen were prepared for flow cytometric sorting using the BSST protocol [1] as adapted for high speed sorting [3] except that all spermatozoa (oriented and non- oriented) were sorted and collected into a single tube. These spermatozoa are described as flow cytometric sorted sperm (FCS sperm). Briefly, Hoechst 33342 (9 µM) (Sigma Chemical Co., Alcobendas, Spain) was added to 30 ml of extended

semen, following by dark incubation for 1h at 35°C. After incubation, samples were filtered though a 60μ nylon mesh filter to remove debris or clumped sperm.

The stained spermatozoa were sorted using an EPICS Altra high speed flow sorter (Beckman Coulter Corporation, Miami, FL. USA) operating at 3.655 kg/cm^2 and modified as described by Johnson and Pinkel [18] with a Model 90C-6, 6-watt argon laser operated in the ultraviolet (351,364 nm) at 175 mW (Coherent Lasers, Inc., Santa Clara, CA).

FCS spermatozoa were collected in 50 ml BSA coated plastic tubes containing 5 ml of TEST-yolk (2%) extender [1] containing 10% of boar seminal plasma and stored at 22°C until all spermatozoa were collected. The average time for the sorting procedure was 10h. After collection, spermatozoa were transferred to 10 ml BSA-coated tubes and concentrated by centrifugation at 500 x g for 4 min to 70 million spermatozoa or 140 million spermatozoa in 7.5 ml. Only samples showing more than 65% of motility and 70% of intact acrosomes after sorting were used for insemination.

4.3.2.-. DUI Technology

The protocol used for the DUI was that described by Martinez et al. [10]. DUI took place for each sow in gestation crates and were performed without sedation of the female. After thorough cleaning of the perineal area of the sows, a commercial disposable artificial insemination (AI) catheter 'spirette' (Magapor, Zaragoza, Spain) was inserted through the vagina into the cervix and used to manipulate a specially designed flexible catheter (working length 1.80 m, outer diameter 4 mm, diameter of the inner tubing 1.80 mm). The flexible catheter was inserted through the spirette, moved through the cervical canal and propelled forward along one uterine horn until all of its length had been inserted. Before inseminations, the inner tubing of the flexible catheter was rinsed with diluent and refilled with approximately 2 ml of diluent at 22°C. Predetermined insemination doses of spermatozoa in a volume of 7.5

ml at 22°C were flushed into one uterine horn using a 10 ml disposable syringe attached to the inner tubing of the flexible catheter. Then, an extra 2 ml of diluent alone was used to force all remaining spermatozoa out of the flexible catheter and into the uterine horn.

4.3.3. Experimental design

Experiments 1 and 2 were carried out into a single commercial farm housing 3.800 sows.

4.3.3.1. Experiment 1: Deep Intrauterine Insemination in sows with induced ovulation.

A total of 280 cross bred sows (2-6 parity) were weaned at 21.01 ± 0.04 days. Estrus was induced by injection of each female intramuscularly with 1250 IU equine chorionic gonadotrophin (eCG) (Folligon, Intervet International B.V., Boxmeer, The Netherlands) 24 h after weaning followed 72 h later with 750 IU human chorionic gonadotrophin (hCG) (Chorulon, Intervet International B.V., Boxmeer, The Netherlands). Estrous detection was performed once a day (7:00 am), beginning 2 days after eCG injection, by allowing females nose-to-nose contact with a mature boar and by applying back pressure. Sows that exhibited a standing heat reflex were considered to be in estrus and inseminated, but only if heat was detected within 24 h of hCG injection. Inseminations using the DUI technology were performed as described at 38h after hCG administration.

Each week sows with induced ovulation were divided in four groups. The inseminations groups were Group A (FCS-low): 70 million of FCS spermatozoa (n=46) and Group B (FCS-high): 140 of FCS spermatozoa (n=45); Control groups were Group C (non-FCS-low): 50 million of non-FCS spermatozoa (n=47) and Group

D (non-FCS-high): 140 million of non-FCS spermatozoa (n=49). Pregnancy was diagnosed two times at 24-28 days and 50-55 days after insemination by transcutaneous ultrasonography (Pie Medical, Maastricht, The Netherlands). All pregnant animals were allowed to carry litters to term and farrowing rates and litter size were obtained.

4.3.3.2.. Experiment 2: Deep Intrauterine Insemination in sows with spontaneous ovulation.

Cross bred sows (2-6 parity) were weaned at 21.35 ± 0.07 days (n=140). Estrous detection was performed twice per day, beginning 3 days after weaning, as described before. Sows that exhibited a standing heat reflex were considered to be in estrus. The ovaries of sows were examined 33-35 h after the onset of estrus by transrectal ultrasonography using a 5.0 MHz multiple scan angle transducer for the presence of pre-ovulatory follicles. Only sows showing multiple pre-ovulatory follicles (diameter of antrum > 6 mm) [19] were selected for insemination. Inseminations were carried out within 1 hour after ultrasonography.

Sows were divided into the following experimental groups: Group A (FCS-low): 70 million of FCS spermatozoa (n=26) and Group B (FCS-high): 140 million of FCS spermatozoa (n=26). Control groups were Group C (non-FCS-low): 70 million of non-FCS spermatozoa (n=26) and Group D (non-FCS-high): 140 million of non-FCS spermatozoa (n=25).

Eighth to ten h after insemination, transrectal ultrasonography was done again to scan a decrease greater than 20% in the number of follicles on that ovary as signal of ovulation. Pregnancy rates, farrowing rates and litter size were evaluated as described above.

4.3.4. Statistical Analysis

Data is expressed as percentages or means ± SEM and differences were considered to be significant at $p<0.05$. The percentage of sows within each insemination group for pregnancy and farrowing was compared using a Chi square test with Yate´s correction. The effect of the source and number of spermatozoa inseminated on the litter size was analyzed using the procedure GLM (ANOVA) of SPSS 9.0/PC statistics package (SPSS Inc. Chicago, IL).

4.4. Results

4.4.1 Experiment 1

The data shown are from 10 consecutive weeks. In total, 200 sows were hormonally treated to induce estrus. Thirteen sows (6.5%) showed no obvious signs of estrus at 24 h around the time of hCG injection and were excluded from the study.

Pregnancy rates, farrowing rates and litter size were not significantly different between boars (data not shown) ($P>0.05$), so the data were pooled. Pregnancy rates, farrowing rates and litter size are shown in Table 1.

Sows inseminated with FCS-low group had a lower but no significantly different pregnancy rate (45.6%) than sows inseminated with FCS-high group (54.3%). Both groups were significantly lower ($P<0.05$) than the controls, non-FCS-low (80.8%) and non-FCS-high (85.7%). There was no significant difference in pregnancy rates between sows inseminates with non-FCS-low and those inseminated with non-FCS-high groups.

Table 1. Pregnancy rates, farrowing rates and litter size in sows with induced ovulation following insemination with 70 million of flow cytometric sorted spermatozoa (FCS-low), 140 million of flow cytometric sorted spermatozoa (FCS-high), 70 million of non-FCS spermatozoa (non-FCS-low) and 140 million of non-FCS spermatozoa (non-FCS-high).

Treatment Group	Number of sows inseminated	Pregnancy Rates (%)	Farrowing Rates (%)	Litter Size (n ± SEM)
FCS-Low	46	45.6[a]	39.1[a]	8.7 ± 1.3
FCS-High	45	54.3[a]	46.6[a]	9.2 ± 0.9
Non-FCS-low	47	80.8[b]	78.7[b]	9.8 ± 0.3
Non-FCS-high	49	85.7[b]	85.7[b]	9.9 ± 0.4

Values within columns with different superscripts are significantly different ($p<0.05$)

Three (14.2%) and four (16%) sows lost their pregnancy between the days 24-28 and 50-55 (positive for pregnancy in the first ultrasound evaluation and negative for pregnancy in the second ultrasound evaluation) for FCS-low and FCS-high groups, respectively. However, only one (2.6%) and zero (0%) sows lost their pregnancy after day 26 for non-FCS-low and -high groups, respectively. Therefore, the differences observed in pregnancy rates between sorted and unsorted spermatozoa groups were increased when the farrowing rates were analysed ($p<0.05$). Besides, FCS-high group (46.6%) was higher but not significantly higher the FCS-low group (39.1%). By contrast, the flow cytometric process and the number of spermatozoa inseminated did not affect the total number of piglets born.

4.4.2. Experiment 2

The data shown are from 8 consecutive weeks. In total, 140 sows were ultrasonographically diagnosed for follicular size. Thirty seven sows (26.4%) showed no multiple follicles with more than 6 mm at 36 hours after onset of estrus and were excluded from the study. Fifteen sows (15/103; 14.5%) showed no decrease greater than 20% in the follicle number 8-10h after insemination and were excluded from the study. Pregnancy rates, farrowing rates and litter size were not significantly different between boars ($P>0.05$) (data not shown), so the data were pooled. Pregnancy rates, farrowing rates and litter size are shown in Table 2.

There was no difference in pregnancy rates between control groups (non-FCS-low=17/22, 77.2% and non-FCS-high=18/21, 85.7%). Regarding the FCS spermatozoa groups, significantly lower pregnancy rates were seen using both low and high number of FCS spermatozoa (FCS-low=7/20, 35%; FCS-high=10/25, 40%). As described in Experiment 1, sows inseminated with FCS spermatozoa showed a higher pregnancy loss than those inseminated with control spermatozoa. The flow cytometric process and the number of spermatozoa inseminated did not affect the total number of piglets born, although a tendency to small litter size was observed in sows inseminated with sorted spermatozoa.

Table 2. Pregnancy rates, farrowing rates and litter size in sows with spontaneous ovulation following insemination with 70 million of flow cytometric sorted spermatozoa (FCS-low), 140 million of flow cytometric sorted spermatozoa (FCS-high), 70 million of non-FCS spermatozoa (non-FCS-low) and 140 million of non-FCS spermatozoa (non-FCS-high).

Treatment group	Number of sows inseminated	Pregnancy rates (%)	Farrowing rates (%)	Litter Size (n± SEM)
FCS-Low	20	35[a]	25[a]	8.1 ± 1.3
FCS-High	25	40[a]	32[a]	8.2 ± 1.1
non-FCS-low	22	77.2[b]	77.2[b]	9.1 ± 0.9
non-FCS-high	21	85.7[b]	80.9[b]	9.5 ± 0.5

Values within columns with different superscripts are significantly different ($P<0.05$)

4.5. Discussion

Currently, only 10 to 15 million sperm on the average can be sorted into X and Y populations per h using high speed flow sorting [3] using the newest sperm sorting modified equipment. This yield is much improved over yields achieved with standard speed sorting (250,000/h) [2, 20] but still too slow to be economical for routine AI of swine. In our view, the use of deep uterine insemination provides the best opportunity for overcoming the high numbers of spermatozoa required for routine AI in the pig. In this study, we have used semen sorted by bulk methods. This process subjects sperm to all aspects of flow sorting (BSST) and permits rapid accumulation of large numbers of sorted spermatozoa without sorting them into separate X and Y populations.

The effects of sorting on sperm has been described in detail by Maxwell and Johnson [9] and Maxwell et al. [21] when they observed that the flow sorting of

spermatozoa alters the membrane status of spermatozoa as assessed by chlorotetracycline fluorescence. In the same way, Parrilla et al [22] revealed important differences in the motility patterns related to hyperactivated motility in flow sorted boar sperm. These spermatozoa have a short lifespan [23, 9]. Martinez et al., [10] described a specially designed disposable flexible catheter that allows to inseminate in the anterior third of one uterine horn. DUI could be an useful tool in the insemination of low numbers of sperm with compromised membranes like sorted spermatozoa are. However, placing the sperm in proximity to the oocytes needs to be done at the proper time (ovulation time) to maximize pregnancy rates. Since the use of the DUI catheter gives proper placement of the sperm, Experiment 1 was designed to place the sperm in conjunction with ovulation time.

Hühn et al. [24] observed a clinical estrus in >95% of sows treated with eCG with a mean interval of 4.5 days between eCG administration and estrous onset. These results are in accordance with the results described in Experiment 1 where only 6.5% of sows treated with eCG showed no signs of estrus three days after the hormone administration. With the use of hCG 72 h after eCG to induce final follicular maturation and accurately time the moment of ovulation [25], we were able to inseminate at a fixed-time, 36h after hCG administration.

In spite of the synchronization methodology used in Experiment 1, inseminations using FCS spermatozoa reduced fertility levels when compared with controls (diluted spermatozoa) in sows with induced ovulation. These results could be explained on the basis that FCS spermatozoa could be potentially damaged by the fluorochrome Hoechst 33342, flowing pass through the laser beam, the projection into the collection tube and the high dilution rate inherent in this methodology [3, 20, 26]. Besides, the time needed from the beginning of the flow cytometric process to the time of DUI (10 h) could generate sublethal injuries in the less stable FCS spermatozoa, decreasing their fertilizing ability. However, the success of the Experiment 1 is the low but reasonable pregnancy rates (45.6-54.3%) achieved with low numbers of sorted spermatozoa inseminating only one time. Johnson et al [27] reported only one sow

pregnant from seven sows inseminated into the lower end of the uterine horn with 60-400 million sorted spermatozoa. Deep insemination into the uterine horn used in this study is likely to be more effective than insemination into the lower uterine horn when FCS are used.

About 15% of the sows lost their pregnancy between days 22-25 and 50-55 when FCS spermatozoa are used in contrast with 1% of pregnancy loss in sows inseminated with untreated spermatozoa. It was also clear that in some sows that lost their pregnancies, the embryonic vesicles were smaller by day 24-25 than the embryonic vesicles in sows than did not lose their pregnancies (data not shown). Therefore, farrowing rates were lower than pregnancy rates when FCS spermatozoa were inseminated. Johnson et al. [1] in rabbits and Johnson [2] in swine showed that the embryo survival rates decrease using flow-sorted spermatozoa. In the same way, McNutt and Johnson [28], Seidel et al. [12] and Buchanan et al [13] observed similar results in rabbits, cattle and mares respectively. By contrast Seidel et al [29] reported only one pregnancy loss from 34 pregnant heifers at the day 64-67. Further experiments are needed to fully understand the reason for the embryonic loss and to exclude DNA damage in some spermatozoa, paternal contribution to the embryos.

The litter size in sows inseminated with FCS spermatozoa showed a tendency to be lower than when non-FCS spermatozoa were inseminated although no statistically differences were observed. Increasing the number of animals involved per treatment group, litter size could be statistically different.

Vazquez et al. [30] showed that it is possible to obtain high pregnancy and farrowing rates in sows with spontaneous ovulation when DUI was used with low numbers of diluted spermatozoa. We determined that it was possible to expect imminent spontaneous ovulations in sows with follicles larger than 6 mm and to inseminate sows that presented these kind of follicles. However, although the ovulation occurred in a high percentage (86.5%) of these sows in a 8-10h period (between the first and second transrectal ultrasonography), the pregnancy rates and farrowing rates dropped to unreasonable rates when sows with spontaneous ovulation

were inseminated using DUI with FCS spermatozoa. However, farrowing rates obtained from inseminating control spermatozoa were reasonably high (77.2 and 80.9% for non-FCS-low and non-FCS-high, respectively). The most important reason that could help to explain these results is that the variability in the onset of ovulation in relation to the time of insemination in high in contrast with sows with induced ovulation where the ovulations occurred 37-40h after the hCG injection. It is note worthy too, that Experiments 1 and 2 were not carried out simultaneously, although pregnancy rates were similar between experiments when control spermatozoa were inseminated.

With the best of the current technology on sperm sorting [3], it would suggest that a five hours sort would be required for attaining sexed piglets (sorting sperm specifically for X or Y). There is no doubt that a five hour sort limits the practical use of DUI in conjunction with sexed sorted sperm, however the use of DUI and the BSST to produce piglets of the desired sex is clearly reality for specialized swine management situations.

In conclusion, this is the first report showing that the DUI Technology can be successfully used to produce piglets from sperm flow cytometrically sorted by the BSST. Although fertility of FCS spermatozoa is lower than for non-FCS sperm, results obtained inseminating 140 million of FCS spermatozoa in sows with induced ovulation can be considered as acceptable. However, further experiments are needed with sperm sorted into X and Y bearing populations to determine the true feasibility of the DUI for producing litters of pigs pre-selected for sex. It is apparent that the DUI Technology will be critical to the application of sex sorted spermatozoa under standard conditions

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Experiencia 5

Hoechst 33342 stain and u.v. laser exposure do not induce genotoxic effects in flow-sorted boar spermatozoa

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5.1 Abstract

Sex selection by flow cytometry/cell sorting involves the staining of spermatozoa with Hoechst 33342 in combination with the impact of a u.v. laser beam, two potentially mutagenic agents. A phenotypic and cytogenetic study in lymphocytes of piglets born after insemination with spermatozoa stained with Hoechst 33342, and from piglets obtained from stained-sorted spermatozoa was performed to evaluate the genotoxic effect of Hoechst 33342 staining and u.v. laser irradiation on the offspring. Lymphocytes from piglets born after insemination with unstained spermatozoa, but from the same ejaculate, were used as a control group. Peripheral blood lymphocytes from these piglets were cultured following a standard cell culture protocol. Cells were then collected by centrifugation, subjected to hypotonic solution and fixed and dropped onto slides. Sister Chromatid Exchanges (SCEs) and chromosome aberrations (CAs: including chromosome and chromatid breaks) per cell were scored in 50 second division metaphase spreads from each donor. Reproductive parameters and litter performance of all inseminations performed were also recorded in all groups. Data were analyzed by ANOVA. No significant increase ($P>0.05$) of SCE and CA frequencies were observed in piglets born from stained spermatozoa, or from stained-sorted with respect to controls (untreated sperm). The results indicate that no mutagenic effect on spermatozoa, expressed as increases in the incidence of abnormalities in the resulting offspring, and also as increases in SCE and CA frequencies on lymphocytes from these individuals, was induced by the staining of boar spermatozoa with Hoechst 33342, nor by combination of the staining with laser impact during flow cytometry.

5.2 Introduction

Flow cytometrically sorted X- and Y- chromosome bearing spermatozoa have been successfully used to obtain offspring in domestic animals and humans using several assisted reproductive technologies (reviewed by Johnson, 2000). Flow cytometric sorting is widely accepted as a safe technique in practice, in the absence of phenotypic evidence that suggests otherwise. As far as we know, the only disadvantageous effects described when flow sorted spermatozoa are used for insemination are the short fertile life span of the spermatozoa as well as the loss of embryos during gestation, expressed as a reduced litter size in both rabbits (Johnson et al., 1989) and pigs (Johnson, 1991; Vazquez et al., 2003). However, the reason for this reduction remains unclear.

It is well known that Hoechst 33342 could have toxic and mutagenic effects upon certain cell types (Durand and Olive, 1982), and also that u.v. light produces an increase in chromosome structural abnormalities in mouse spermatozoa (Matsuda and Tobari, 1988). Concerns have been raised that simultaneous use of both agents may affect the genetic safety of sperm selection by flow cytometry (Ashwood-Smith, 1994; Munné, 1994; Gardiner-Garner, 1999) and, consequently, the necessity to evaluate DNA damage on the sorted spermatozoa has become a priority in spite of the absence of congenital abnormalities (Morrel and Dresser, 1989). However, no cytogenetic studies about potentially mutagenic effect of flow cytometric sorting technology on the animals born after inseminations with spermatozoa processed with this technology have been reported.

Evaluation of the increases in baseline frequencies of cytogenetic endpoints as sister chromatid exchanges (SCEs) and chromosome aberrations (CAs) has been used for many years to measure the possible mutagenic and carcinogenic effect when cells, animal or human, are exposed to known genotoxic agents (Perry and Evans, 1975; Latt et al., 1981; Albertini et al., 2000).

While it has been determined that SCE is an ideal method for evaluating the genotoxic potential of those substances that induce DNA damage or interfere with DNA metabolism or repair, CA has been more related with those substances that directly break the backbone of DNA or significantly distort the DNA helix (Carrano and Natarajan, 1988).

Consequently, the cytogenetic analysis using SCE and CA of animals born after inseminations with spermatozoa stained with Hoechst 33342 and/or flow cytometrically sorted could represent an ideal means of determining the safety of this sex selection procedure.

The aim of the present study was to evaluate the phenotypic and cytogenetic normalcy of piglets born after inseminations with Hoechst-33342 stained or stained and flow cytometrically -sorted boar spermatozoa.

5.3 Material and Methods

All reagents used in this study were provided by Sigma-Aldrich Co. (Alcobendas, Madrid), unless otherwise stated.

5.3.1. Animals

All animal experiments were approved by the ethical committee for animal experiments of the University of Murcia, Spain.

Animals were obtained from a commercial pig farm in Murcia (Spain). All males and females used for artificial insemination (AI) were sexually mature crossbred pigs. Sows (parity of two to six) were selected on the day of weaning and allocated individually to crates in a mechanically ventilated confinement facility. Mature boars of proven fertility were housed in individual pens in a controlled environment

(23°±2°C). Animals received a commercial diet according to their reproductive condition, water being available ad libitum. Piglets generated from matings between these breeding animals were allocated into groups in a mechanically ventilated confinement facility and fed with a commercial ration twice a day, water being available ad libitum.

5.3.2. Semen collection and processing.

Sperm-rich ejaculate fractions were obtained from five boars using the gloved-hand method, pooled and extended in Beltsville Thawing Solution (BTS; 205.0 mmol glucose, 20.39 mmol NaCl, 5.4 mmol KCl, 15.01 mmol $NaHCO_3$ and 3.35 mmol EDTA (Johnson et al., 1988)) to 30 x 10^6 spermatozoa/ml. Shortly after collection, the semen samples were evaluated and used if they met the minimum criteria: motility >80%, viability > 85%, acrosomal abnormalities < 10%, abnormal sperm morphology < 15% (Vazquez et al., 1997).

Spermatozoa were prepared using the Beltsville sperm sorting technology protocol as adapted for high speed sorting (Johnson et al., 1999) except that all spermatozoa (oriented and non-oriented) were sorted into a single tube. Extended spermatozoa were incubated in the dark with Hoechst 33342 (0.3 µM /1x10^6 spermatozoa) for 1h at 35°C. After incubation, samples were filtered through a 30 µm nylon mesh filter to remove debris or clumped spermatozoa. These spermatozoa were sorted using an EPICS Altra high speed flow sorter (Beckman Coulter Corporation, Miami, FL, USA) operating at 42 p.s.i. and modified as described Johnson and Pinkel (1986) with a Model 90C-6, 6 – watt argon laser operated in the u.v. light (351,364 nm) at 175 mW (Coherent Lasers, Inc., Santa Clara, CA, USA). Flow cytometrically sorted spermatozoa (FCS sperm) were collected in 50 ml BSA-coated plastic tubes containing 5 ml of TEST-yolk (218 mmol TES, 56.1 mmol TRIS, 33.2 mmol glucose and 2% v/v of fresh egg yolk) extender (Johnson et al., 1989) and 10 % of boar seminal plasma. FCS sperm were stored at 22°C until they were used. Spermatozoa were transferred to 10 ml BSA-coated tubes and concentrated by centrifugation at 500

x g for 4 min to 150 million spermatozoa in 7.5 ml. Only samples showing more than 65% motility and 70% intact acrosomes after sorting were used for insemination.

5.3.3. Artificial insemination

AI was carried out, depending on the experiment, intracervically using an AI catheter (Minitüb Tiefenbach, Germany) or into the depth of one uterine horn using the deep intrauterine insemination technique (DUI) (Martinez et al., 2002).

Standard AI into the cervix was performed in sows with spontaneous ovulation. Detection of oestrus was performed twice per day, beginning 3 days after weaning by allowing females nose-to-nose contact with a mature boar and by applying back pressure. Sows that exhibited a standing heat reflex were considered to be in oestrus and inseminated. AI into the cervix was performed twice, at 0 and 24 h after the onset of standing heat.

Since the membranes of FCS spermatozoa may be compromised, we considered it would be advantageous to induce ovulation in conjunction with the use of DUI. Oestrus was induced by injection of each female intramuscularly with 1250 IU equine chorionic gonadotrophin (Folligon, Intervet International B.V., Boxmeer, The Netherlands) 24 h after weaning followed 72 h later with 750 IU human chorionic gonadotrophin (hCG) (Chorulon, Intervet International B.V., Boxmeer, The Netherlands). DUI was performed in each sow in gestation crates without sedation, 38 h after hCG administration. After thorough cleaning of the perineal area of the sow, a commercial AI catheter was inserted through the vagina into the cervix and used to manipulate a specially designed flexible catheter (working length 1.80 cm, outer diameter 4 mm, diameter of the inner tubing 1.80 mm). The flexible catheter was inserted through the spirette, moved through the cervical canal and propelled forward along one uterine horn until all of his length had been inserted. Before insemination the inner tubing of the flexible catheter was rinsed with BTS and refilled with approximately 2 ml of BTS at 22°C. Insemination doses at 22°C were flushed into one

uterine horn using a 10 ml disposable syringe attached to the inner tubing of the flexible catheter. AN extra 2 ml BTS alone was used to force all remaining spermatozoa out of the flexible catheter and into the uterine horn.

5.3.4. Evaluation of reproductive parameters

Pregnancy was diagnosed at 24-28 days after AI and 15 days later, by transcutaneous ultrasonography (Pie Medical, Maastricht, The Nethelands). All pregnant animals were allowed to go to term. Farrowing rates and litter sizes were registered.

5.3.5. Evaluation of the normality of offspring

5.3.5.1. Phenotypic evaluation.

Numbers of live, dead, mummified and morphologically abnormal piglets were registered in all litters. All piglets born live were weighed within 2 h after birth. Each pig was subjected to a daily health inspection, during the first fifteen days, and the following conditions noted: ability to stand, unusual discharges from the mouth, bowels, urethra, or vagina, eyes or nose. All piglets were weaned at 21 days of age. Weights at 21, 42 and 92 days were registered.

5.3.5.2. Cytogenetic evaluation.

SCEs and structural CA were used as biomarkers for the evaluation of DNA damage of lymphocytes of piglets born after insemination of the sows with stained or stained and sorted spermatozoa.

Peripheral blood was aseptically collected from piglets between 3 and 4 months of age (approximately 40-50 Kg weight). Cell suspensions were cultured following the protocol described for goat metaphases by Di Berardino et al. (1996) with minor modifications. Briefly, aliquots of 0.5 ml whole blood containing 6 x 10^6 lymphocytes were added to each culture flasks containing 8 ml of RPMI 1640 medium without L-glutamine (Gibco Life Technologies, Barcelona), including 1 ml of fetal bovine serum, 0.1 ml of L-glutamine (Gibco), 50 µl of antibiotic/antimycotic solution, and 0.1 ml of Pokeweed mitogen (Lectin from *Phytolacca Americana*) to stimulate mitogenetic activity . All cultures were allowed to grow for 68 h at 38.5 °C. After 48 h from initiation, 0'1 µg/ml of bromodeoxyuridine (BrdU) was added to each culture flask. This concentration of BrdU was the optimal dose obtained in a preliminary study to determine a concentration of BrdU sufficient for sister chromatid differentiation and yet having a minimal effect in the baseline frequencies of SCEs (I Parrilla, J M Vázquez, C Cuello, M A Gil, J Roca, D Di Berardino, & E A Martínez, unpublished observations). Cultures without BrdU were included in order to analyse the frequencies of CAs.

The cultures were protected from the light and allowed to grow for an additional 20-22 h at 38.5°C. Colcemid (Gibco), at 0.1 µg/ml of final concentration, was added for the final 15 min prior to harvesting. Harvested cells were collected by centrifugation (370g/10min), subjected to hypotonic solution (75 mM KCl) for 20 min, and fixed in methanol/acetic acid (3:1 v/v). After fixation, the metaphases were dropped onto clean microscope slides and air dried. Air-dried slides were stained with acridine orange at 0.1% (w/v) in phosphate buffer (pH= 7) and sealed with paraffin. Samples were examined under a fluorescence microscope (Leica DMRB Fluo; Heerbrugg, Switzerland) and metaphases were stored by digital photography. SCE and CA data were obtained from the analysis of 50 well-spread metaphases in second division bearing 38 chromosomes (total number of chromosomes in pig cells) and randomly scored per sample. SCEs was counted each time that two adjacent segments of one of the chromatids in a chromosome were stained differently and CA was

counted each time that a discontinuity or displacement greater that the width of the chromatid arm in one or both of the chromatids was observed. To avoid possible individual bias, all scoring was performed by the same investigator.

5.3.6- Experimental design

In experiment 1, the genotoxic effect of the Hoechst 33342 staining on boar spermatozoa was evaluated. A total of 45 and 44 sows were intracervically inseminated with 3 x 10^9 unstained (control) and Hoechst 33342 stained spermatozoa, respectively, in a volume of 80 ml. Evaluation of reproductive parameters and piglets phenotypic characteristics was carried out in all sows inseminated and all piglets born, respectively. Cytogenetic analysis was performed in eight randomly selected piglets born after insemination with unstained spermatozoa (four piglets, two males and two females) and stained spermatozoa (four piglets, two males and two females).

In experiment 2 the genotoxic effect of the Hoechst 33342 staining followed by the u.v. laser impact on sorted boar spermatozoa was evaluated. A total of 30 and 28 sows with induced ovulation were deeply inseminated with 150 x 10^6 stained and sorted or unstained and unsorted (control) spermatozoa, respectively in a volume of 7.5 ml. Evaluation of reproductive parameters and piglets phenotypic characteristics was carried out in all sows inseminated and all piglets born, respectively. Cytogenetic analysis was performed in eight randomly selected piglets born after inseminations with unstained spermatozoa (four piglets, two males and two females) and stained and sorted spermatozoa (four piglets, two males and two females), respectively.

5.3.7.- Statistical Analysis

Data are expressed as percentages or means ± S.E.M .and differences were considered to be significant at $P<0.05$. The percentage of sows within each insemination group for pregnancy and farrowing rates was compared using a chi-square test with Yate´s correction. Litter size, live, low viable, splay-leg, mummified

piglets, SCEs and CA differences was analysed using the procedure GLM (ANOVA) of SPSS 11.5/PC statistics package (SPSS Inc., Chicago, IL, USA).

5.4. Results

5.4.1 Experiment 1: Evaluation of the genotoxic effect of Hoechst 33342 on boar spermatozoa.

The data shown are from 8 consecutive weeks. Reproductive parameters for the inseminations performed in this experiment are shown in Table 1. Pregnancy rates, farrowing rates and litters size did not significantly differ ($p>0.05$) from control group when Hoechst 33342 stained spermatozoa were used for AI.

Table1. Pregnancy rates, farrowing rates and litter size in sows inseminated with $3x10^9$ of Hoechst 33342 stained or unstained (control) spermatozoa. Values for litter size are means ± S.E.M.

Treatment Group	N° of sows inseminated	Pregnancy Rates (%)	Farrowing Rates (%)	Litter Size *(n)*
Stained	44	86.3	84.0	10.8 ± 2.9
Unstained	45	84.4	82.2	11.1 ± 2.3

There was no difference in the number of piglets born alive (9.97 ± 3.20 vs 9.97 ± 2.72); low viable piglets (0.39± 0.63 vs 0.51V0.83); mummified piglets (0.21 ± 0.41 vs 0.27 ± 0.45) and in the number of piglets suffering splay leg (0.28 ± 0.51 vs 0.35 ± 0.78) for piglets born after insemination with unstained and Hoechst 33342 stained spermatozoa respectively ($P>0.05$).

Birth weight and 21-day weaning weight means for piglets born with stained spermatozoa were established at about 1.48 ± 0.04 and 5.65 ± 0.2 kg respectively, and no significant variations ($P> 0.05$) with respect to the control group (1.36 ± 0.03 kg at

the day of birth and 5.72 ± 0.3 kg at 21 days of weaning) were observed. Similar results were found at 42 and 92 days with weight means of 10.21 ± 0.6 and 47.50 ± 1.3 and 10.18 ± 0.7 and 47.23 ± 1.5 for piglets born from unstained and Hoechst 33342-stained spermatozoa.

An average rate of 3.1 ± 0.21 SCEs/metaphase and 2.8 ± 0.16 SCEs/metaphase was found in lymphocytes from piglets born after insemination with unstained (control) and Hoechst 33342-stained spermatozoa, respectively. This small difference was within the range of biological variability and did not reach statistical significance ($P>0.05$) (Table 2). Differences in SCEs frequencies within piglets in the same group were observed but these differences did not reach statistical significance ($P>0.05$). No interaction ($P>0.05$) between the sex of the piglets and number of SCEs per cell was observed.

CAs in metaphase analyse of lymphocytes revealed mainly chromatid breaks. Percentages of metaphases with CAs (in one or two chromatids) were low, ranging from an average rate of 0.05 ± 0.01 CAs/metaphase and 0.04 ± 0.01 CAs/metaphase for control and Hoechst 33342-stained spermatozoa, respectively. The sex of the piglets did not affect the CA frequencies ($P>0.05$).

Table 2. Mean ± S.E.M rates of SCEs and CAs per cell in the lymphocytes of piglets born after insemination with $3x10^9$ Hoechst 33342-stained or unstained (control) spermatozoa. Each entry is the mean ± S.E.M. for 50 metaphase cells.

Donor	Group	Sex	SCEs	CAs
1	Control	male	3.38±0.37	0.12±0.05
2	Control	male	2.72±0.27	0.04±0.03
3	Control	female	2.92±0.28	0.02±0.02
4	Control	female	3.48±0.45	0.04±0.03
5	Stained	male	2.40±0.31	0.16±0.05
6	Stained	male	3.08±0.53	0.02±0.02
7	Stained	female	3.12±0.36	0.01±0.01
8	Stained	female	2.60±0.35	0.00±0.00
Mean	Control		3.12±0.21	0.05±0.01
Mean	Stained		2.80±0.16	0.04±0.01

5.4.2. Experiment 2: Evaluation of the genotoxic effect of Hoechst 33342 followed by the u.v. laser impact on sorted boar spermatozoa.

The data shown are from 15 consecutive weeks. Reproductive parameters for the deep intrauterine inseminations performed in this experiment are shown in Table 3. Significantly lower pregnancy and farrowing rates and litter size were seen using FCS spermatozoa when compared with control spermatozoa ($P<0.05$).

Table 3. Pregnancy rates, farrowing rates and litter size in sows inseminated with 150 x 10^6 FCS or unstained and unsorted (control) spermatozoa. Values for litter size are means ± S.E.M.

Treatment Group	Nº of sows inseminated	Pregnancy Rates (%)	Farrowing Rates (%)	Litter Size (n)
FCS	30	43.3 [a]	36.6 [a]	8.1 ± 1.9 [a]
control	28	89.2 [b]	82.1 [b]	10.9 ± 1.6 [b]

Values within columns with different superscripts are significantly different (P<0.05)

No variations for low viability piglets (0.25±0.33 vs 0.42±0.51); mummified piglets (0.14±0.34 vs 0.19± 0.32) and in the number of piglets suffering splay leg (0.34± 0.43 vs 0.43± 0.65) were found between piglets in the control group and those obtained after DUI inseminations with stained and sorted spermatozoa respectively.

Birth-weights and 21 day weaning-weights means for piglets born in the control group were established at about 1.46 ± 0.03 Kg and 5.65 ± 0.4 Kg respectively, and no significant variations (P>0.05) with respect to the piglets born after inseminations with FCS spermatozoa (1.52 ±0.03 Kg at the day of birth and 5.72± 0.2Kg at 21 days of weaning) were observed. Similar results were found at 42 and 92 days with weight means of 10.89± 0.5 and 48.12± 1.6 and 10.57± 0.8 and 47.59± 1.2 for piglets born from control and FCS spermatozoa.

An average rate of 2.6 ± 0.19 SCEs/metaphase and 2.6 ± 0.26 SCEs/metaphase was found in lymphocytes from piglets born after insemination with control and FCS spermatozoa respectively. As occurred in experiment 1, a small difference was found within the range of biological variability and did not reach statistical significance (P>0.05) (Table 4).

CAs in metaphase analyse of lymphocytes also revealed mainly chromatid breaks. Percentages of metaphases with CAs (in one or two chromatids) were low ranging from an average rate of 0.05 ± 0.01 CAs/metaphase and 0.06 ± 0.02 CAs/metaphase for control and FCS spermatozoa, respectively.

No significant variations ($p>0.05$) were found in SCEs and CA frequencies between males and females.

Table 4. Mean ± S.E.M rates of SCEs and CAs per cell in the lymphocytes of piglets born after insemination with 150 x 10^6 FCS spermatozoa or unstained and unsorted (control) spermatozoa. Each entry is the mean ± S.E.M. for 50 metaphase cells

Donor	Group	Sex	SCEs	CAs
1	Control	male	2.34±0.25	0.05±0.04
2	Control	male	2.65±0.31	0.03±0.03
3	Control	female	2.41±0.37	0.09±0.05
4	Control	female	3.23±0.41	0.04±0.03
5	FCS	male	2.36±0.27	0.04±0.04
6	FCS	male	2.46±0.34	0.06±0.03
7	FCS	female	3.16±0.32	0.10±0.05
8	FCS	female	2.44±0.37	0.04±0.03
Mean	Control		2.65±0.19	0.05±0.01
Mean	FCS		2.60±0.26	0.06±0.01

5.5. Discussion

Considerable reservations concerning the genetic safety of a process that involves Hoechst 33342 spermatozoa staining in combination with u.v. laser impact have been expressed by several authors (Ashwood-Smith, 1994; Munné, 1994), especially after the successful application of this technology to the separation of

human spermatozoa (Johnson et al. 1993). It is well known that both Hoechst 33342 and u.v. laser are toxic and/or mutagenic when used in somatic cells (Durand and Olive, 1982; Sinha and Hader, 2002). However, to the best of our knowledge, no data concerning the mutagenic effects of these agents on boar spermatozoa as required for preselecting the sex by flow cytometry sorting procedure are available.

We evaluated two steps of the flow cytometric sorting procedure that could be potentially harmful to the functional and chromosomal integrity of spermatozoa. First, the individual effect of the Hoechst 33342 on the spermatozoa and, secondly, the additive effect of the u.v. laser irradiation on the Hoechst 33342-stained spermatozoa.

The dosage of Hoechst 33342 required for staining spermatozoa before sorting by sexual chromosome did not affect pregnancy rates at 24-28 days and neither farrowing rates or litter size. These findings are not compatible with damage to the sperm chromatin integrity because it has been demonstrated that when the chromatin is compromised, failures in fertilization, early embryonic losses and spontaneous abortions are observed (Evenson, 1999). This innocuous effect of Hoechst 33342 on boar spermatozoa under our experimental conditions is in agreement with those obtained earlier (Vazquez et al., 2002). In the same way, piglets obtained after AI with Hoechst 33342-stained spermatozoa appeared to be no different from controls, either in the presence of congenital abnormalities or their growth at 21, 42 and 92 days, reinforcing the apparent innocuous effect of Hoechst 33342 on boar spermatozoa.

As far as we know, no data about cytogenetic analysis in piglets born from Hoechst-33342 staining spermatozoa have previously been reported. Genotoxicity endpoints, such as SCEs and CAs, are widely used as biomarkers of exposure to DNA-damaging agents. SCE evaluation is considered to be a more sensitive cytogenetic method than CA for evaluating genotoxic potential of a variety of mutagenic and carcinogenic agents (WHO 1993). However, one cytogenetic analysis alone is not generally accepted as sufficient evidence to classify an agent as mutagenic (WHO 1985). Thus, and taking into account that the two genetic endpoints used here are not

mutually exclusive but complementary, and can respond with different sensitivities to the suspect agent, they have been included in the present study.

No significant increase in baseline frequencies of SCEs/cell and CAs/cell was found in lymphocytes from piglets born after inseminations with Hoechst 33342-stained spermatozoa compared with lymphocytes of animals from the control group. These findings suggested that Hoechst 33342 does not induce DNA damage; this was expected since this DNA specific dye has been defined as a non-intercalating agent and binds to the minor groove of the adenine-thymine regions of the DNA helix (Johnson & Schulman, 1994). In addition, the extreme degree of condensation of DNA in the sperm by substitution of histones by protamines increases its protection against foreign influences (Tanphaichtr et al., 1978; Rodriguez-Martinez et al., 1990).

Variations in the number of SCEs/cell among individuals within the same group, regardless of the sex, were found but not at significant levels. This slight variation can be mainly attributed in our experiment to the concentration of BrdU relative to the number of lymphocytes in the culture which is an individual factor (Carrano et al., 1980). Since the presence of BrdU in the culture medium is necessary to achieve chromatid differentiation for disclosure of the exchanges, 0.1 μg/ml was found to be the limit in our experimental conditions for discriminating sister chromatid differential staining as observed earlier for several ruminant species (Di Berardino et al. 1996, 1997).

On the other hand, when Hoechst-33342 stained spermatozoa were passed through the u.v. laser beam of the flow cytometer, pregnancy rates, farrowing rates and litter size obtained after DUI insemination were significantly lower than those obtained for control group (unstained and unsorted spermatozoa). It is well known that the fertile life span of spermatozoa is considerably reduced by flow cytometric sorting (Vazquez et al., 2003). However, the reason for this reduction remains unclear and no in-depth explanation has yet been given. The exposure of the Hoechst 33342-stained spermatozoa to the laser and/or the passage of the spermatozoa through the flow cytometer (including the high dilution rate, the projection into the collection tube or

the post-sort centrifugation required for in vitro or in vivo insemination) are highlighted as the two main sources of the loss of the reproductive yield. If the damage were more related to DNA changes, the fall in the reproductive parameters should be associated with an increased rate of abortions or congenital abnormalities as well as with an increase of SCEs and CAs in the piglets. By contrast, if the damage were more related to alterations in functionality of spermatozoa shortening the fertile life span of the cells, a decrease in the reproductive index alone, without other alterations associated, would be expected.

It could be argued that the u.v. laser beam may be having an impact on some of the spermatozoa because u.v. radiation induces two of the most abundant mutagenic and cytotoxic DNA lesions in somatic cells (Sinha and Hader, 2002). In addition, a reduction in the development of embryos has been observed after inseminating flow-sorted spermatozoa in pigs (Johnson, 1991), rabbits (McNutt & Johnson, 1996) and cows (Cran et al, 1993). DNA-damaged spermatozoa can fertilize oocytes at the same rate as intact DNA sperm but embryonic development is significantly decreased (Ahmadi & NG, 1999). However, more recent studies undertaken with bovine or porcine zygotes showed that sorting stained spermatozoa with the new high speed flow sorter did not affect embryo development in blastocysts (Gurhrie et al., 2002; Zhang et al., 2003). We must take account, that if the u.v. laser has any effect, the duration of exposure of the stained sperm to u.v. laser light using the current high speed sperm sorting procedure is much shorter than the exposure time necessary when standard speed flow-sorting cytometers were used. Consequently any presumed u.v. damage of spermatozoa would be expected to be less with this shorter u.v. light exposure (Guthrie et al., 2002). In addition, it is also important to note that the high degree of condensation of the sperm chromatin, in comparison with other cells, makes DNA highly resistant to physical or chemical agents (Lopes et al., 1998), and thus could represent an optimal protection from u.v. laser impact. Moreover, the safety of the procedure is reinforced by phenotypic and cytogenetic analyse done in our experiments. The normalcy of piglets obtained from our experiment as well as in

earlier studies (Johnson 1991; Rath et al.,1997, 1999; Abeydera et al., 1998; Vazquez et al., 2003) allows us to deduce that no severe DNA damage resulting from the combination of staining and laser beam is induced in boar spermatozoa and thus no genetic abnormalities are expected in the offspring. Moreover, no deviations from SCE and CA baseline frequencies were found for these piglets with respect to the control group.

On the other hand, it could also be argued that the sorting process itself could induce alterations in membrane status, changes in motility patterns, thereby reducing the fertile life span of the spermatozoa regardless of the Hoechst 33342 staining or u.v. laser impact effect (Johnson, 1995; Maxwell et al., 1997; Maxwell and Johnson, 1999; Parrilla et al., 2001). In truth, a reduction in the fertility indexes without any increase in lost pregnancies, abortions or morphological abnormalities should be obtained as observed in our experiments where, in addition, the cytogenetic analysis did not shown deviations from baseline levels according to the phenotypical normalcy of the offspring.

In conclusion, this is the first report confirming the absence of *in vivo* genotoxic effect of the flow cytometry-sorted semen technology, based not only on the lack of phenotypic evidence but also on the absence of increases in the frequencies of mutagenic indexes in the offspring. Further analyses on specific genes in boar spermatozoa should be undertaken to increase our knowledge about of the genetic safety of this procedure.

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3.- Discusión General

El desarrollo de la técnica de selección y separación espermática, mediante citometría de flujo, en función del contenido en ADN es, sin duda, uno de los avances más importantes dentro de la tecnología de la reproducción. En el ganado porcino, la utilización de esta tecnología para la predeterminación del sexo de la descendencia podría resultar altamente beneficiosa ya que determinaría una aceleración en los programas de mejora genética, así como una mayor facilidad en el manejo de las explotaciones y un aumento en la eficiencia de la producción. Son numerosos los estudios que describen la obtención de descendencia del sexo deseado tras la utilización de espermatozoides de verraco separados y aplicados mediante inseminación quirúrgica (Johnson, 1991), o bien utilizados para FIV (Rath y cols., 1997, 1999; Abeydeera y cols., 1998), e ICSI (Probst y Rath, 2003) seguidas de transferencia de embriones. Sin embargo, antes de su implementación aún deben investigarse algunos aspectos relacionados con esta tecnología y que hasta el momento han permanecido inéditos.

La utilidad de la citometría de flujo como método de separación espermática para conseguir la predeterminación del sexo de la descendencia quedó demostrada en el año 1989 con la obtención por parte de Johnson y colaboradores de los primeros animales de sexo deseado tras la aplicación de la técnica en conejos. Desde entonces y hasta nuestros días son numerosos los artículos publicados describiendo la obtención de resultados similares en diferentes especies (Johnson, 1991; Cran y cols., 1993, 1997; Catt y cols., 1996; Buchanan y cols., 2000).

Aunque de forma general el procedimiento de separación espermática a alta velocidad (Johnson y Welch, 1999) está estandarizado en cuanto a protocolos de tinción y manipulación de los espermatozoides antes y después de la separación se deben realizar pequeñas modificaciones en diferentes puntos del proceso según la especie debido, entre otros, a las diferencias respecto al grado de compactación de la cromatina nuclear que existen entre diferentes especies (Garner y cols., 1983; Gardiner-Garden, 1999). Estas diferencias pueden condicionar la penetración del Hoechst 33342 dando lugar a una tinción deficiente que influirá de manera definitiva sobre la eficiencia del proceso de separación (Johnson, 2000), haciendo necesario adaptar los protocolos de tinción de forma especifica a cada especie. Del mismo modo, pequeñas diferencias en la morfología de la cabeza espermática entre especies provocan diferencias en las fluorescencias emitidas (Garner y cols., 1983) condicionando la identificación de los espermatozoides orientados y de las poblaciones de espermatozoides X e Y e influyendo por tanto sobre la calidad de la separación. La mayoría de los mamíferos domésticos presentan una morfología aplanada de la cabeza espermática lo que permite una clara diferenciación entre el la fluorescencia emitida por el perfil (mayor fluorescencia) y la cara plana de la misma (menor fluorescencia) que será recogida en el detector situado lateralmente permitiendo una fácil identificación de los espermatozoides orientados; la morfología más redondeada de los espermatozoides humanos hace esta diferenciación más difícil complicando la identificación de los espermatozoides correctamente orientados.

Sin embargo, la mayor o menor facilidad en la identificación de la población no solo depende del tipo de muestra, sino también del correcto alineamiento y enfoque óptico del sistema; estos ajustes son imprescindibles para obtener la resolución requerida permitiendo la obtención de unos rendimientos óptimos. Para ajustar los citómetros de alta velocidad se recomienda la utilización, en primer lugar de esferas fluorescentes, las cuales al ser uniformes deben dar lugar a coeficientes de variación de 1%, permitiendo ajustes muy finos del equipo. A continuación, el ajuste debe proseguirse con cabezas de espermatozoides pertenecientes a una especie donde exista una diferencia importante en la cantidad de ADN entre espermatozoides que

transportan un cromosoma X e Y. La utilización de cabezas espermáticas permite modificar y adecuar los parámetros del equipo de acuerdo al tipo de muestra que se vaya a procesar. Normalmente se utilizan cabezas espermáticas de toro cuya diferencia en el contenido de ADN (3'9%) y morfología las convierte en una buena herramienta para el ajuste de los equipos para, finalmente, realizar el último ajuste con cabezas de la especie que se pretende separar. Debido a que el trabajo de esta memoria ha sido realizado en espermatozoides de verraco y a que esta especie presenta una diferencia muy pequeña entre espermatozoides X e Y en relación a la cantidad de ADN, era necesario realizar un ajuste previo con cabezas espermáticas de otra especie. Aunque se podía realizar el ajuste del citómetro con cabezas de espermatozoides de toro, se eligió estudiar una especie, hasta este momento inédita en citometría, que podía presentar tan buenos resultados como los espermatozoides de toro. De este modo y en la primera experiencia realizada en este trabajo se utilizaron espermatozoides de caprino por las siguientes razones: 1) debido a la proximidad filogenética con otras especies como el ovino y el bovino era de esperar que las diferencias entre los espermatozoides X e Y de caprino estuvieran cercanas a las que presentan estas especies (4'2 % y 3'9 %, respectivamente) facilitando la calibración y puesta a punto del procedimiento bajo nuestras condiciones de trabajo y 2) la ausencia de datos acerca del contenido en ADN de los espermatozoides de esta especie hacía interesante el estudio de los mismos, mas aun si se tiene en cuenta la importancia que tiene dentro del sector ganadero y, en especial, en el campo de la transgénesis para el aislamiento de proteínas recombinantes a partir de leche de animales transgénicos (Balsadarre y cols., 2003). En los últimos años la utilización de cabras transgénicas como fuente de ciertos fármacos, secretados en la leche de estos animales merced a modificaciones en su dotación genética, se ha convertido en una posibilidad muy atractiva ya que el porcentaje de animales que expresan el gen de interés es más alto que en otras especies utilizadas con el mismo fin (Baguisi y cols., 1999). En este caso, resulta evidente la gran utilidad que tendría la realización de inseminaciones con espermatozoides portadores del cromosoma X exclusivamente (Seidel 2003).

En este trabajo se establece por primera vez la diferencia en el contenido de ADN entre los espermatozoides de caprino portadores del cromosoma X y los portadores del cromosoma Y. La diferencia quedó establecida en un 4'4 % situándose, como era de esperar, muy próxima al 4'2 % existente entre los espermatozoides X e Y de morueco (Johnson y Pinkel, 1986). Esta diferencia da lugar a la obtención de histogramas donde los picos correspondientes a las poblaciones de espermatozoides X e Y son fácilmente identificables ya que se encuentran separadas por una hendidura profunda. La clara definición de ambas poblaciones merced a la profundidad de la hendidura entre los picos, nos da una idea de la facilidad a la hora de separar los espermatozoides X de los Y, ya que permite seleccionar poblaciones mas amplias y definidas (Johnson, 2000). Gracias a estas diferencias en el contenido de ADN se puede realizar en esta especie separaciones espermática a alta velocidad, determinando una eficiencia óptima del procedimiento y posibilitando la utilización a nivel práctico de los espermatozoides separados en combinación con otras técnicas reproductivas, lo cual incrementaría notablemente la eficiencia económica y biológica de la producción en ganado caprino, como ya ha sido descrito en el caso de explotaciones de bovino (Seidel y Johnson, 1999; Doyle, 2000). Además, la gran diferencia en cuanto al contenido de ADN encontrada entre los espermatozoides X e Y de esta especie hace posible la utilización de los mismos como herramienta para comprobar el correcto ajuste del citómetro de flujo antes de cada proceso de separación incluso cuando espermatozoides de otras especies van a ser separados o analizados. En este sentido y coincidiendo con lo anteriormente expuesto Johnson y Welch (1999) aseguran que el ajuste del citómetro resulta fundamental a la hora de optimizar los rendimientos del proceso y que los parámetros del sistema deberán estar ajustados según las particularidades de los espermatozoides con los que se vaya a trabajar.

Como conclusión de esta primera experiencia, podemos afirmar que la diferencia en la cantidad de ADN entre los espermatozoides de caprino portadores del cromosoma X y los portadores del cromosoma Y permite una clara identificación de ambas poblaciones espermáticas, lo que a la hora de la separación mediante citometría de flujo permitirá la obtención de unos rendimientos óptimos haciendo esta tecnología

muy atractiva tanto para los ganaderos del sector como para el campo de la investigación transgénica. Asimismo los resultados obtenidos demuestran la correcta calibración del citómetro empleado y la utilidad de estos espermatozoides como instrumento para el ajuste y alineamiento de los equipos de separación espermática.

De manera general se acepta que la observación del sexo de la descendencia, obtenida tras la inseminación con espermatozoides sexados, es el mejor método para comprobar la eficiencia del mismo (Johnson y cols., 1989; Johnson, 1991; Cran y cols., 1995; Fugger y cols., 1998). Sin embargo, conocer la eficacia del proceso de separación antes de su utilización mediante diferentes técnicas reproductivas resulta esencial para hacerlo económicamente rentable (Welch y Johnson, 1999). En primer lugar porque permite la optimización de los costes asociados con la aplicación de los espermatozoides sexados en la realización de experiencias in vivo, ya que estas solo tendrán lugar cuando las purezas obtenidas en la separación se consideren adecuadas. Y en segundo lugar, porque la seguridad de que la descendencia obtenida va a ser del sexo deseado supone la rentabilización al máximo del proceso permitiendo la planificación de la producción de una explotación determinada según las exigencias del mercado en ese momento.

El rendimiento del proceso de separación por citometría de flujo, expresado como el número de espermatozoides separados por unidad de tiempo, depende en gran medida del tamaño de la región que define la población seleccionada (Johnson y Welch, 1999). Un aumento de un 15 % en el tamaño de la región de separación representa un incremento de alrededor del 20 % en los rendimientos del proceso, pero supone una disminución de al menos un 10 % en la pureza obtenida (Johnson, 1997). Aunque la obtención de bajos rendimientos con altas purezas sea viable a la hora de la utilización de estos espermatozoides en FIV, resulta del todo insuficiente cuando nos referimos a su aplicación en porcino mediante inseminación intrauterina profunda. La necesidad de mantener el equilibrio entre el número de espermatozoides separados y la pureza de los mismos ha determinado que desde la introducción de la citometría de flujo como técnica de separación espermática se hayan desarrollado diferentes métodos para la verificación de las purezas de las muestras separadas (Welch y Johnson, 1999).

El reanálisis por citometría de flujo de las muestras separadas es uno de los métodos desarrollados para comprobar el grado de pureza obtenido (Johnson y cols., 1987; 1989). Sin embargo, supone la utilización de la misma instrumentación que en el proceso de separación, y por tanto no resulta un procedimiento independiente. Además, hay que tener en cuenta que cuando las diferencias en el contenido de ADN entre los espermatozoides X e Y son muy pequeñas, como ocurre en el caso de los humanos (2.8%) o incluso en porcino, este método no puede ser aplicado con la suficiente precisión (Johnson y cols 1993; Fugger y cols., 1998).

La amplificación mediante PCR del ADN de una célula de forma individualizada ha sido también utilizada como método para la determinación de la pureza de las muestras separadas (Welch y cols., 1995). Para ello, es necesario recoger las células separadas de forma individual, de manera que podamos realizar una amplificación de fragmentos específicos de los cromosomas sexuales, que nos indicará, en esa célula concreta, la presencia del cromosoma X o Y. Los resultados obtenidos mediante este procedimiento son similares a los obtenidos mediante el reanálisis por citometría de flujo, obteniéndose en ambos casos purezas superiores a un 90 % para espermatozoides portadores del cromosoma X (Welch y Johnson, 1999). A pesar de que estos resultados demuestran la utilidad de esta técnica, el trabajo necesario para la realización de reacciones de PCR de forma individual en células separadas, la hace inviable como método rutinario para la estimación de las purezas obtenidas en los procedimientos de separación.

El porcentaje de espermatozoides X o Y presentes en una muestra tras el proceso de separación puede ser también determinado mediante la aplicación de técnicas de FISH (Fluorescent *in situ* hibridization), las cuales permiten identificar secuencias de ADN pertenecientes de forma especifica a determinados cromosomas. La FISH ofrece la oportunidad de utilizar una técnica totalmente independiente para la evaluación de las purezas de las poblaciones separadas, lo cual es su ventaja más importante sobre el reanalisis por citometría de flujo. Además, esta técnica ofrece una evaluación cualitativa y cuantitativa de la muestra, ya que identifica específicamente determinados cromosomas indicando si están o no presentes y además nos permite

contar el número concreto de espermatozoides que poseen esta marca específica (Welch y Johnson, 1999). Esta técnica en alguna de sus variantes ha sido utilizada en espermatozoides separados de toro (Hassane y cols., 1999; Piumi y cols., 2001), de verraco (Kawarasaki y cols., 1995; 1996) y también espermatozoides humanos (Johnson y cols., 1993) resultando particularmente útil en aquellas especies donde la diferencia de ADN entre los espermatozoides X e Y es muy pequeña, como es el caso de los espermatozoides de humano.

En una reacción de FISH, el elemento mas importante es la sonda fluorescente de ADN, la cual puesta en contacto con el material nuclear del espermatozoide dará lugar a una marca brillante fácilmente identificable mediante microscopia de fluorescencia. Estas sondas se encuentran disponibles deforma comercial solamente en la especie humana. En el caso concreto de espermatozoides separados de cerdo, se han utilizado sondas indirectas especificas de cromosoma Y, para la detección de la presencia o no de este cromosoma, combinadas con sondas indirectas especificas de cromosoma 1, que dan lugar a una marca fluorescente en todos los espermatozoides hibridados y que se utiliza como control de la eficiencia de la reacción de hibridación (Kawarasaki y cols., 1998). Aunque la utilidad de estas sondas indirectas ha quedado demostrada, el hecho de que impliquen entre uno y tres pasos de detección antes de la visualización de las señales fluorescentes supone un inconveniente respecto a las sondas llamadas directas las cuales posibilitan una visualización directa de la muestra tras el proceso de hibridación.

En la realización de esta experiencia se han utilizado sondas directas, especificas para cromosomas 1 e Y porcinos, marcadas fluorescentemente mediante el método de desplazamiento de cortes (nick translation), definido como un método homogéneo de marcaje ya que incorpora los nucleótidos marcados fluorescentemente al azar a lo largo de la longitud del fragmento de ADN que constituye la sonda (Rigby y cols., 1977; Langer y cols., 1981). Las sondas obtenidas presentan una sensibilidad elevada, lo que se traduce en la obtención de una eficiencia de hibridación (porcentaje de espermatozoides con señal fluorescente para cromosoma 1) próxima al 98 %. En nuestro caso el método de desplazamiento de cortes se convierte en una herramienta

extremadamente útil ya que las manipulaciones que se realizan sobre el ADN que se va a marcar son mínimas reduciéndose notablemente las posibilidades de contaminación. De igual manera la utilización de este sistema de marcaje da lugar a sondas que originan marcas fluorescentes muy claras obteniéndose hibridaciones limpias y fáciles de evaluar.

El tiempo necesario para llevar a cabo la reacción, sobre todo si hay que fabricar las sondas como ocurre en el porcino, y el elevado coste de los reactivos necesarios, son los dos inconvenientes principales de este método. Sin embargo, es una técnica que permite la preparación de numerosas muestras a la vez y esto, a pesar del tiempo necesario para su realización, hace que pueda ser aplicable de forma eficiente en laboratorios que se dediquen a la comercialización de semen separado (Welch y Johnson, 1999).

La calidad de las señales fluorescentes proporcionadas por estas sondas directas posibilitó, además, el análisis de los porcentajes de anomalías numéricas correspondientes a los cromosomas 1 e Y, situándose en ambos casos por debajo del 0'1 % y coincidiendo con los resultados obtenidos por otros autores referentes a la presencia de aneuploidias en espermatozoides de verraco (Rûbes y cols., 1999).

Una vez que los resultados demostraron la utilidad de las sondas directas marcadas mediante desplazamiento de cortes para hibridar espermatozoides de porcino, se evaluaron las purezas obtenidas en poblaciones espermáticas separadas de acuerdo a criterios de selección más o menos estrictos establecidos en función de tamaños más o menos grandes de las regiones de selección. Como era de esperar las purezas mas altas (entre un 89'5 y un 93'0 %) se obtuvieron utilizando criterios de selección estrictos correspondientes a tamaños de ventana reducidos, confirmando la fiabilidad y eficacia del equipo utilizado y evidenciando la estrecha relación existente entre la pureza de una muestra espermática separada y el tamaño de la población seleccionada, y por tanto entre esta pureza y el rendimiento del proceso, como fue descrito por anteriormente por Johnson (1997). En la especie porcina ni siquiera la utilización de ventanas de selección amplias, comprometiendo las purezas, resulta suficiente para obtener el número de espermatozoides separados necesarios para una

inseminación artificial tradicional. Sin duda la utilización de sistemas que permitan la utilización de un reducido número de espermatozoides separados de verraco manteniendo unas tasas de fertilidad aceptables, puede ser fundamental para alcanzar unos rendimientos óptimos de la técnica de separación espermática por citometría de flujo en producción porcina.

En conclusión, los resultados de esta experiencia demuestran que la FISH con sondas directas de ADN construidas mediante amplificación por PCR de fragmentos de ADN específicos de cromosomas porcinos y posterior marcaje de los mismos mediante desplazamiento de cortes, representa una herramienta de gran utilidad para la evaluación de las purezas obtenidas tras el proceso de separación. La sencillez del protocolo de marcaje y la calidad de la señal fluorescente obtenida demuestran que el desplazamiento de cortes es un método ideal para la construcción de sondas fluorescentes que serán utilizadas en procesos de FISH destinados a comprobar la eficacia del proceso de separación espermática por citometría de flujo en porcino. Asimismo, estas sondas permiten la identificación de aneuploidias u otras alteraciones cromosómicas en espermatozoides de porcino. La evaluación del sexo en embriones y la determinación del correcto desarrollo de los mismos debería ser también posible mediante la aplicación de esta técnica de FISH directa.

La velocidad de separación de los equipos actuales puede alcanzar los 5000-6000 espermatozoides por segundo (Seidel, 2003), lo cual significa unos rendimientos de hasta 18 millones de espermatozoides por hora. Aunque estos rendimientos en la separación se han incrementado exponencialmente en los últimos años gracias a los avances técnicos, la obtención de un número de espermatozoides separados de verraco adecuado supone periodos de separación más o menos prolongados. Además a este tiempo de separación habrá que añadirle el tiempo necesario para trasladar los espermatozoides separados desde el laboratorio al lugar de inseminación. Todo esto determina la necesidad de conocer la influencia del tiempo de conservación sobre el potencial fecundante de la población resultante, para de este modo optimizar las condiciones de almacenamiento, en medio líquido, de los espermatozoides separados

con el fin de mantener la viabilidad y capacidad fecundante de los mismos a niveles aceptables.

Aunque existen experiencias previas que mostraban una disminución de la motilidad tras 4 horas de conservación (Maxwell et al, 1997), bajo nuestras condiciones experimentales este efecto no se observó hasta las 10 horas de conservación. La adición a los medios de recogida de componentes como la yema de huevo o el plasma seminal, que actúan como agentes protectores de membrana, ayuda a estabilizar las membranas espermáticas (Maxwell y Johnson, 1997, 1999). Sin embargo, esta protección desapareció tras largos periodos de almacenamiento (10 h) observándose un aumento del porcentaje de células dañadas, reduciéndose la motilidad y viabilidad de las mismas y viéndose comprometida su capacidad fecundante. A pesar de lo cual, es necesario señalar, que la disminución en ambos parámetros no es dramática, manteniéndose los porcentajes de espermatozoides mótiles y con la membrana intacta por encima de aquellos descritos por Maxwell y cols. (1997 y 1998) en espermatozoides de verraco separados y evaluados inmediatamente después de su recogida. Estos resultados pueden ser debidos a que actualmente el desarrollo de la separación espermática de alta velocidad (Johnson y Welch, 1999) permite obtener un mayor numero de espermatozoides separados por unidad de tiempo aumentando la concentración de los mismos y disminuyendo los daños inducidos por el proceso de separación dando lugar por tanto a niveles óptimos de funcionalidad de los espermatozoides separados.

A pesar de que el porcentaje total de espermatozoides separados mótiles no varió hasta las 10 horas de almacenamiento, si lo hicieron los patrones de motilidad de los mismos. En los espermatozoides recién separados se observó un aumento significativo en el Dance y la ALH, así como en la VCL y la VSL, parámetros que son indicadores de la angularidad y linealidad del movimiento espermático, respectivamente. Para encontrar una explicación a estos cambios se debe tener en cuenta que durante su paso por el citómetro de flujo los espermatozoides son diluidos y procesados utilizando únicamente medios isotónicos y sin presencia de ningún componente que ejerza de protector de membrana, lo cual presumiblemente elimina los

antioxidantes naturales y otros componentes beneficiosos presentes en el plasma seminal requeridos para el mantenimiento de la integridad y funcionalidad espermática (Maxwell y Johnson, 1999), induciendo además en estos espermatozoides un estado similar al que ocurre durante la capacitación espermática, efecto que ha sido observado y descrito por numerosos autores en espermatozoides separados de diferentes especies (Maxwell y Johnson, 1999 y 1997; Maxwell y cols. 1997 y 1998; Catt y cols., 1997). Un aumento en los parámetros de motilidad espermática ha sido relacionado con el inicio del estado de capacitación (Ho y Suarez, 2001) y por tanto podría ser que los cambios en la motilidad de los espermatozoides separados indicaran el comienzo de este proceso en dichos espermatozoides. Los resultados de motilidad obtenidos en el presente estudio coinciden plenamente con esta hipótesis. Estos resultados fueron reforzados por los resultados obtenidos tras la incubación de los espermatozoides con PNA, lectina que detecta los primeros signos de la exocitosis acrosómica, tan pronto como empieza a producirse la fusión entre los poros de la membrana plasmática y la membrana acrosomal (Gadella y Harrison, 2000), observándose un aumento significativo en el número de espermatozoides con el acrosoma reaccionado cuando se compararon con los pertenecientes al grupo control. En esta misma línea, un estudio reciente (Hollinshead y cols., 2004) demuestra que los espermatozoides separados de morueco progresan más rápidamente hacia el estado de reacción acrosómica que aquellos no separados. Sin embargo en nuestro caso no se ha observado un incremento significativo en el porcentaje de espermatozoides reaccionados relacionado con un aumento en el tiempo de almacenamiento. Como explicación a estos resultados debe ser señalado que probablemente los espermatozoides de verraco sean menos susceptibles al proceso de separación espermática mediante citometría de flujo que los espermatozoides de morueco, de la misma manera que son menos susceptibles que estos últimos a los daños inducidos por las altas diluciones (Ashworth y cols., 1994).

Sin embargo, estos resultados también demuestran que este movimiento hiperactivo puede ser revertido tras 2h de almacenamiento a temperatura ambiente de los espermatozoides en el medio de recogida descrito anteriormente, alcanzándose

valores para los parámetros de motilidad analizados (Dance, ALH, VCL, VSL) similares a los obtenidos en el grupo control (diluidos no separados) sin observarse un incremento en el porcentaje de espermatozoides con reacción acrosómica. La presencia de plasma seminal en el medio de recogida (10%) y la capacidad de éste para estabilizar las membranas espermáticas y revertir el estado de capacitación de los espermatozoides de verraco separados (Maxwell y Johnson, 1997) parecen ser los responsables del retorno a patrones normales de motilidad.

La motilidad, la viabilidad y el estado acrosomal son parámetros capaces de evaluar el impacto que el proceso de separación por citometría de flujo puede tener sobre los espermatozoides. Sin embargo resultan insuficientes a la hora de predecir la fertilidad de los mismos. La realización de experiencias de fecundación *in vitro* homologa utilizando ovocitos de porcino tanto inmaduros (Martínez y cols., 1993) como madurados *in vitro* (Xu y cols., 1996) es, en la actualidad, el mejor método para la evaluación de la fertilidad espermática, ya que son numerosos y variados los procesos fisiológicos y químicos que deben tener lugar para conseguir una fecundación (capacitación; reacción acrosómica; reconocimiento, unión y penetración de la zona pelúcida y finalmente unión a la membrana plasmática del ovocito). Teniendo en cuenta que la fertilidad de los espermatozoides separados puede disminuir durante el almacenamiento, en nuestra opinión resulta fundamental conocer la evolución de esta capacidad fecundante a lo largo de diferentes periodos de conservación.

La capacidad de penetración fue en líneas generales menor para los espermatozoides separados (excepto para aquellos almacenados 2 h tras la separación) que para los del grupo control (diluidos no separados). Estos resultados no eran esperados ya que experiencias anteriores (Rath y cols., 1999) habían descrito la necesidad de disminuir el número de espermatozoides separados en un sistema de FIV ya que debido a su estado de capacitación, el índice de polispermia era mucho mayor. La presencia de plasma seminal en el medio de recogida de los espermatozoides separados, y el hecho demostrado de que este componente sea capaz de reducir las tasas de penetración *in vitro* (Maxwell y cols., 1998) pueden ser los causantes de la disminución en la capacidad fecundante de los espermatozoides separados. Las

primeras evidencias de penetración de los ovocitos madurados *in vitro* se obtuvieron tras 2 h de cocultivo con los espermatozoides separados obteniéndose, a este tiempo, entre un 5 y un 25 % de penetración para aquellos espermatozoides almacenados durante 10 y 2 h respectivamente. Las tasas máximas de penetración se obtuvieron a las 18 horas de cocultivo para todos los grupos analizados, aunque las diferencias con las tasas obtenidas a las 6 h de cocultivo no fueron significativas con excepción de aquellos espermatozoides almacenados durante 10 h que alcanzaron el nivel máximo de penetración a las 18 h. Estos resultados referentes al tiempo de penetración inicial coinciden con los obtenidos en experiencias previas realizadas in vivo (Hunter, 1968), pero difieren de los obtenidos en otros estudios realizados *in vitro* que establecen un tiempo mínimo de cocultivo de 3 h para obtener penetración (Martínez y cols., 1996); posiblemente estas diferencias sean debidas a que en este último caso se utilizaron ovocitos inmaduros y espermatozoides no separados.

Sin duda, lo mas llamativo de esta experiencia es el hecho de que los espermatozoides separados y conservados durante 2 h tengan mayores tasas de penetración y mayor numero de espermatozoides por ovocito que aquellos almacenados durante 0, 5 y 10 h. Las diferencias con los espermatozoides sometidos a periodos más largos de conservación (5 y 10 h) pueden ser atribuidas a una debilidad de los mismos que reduciría el número de espermatozoides capaces de penetrar; sin embargo esta hipótesis no resulta valida para aquellos espermatozoides puestos en contacto con los ovocitos inmediatamente después de la separación. La explicación en este caso podría estar basada en la desorganización de la membrana plasmática del espermatozoide producida por el paso del mismo a través del citómetro de flujo, afectando a los componentes de la membrana, incluyendo las glicoproteinas relacionadas con el reconocimiento y penetración del ovocito. Las 2 h de almacenamiento, antes de su utilización en FIV, en presencia de determinadas sustancias protectoras pueden ejercer un efecto de reorganización de membranas y por tanto restaurar la capacidad fecundante produciendo un incremento en el número de espermatozoides capaces de penetrar. No obstante son necesario nuevos experimentos para explicar estos resultados. Si este razonamiento fuese correcto un periodo de

almacenamiento de 2h de los espermatozoides separados debería ser incluido como un paso previo necesario en los protocolos de aplicación de estos espermatozoides.

En resumen y desde un punto de vista práctico, los resultados del presente estudio muestran la necesidad de depositar los espermatozoides separados lo más cerca posible del momento de la ovulación y del lugar de fecundación cuando sean requeridos periodos de conservación relativamente largos (5, 10 h), ya que la capacidad de penetración de estos espermatozoides empieza a disminuir a partir de las 5 h tras la separación. Este hecho podría explicar la reducción en la fertilidad de los espermatozoides separados observada cuando son requeridos largos tiempos de separación para obtener una dosis de inseminación. Sin embargo, y en base a los datos obtenidos, debe ser señalado que un tiempo de almacenamiento de 2 h aumenta la capacidad fecundante de los espermatozoides separados, por lo que se deduce que cortos periodos de conservación podrían ser beneficiosos cuando los espermatozoides vayan a ser utilizados in vivo o *in vitro*.

Los resultados obtenidos en la experiencia anterior indican que los espermatozoides separados deberían ser depositados, literalmente, lo más cerca posible del lugar de fecundación durante el tiempo de ovulación. A este respecto, la inseminación intrauterina profunda (DUI) podría resultar extremadamente útil para facilitar la penetración de estos espermatozoides que presentan las membranas alteradas ya que, mediante esta técnica de inseminación, son depositados cerca de los ovocitos. La inseminación intrauterina profunda (Martínez y cols., 2001; 2002) ha sido de extremada ayuda en la inseminación de espermatozoides "débiles" como son los espermatozoides congelados (Roca y cols, 2003). Además, también parece importante, a priori, realizar la inseminación lo más próxima posible al momento de la ovulación con el objetivo de maximizar las tasas de gestación obtenidas. Asumiendo que la utilización de la DUI permite dejar los espermatozoides en el lugar adecuado, se diseño una experiencia con la idea de realizar la inseminación acorde al momento de ovulación, para lo cual se sometieron las cerdas a un tratamiento de inducción de celo e inducción de la ovulación. En este experimento y con el objetivo de obtener un mayor número de espermatozoides para realizar las inseminaciones no se separaron en

poblaciones X e Y, sino que se recogieron todos los que pasaban por delante del láser incluyendo tanto los orientados como los no orientados.

Respecto al tratamiento de inducción de celo con eCG, solo un 6'5 % de los animales tratados no mostraron síntomas evidentes de celo 3 días después de la administración de la hormona, lo cual supone una eficiencia elevada de este tratamiento y coincide con resultados obtenidos en experiencias previas realizadas con esta hormona (Hühn, 1996). La hormona hCG fue administrada a los animales 72 horas después de la eCG, para inducir la maduración total de los folículos y el momento de la ovulación con la máxima precisión posible (Brüssow y cols., 1996), permitiendo que todas las cerdas fueran inseminadas a tiempo fijo, 36 h después de la inyección de la hCG.

A pesar de los óptimos resultados del tratamiento de inducción de celo y ovulación, la fertilidad obtenida tras la inseminación realizada con los espermatozoides sometidos al proceso de separación espermática fue más baja que la obtenida con los espermatozoides del grupo control (espermatozoides diluidos). El daño potencial que sufren los espermatozoides sometidos a este proceso de separación (Johnson y Welch, 1999; Johnson, 2000; Guthrie y cols., 2002) puede ser la causa de esta reducción en la fertilidad. Además, las aproximadamente 10 horas necesarias para conseguir el número suficiente de espermatozoides separados para una DUI, determinan una gran heterogeneidad de la población final que constituirá la dosis de inseminación, lo cual podrían suponer un mayor daño en estos espermatozoides ya debilitados por el proceso de separación, influyendo directamente sobre el resultado de la inseminación. Sin embargo, los porcentajes de gestación obtenidos en esta experiencia (45'6 y 54'3%), si bien son bajos respecto a los obtenidos en el grupo control (80'8 y 85'7 %), representan un éxito debido al bajo número de espermatozoides separados utilizado sobre todo si tenemos en cuenta que solo se realizó una inseminación. Esta ha sido la primera vez que se ha obtenido, en el ganado porcino, descendencia viva tras la inseminación por vía no quirúrgica de espermatozoides separados mediante citometría de flujo.

Alrededor de un 15% de las hembras inseminadas con espermatozoides sometidos al proceso de separación, perdieron su gestación entre los 22-25 días o entre los 50-55, lo cual contrasta de forma llamativa con el 1% de perdida gestacional encontrado en el grupo control. El número de partos fue, por tanto, menor cuando se utilizaron espermatozoides separados en las inseminaciones. En este sentido estudios previos realizados en conejos y cerdos (Johnson y cols., 1989; Johnson, 1991) ya mostraban una reducción en la supervivencia de los embriones obtenidos con espermatozoides separados. También se han descrito resultados similares por diferentes autores en conejas, vacas y yeguas (McNutt and Johnson, 1996; Seidel y cols., 1997; Buchanan y cols., 2000). Sin embargo resultados contradictorios fueron obtenidos por Seidel y cols., (1998) en un trabajo realizado en vacuno, en el cual describen la perdida únicamente de una gestación a los 64-67 días de la inseminación frente a 33 que llegaron a termino. Sin duda investigaciones más detalladas a este respecto son necesarias para conocer en profundidad la causa de esta perdida embrionaria y para excluir la posibilidad de que sea debida a un daño en el ADN espermático, de los espermatozoides separados, que pudiera determinar una alteración genética en el embrión haciéndolo inviable.

El número de animales nacidos por camada fue ligeramente más bajo en las hembras inseminadas con espermatozoides separados, aunque la diferencia no fue estadísticamente significativa. Posiblemente un aumento en el número de animales estudiado por grupo daría lugar a la obtención de diferencias significativas, a nivel estadístico, entre ambos grupos.

Los buenos resultados en cuanto a tasas de gestación y partos obtenidos utilizando el sistema DUI para la aplicación de un reducido número de espermatozoides diluidos en cerdas con celo espontáneo descritos por Vázquez y cols (2001a), determinaron la realización de una segunda experiencia, cuyo objetivo fue evaluar la utilidad de este método de inseminación intrauterina para la inseminación con espermatozoides separados bajo condiciones de campo. Para ello se utilizaron cerdas con celo espontáneo, en las cuales fue posible, mediante ecografía transrectal predecir con bastante exactitud el momento de la ovulación. Se seleccionaron para

inseminación todas aquellas cerdas que presentaban folículos con un diámetro mayor de 6 mm. En un alto porcentaje de cerdas (86'5%) la ovulación tuvo lugar en un periodo de aproximadamente 8 ó 10 horas que transcurrió entre la primera y la segunda ecografía. A pesar de ello las tasas de gestación y partos disminuyeron notablemente tras DUI con espermatozoides separados. Sin embargo, el número de animales gestantes, así como el número de partos fue razonable cuando se utilizaron espermatozoides diluidos no separados (aproximadamente un 80%). La razón fundamental para explicar este descenso en las tasas de gestación y partos respecto a los obtenidos en la experiencia anterior con hembras tratadas hormonalmente, puede ser la variabilidad en el momento de ovulación respecto al momento de realización de la DUI en los animales con celo espontáneo en comparación con las cerdas con celo y ovulación inducida, donde el momento de la ovulación se producía de manera constante entre las 37 y 40 horas tras la administración de hCG.

En la actualidad, y utilizando la mas novedosa tecnología en separación espermática mediante citometría de flujo (Johnson y Welch, 1999), sería necesario invertir alrededor de 5 horas de trabajo para seleccionar y separar espermatozoides X o Y que permitieran, tras DUI, obtener lechones de sexo deseado. No hay duda de que este hecho limita la aplicación práctica rutinaria de la DUI en combinación con los espermatozoides separados en porcino. Sin embargo la combinación de ambas técnicas para la obtención de descendencia del sexo deseado podría ser de interés en determinadas situaciones como por ejemplo la obtención de animales de alto valor genético.

En conclusión, los resultados obtenidos en esta experiencia son los primeros en demostrar que la tecnología de inseminación intrauterina profunda puede ser usada con éxito en la producción de lechones a partir de espermatozoides separados mediante citometría de flujo de alta velocidad. Aunque la fertilidad de los espermatozoides sometidos al proceso de separación se ve comprometida, los resultados obtenidos, al respecto, cuando se utiliza un alto número de estos espermatozoides son aceptables.

El nacimiento de animales del sexo deseado en diferentes especies tras la utilización de espermatozoides X o Y separados por citometría de flujo en

combinación con diferentes técnicas de reproducción asistida ha sido descrita por numerosos autores (revisado por Johnson, 2000). Los individuos obtenidos en estos nacimientos se cuentan por miles (incluyendo los pertenecientes a la especie humana) y en ningún caso se han descrito malformaciones o anormalidades fenotípicas (Seidel y Garner, 2002), lo cual determina que la tecnología de separación espermática mediante citometría de flujo sea aceptada como segura desde el momento en que no existen evidencias que demuestren lo contrario. El único efecto negativo asociado hasta ahora con la utilización de los espermatozoides separados en IA es la reducción en la capacidad fecundante de los espermatozoides, así como una posible pérdida embrionaria durante la gestación que se refleja en una reducción del tamaño de camada (Johnson y cols., 1989; Johnson, 1991). La causa de esta pérdida embrionaria permanece todavía por aclarar y podría estar relacionada con un daño a nivel del ADN del espermatozoide debido principalmente a dos pasos necesarios del proceso de separación por citometría de flujo, como son la tinción con el Hoechst 33342 y el impacto del láser ultravioleta sobre la célula espermática. En este sentido, diferentes autores han planteado dudas acerca de la seguridad genética de un proceso que envuelve la tinción con Hoechst 33342 junto con el impacto de un láser ultravioleta (Ashwood-Smith, 1994; Munné, 1994), sobre todo desde que esta tecnología de separación espermática ha sido aplicada con éxito para la obtención de descendencia en la especie humana (Johnson y cols., 1993; Fugger y cols., 1998). Es bien sabido que el Hoechst 33342 podría tener un efecto tóxico o mutagénico sobre ciertos tipos celulares (Durand y Olive, 1982), al igual que también ha sido descrita la capacidad de la luz ultravioleta de producir un aumento en las alteraciones cromosómicas estructurales de los espermatozoides de ratón (Matsuda y Tobari, 1988). Sin embargo, hasta el momento no se habían realizado estudios acerca del posible efecto mutagénico de estos agentes, el Hoechst 33342 y el láser ultravioleta, sobre los espermatozoides de verraco.

La concentración de fluorocromo utilizada fue la necesaria para conseguir una óptima tinción de los espermatozoides de verraco antes de su separación en base al cromosoma sexual que transporten. Los resultados obtenidos demuestran que esta

concentración de Hoechst 33342 (0'3 μM por millón de espermatozoides) no afecta a las tasas de gestación, así como tampoco al número de partos o al tamaño de las camadas obtenidos tras la utilización de los espermatozoides teñidos, lo cual indica que la tinción no produce alteraciones a nivel de la cromatina espermática ya que, según Evenson (1999), cuando la cromatina del espermatozoide está comprometida se producen fallos en la fecundación, pérdidas embrionarias tempranas y abortos espontáneos que se manifestarían con una reducción en los parámetros reproductivos analizados en esta experiencia. La inocuidad del Hoechst 33342 sobre los espermatozoides de verraco, en las condiciones que se describen en el presente estudio, coincide con resultados previos obtenidos por Vázquez y cols., (2002). En este sentido los lechones nacidos de las IA realizadas con espermatozoides teñidos fueron completamente normales y no presentaron diferencia alguna con aquellos procedentes de espermatozoides no teñidos (grupo control), respecto a la presencia de anormalidades congénitas o al ritmo de crecimiento. Esta normalidad fenotípica de los lechones en ambos grupos refuerza aun más el aparente efecto inocuo del Hoechst 33342 sobre los espermatozoides de verraco.

Posiblemente, la normalidad fenotípica en todos los animales obtenidos a partir de espermatozoides separados por citometría de flujo (Seidel y Garner, 2002) es lo que ha determinado la inexistencia de estudios mas profundos sobre el posible efecto genotoxico del proceso de separación. En el presente trabajo se describe el primer análisis citogenetico de animales nacidos a partir de espermatozoides teñidos con Hoechst 33342, así como de animales obtenidos tras inseminación con espermatozoides teñidos que han sufrido el impacto del láser ultravioleta. El intercambio de cromátidas hermanas (Sister Chromatid Exchanges, SCE) y las aberraciones cromosómicas (chromosome aberrations, CAs) incluyendo roturas cromatídicas y cromosómicas son los dos análisis citogenéticos incluidos en este trabajo. La evaluación de los SCEs es considerada como una prueba citogenética mas sensible que las CAs para la evaluación del potencial efecto toxico sobre el ADN de una gran variedad de agentes mutagénicos y carcinogénicos (WHO, 1993). Sin embargo la realización de un sólo tipo de análisis citogenetico no se acepta de forma

general como evidencia suficiente para clasificar una determinada sustancia como mutagénica (WHO, 1985). Por tanto y teniendo en cuenta que ambas pruebas no son excluyentes entre sí, sino que resultan complementarias y pueden responder con diferente sensibilidad al agente sospechoso fueron incluidas en la realización de esta experiencia. Es mas, ambos análisis han sido ampliamente usados como biomarcadores de la exposición a agentes perjudiciales para el ADN.

En primer lugar se evaluó el numero de SCEs y CAs por célula obtenido en metafases linfocitarias de los lechones nacidos tras las inseminaciones realizadas con espermatozoides teñidos con Hoechst 33342 y los resultados obtenidos demostraron que no existían diferencias significativas respecto a los encontrados en linfocitos pertenecientes a los animales del grupo control obtenidos a partir de espermatozoides no teñidos. Estos resultados sugieren que el Hoechst 33342 no provoca daños a nivel del ADN y podían resultar previsibles desde el momento en que el Hoechst 33342 ha sido definido como un colorante especifico de ADN, ya que se une de forma especifica, sin intercalarse, a los pares de bases adenina-timina presentes en el surco menor de la doble hélice de ADN (Johnson y Schulman, 1994). Por otro lado, el elevado grado de condensación del ADN de los espermatozoides debido a la sustitución de histonas por protaminas supone una barrera protectora frente a agentes externos (Tanphaichtr y cols., 1978; Rodríguez-Martínez y cols., 1990), y por tanto puede jugar un papel relevante en la inocuidad del Hoechst 33342 sobre los espermatozoides de verraco.

La frecuencia de SCEs por célula entre individuos pertenecientes al mismo grupo, independientemente de su sexo, presentó ligeras variaciones sin llegar a niveles estadísticamente significativos, las cuales, bajo estas condiciones experimentales concretas, deben ser atribuidas principalmente a la relación entre la concentración de bromodeoxiuridina (BrdU) presente en el medio y el numero de linfocitos existente, lo cual es un factor individual y dependiente de cada individuo (Carrano y cols., 1980). La utilización de BrdU es necesaria para la diferenciación de las dos cromátidas de un mismo cromosoma y por tanto para la identificación de los SCEs, pero se debe prestar una atención especial a la hora de determinar su concentración en el medio ya que

esta sustancia es por si misma inductora de SCEs (Latt, 1973; Klingerman y cols., 1982). La concentración utilizada en esta experiencia fue de 0'1 μgr/ml, permitiendo una adecuada diferenciación de las cromátidas con un efecto mínimo sobre la frecuencia de SCEs y coincidiendo con las descritas, anteriormente, como concentraciones límites para la correcta diferenciación cromatídica en diferentes especies de rumiantes (Di Berardino y cols., 1996 y 1997).

La utilización, en la cuarta experiencia, de espermatozoides teñidos con Hoechst 33342 y sometidos al impacto del láser durante su paso a través del citómetro, dio lugar a una disminución significativa de todos los parámetros reproductivos analizados (tasa de gestación, tasa de partos y tamaño de la camada) respecto a los obtenidos en el grupo control (espermatozoides no teñidos y no sometidos al paso por el citómetro). Resultados similares han sido descritos en diferentes especies como resultado de la utilización de espermatozoides separados en las inseminaciones (Johnson y cols., 1989; Johnson, 1991,1995; Cran y cols., 1993; Seidel y cols., 1999a; Hollinshead y cols., 2002a y b; Seidel y Garner, 2002; Maxwell y cols., 2003). Las radiaciones ultravioleta inducen dos de las lesiones mas frecuentes en el ADN de células somáticas (Sinha y Hader, 2002), lo que podría hacer pensar que los espermatozoides que reciben el impacto del láser ultravioleta durante la separación mediante citometría de flujo pudieran presentar también alteraciones a nivel del material nuclear. En este sentido una reducción en el desarrollo de los embriones ha sido descrito tras la inseminación con espermatozoides separados de cerdas (Johnson, 1991), conejas (McNutt y Johnson, 1996) y vacas (Cran y cols., 1993), resultados que podrían ser explicados en base al trabajo publicado por Ahmadi y Ng (1999), en el que se asegura que aunque los espermatozoides que presentan lesiones en el ADN son capaces de fecundar los ovocitos con la misma efectividad que los no dañados, el desarrollo embrionario es significativamente mas bajo cuando se utilizan espermatozoides con el ADN alterado. Investigaciones mas recientes llevadas a cabo en cigotos de bovino y porcino demuestran que cuando se utilizan espermatozoides teñidos y separados empleando los nuevos citómetros de alta velocidad el desarrollo embrionario y la capacidad de esos embriones para alcanzar el estadio de blastocisto

no se ven afectados (Guthrie y cols., 2002; Zhang y cols., 2003). Además debe ser tenido en cuenta que actualmente y gracias a la alta velocidad, la duración del tiempo de exposición de los espermatozoides a luz del láser es mucho mas reducida que cuando se utilizaban equipos de separación de velocidad estándar, y por tanto cualquier posible efecto perjudicial que pudiera producirse en el ADN de los espermatozoides debido al impacto del láser ultravioleta debería ser mucho menor (Guthrie y cols., 2002). Por ultimo, la elevada condensación de la cromatina espermática, en comparación con otros tipos celulares, determina que el ADN de los espermatozoides sea altamente resistente frente agentes físicos o químicos (Lopes y cols., 1998), lo cual podría representar una optima protección frente al impacto del láser ultravioleta. Los resultados procedentes de los análisis fenotípicos y genotípicos realizados en esta experiencia suponen la primera evidencia científica de la seguridad genética del proceso de separación espermática mediante citometría de flujo. La normalidad de los lechones nacidos en el presente estudio, así como de los obtenidos en estudios anteriores realizados por diferentes autores (Johnson, 1991; Rath y cols., 1997 y 1999; Abeydeera y cols., 1998) permiten afirmar que la combinación del Hoechst 33342 con el impacto del láser no produce alteraciones relevantes en el ADN de los espermatozoides de verraco separados y que por tanto no deberían existir anomalías genéticas en la descendencia. Los resultados referentes a los niveles de SCEs y CAs observados en las metafases de los animales nacidos tras las inseminaciones con espermatozoides teñidos y sometidos al proceso de separación no difieren significativamente de los encontrados en el grupo control, lo cual confirma lo afirmado anteriormente.

Por otro lado podría ser argumentado que el proceso de separación por si mismo es capaz de inducir alteraciones en el estado de la membrana plasmática de los espermatozoides separados así como cambios en los patrones de motilidad de los mismos determinando una reducción de su vida útil independientemente del efecto producido por la tinción con Hoechst 33342 o por el impacto del láser ultravioleta (Johnson, 1995; Maxwell y cols., 1997; Maxwell y Johnson, 1999; Parrilla y cols., 2001), lo cual determinaría, como se ha señalado anteriormente, una reducción en los

índices de fertilidad sin que esto llevara consigo un incremento en el numero de gestaciones perdidas, en la incidencia de abortos o en la presencia de anormalidades morfológicas en la descendencia, coincidiendo con lo observado en esta experiencia donde la normalidad en los resultados de los análisis citogenéticos coincide con la normalidad fenotípica de los lechones obtenidos.

Como conclusión, hay que señalar que este es el primer estudio que confirma la ausencia de efectos genotóxicos in vivo de la tecnología de separación espermática mediante citometría de flujo, en base no sólo a evidencias fenotípicas, sino también a análisis citogenéticos. La normalidad de la descendencia a nivel fenotípico y la ausencia de un incremento significativo de los parámetros utilizados como indicadores de un posible efecto mutagénico en la descendencia confirman, con la precaución con la que se deben tomar este tipo de afirmaciones, la inocuidad del procedimiento. Investigaciones mas detalladas a nivel de genes concretos en espermatozoides de verraco deben ser llevadas a cabo con el objetivo de aumentar los conocimientos sobre la seguridad genética del proceso.

4.- Conclusiones//Conclusions

4.1.- CONCLUSIONES

1.- La diferencia que existe en el contenido de ADN entre los espermatozoides X e Y de macho cabrio así como las particularidades morfológicas de los mismos permiten una clara diferenciación entre ambas poblaciones espermáticas, determinando la utilidad de los mismos como herramienta para el alineamiento de los citómetros de flujo previamente al análisis y/o separación de espermatozoides de verraco.

2.- La hibridación *in situ* fluorescente directa, llevada a cabo con sondas de ADN especificas de cromosomas porcinos construidas mediante amplificación por PCR y marcadas posteriormente utilizando el método de desplazamiento de cortes (nick translation), ofrece una elevada eficiencia de hibridación dejando constancia de su utilidad en la evaluación del grado de pureza de las poblaciones espermáticas obtenidas tras el proceso de separación en espermatozoides de verraco.

3.- La capacidad de penetración de ovocitos madurados *in vitro* de los espermatozoides de verraco separados disminuye a partir de las 5 horas de conservación sin alteración aparente de la motilidad, viabilidad y estado

acrosomal de los mismos. Además, tiempos cortos de conservación (2 horas), aumentan la capacidad fecundante de los espermatozoides separados.

4.- La tecnología de la inseminación intrauterina profunda puede ser usada para la obtención de lechones a partir de espermatozoides separados, resultando fundamental el empleo de un numero adecuado de estos espermatozoides y un intervalo inseminación-ovulación lo mas corto posible para la obtención de las mejores tasas de gestación.

5.- La normalidad fenotípica y la inexistencia, en la descendencia, de un incremento en los valores de los indicadores de mutagenicidad empleados, demuestran la ausencia de un efecto genotóxico del proceso de separación espermática por citometría de flujo sobre los espermatozoides de verraco.

4.2.- CONCLUSIONS

1.- The large difference in DNA content between X- and Y- chromosome bearing goat spermatozoa as well as the flat shape of this head allows a clear identification of X- and Y- chromosome bearing goat spermatozoa using flow cytoemtric analysis. These properties of goat spermatozoa make it perfect choice for accurate,adjustment and calibration of the flow cytometer.

2.- Two-colour direct FISH with pig chromosome-specific DNA probes prepared by nick translation provides a useful tool for determining purity, with a high specificity, in flow cytometric sex sorted boar spermatozoa The easiness of the labelling procedure and the quality of the fluorescent signal obtained demonstrate that nick translation is an ideal labelling method for the preparation of specific fluorescent porcine DNA probes.

3.- Deposition of flow sorted boar spermatozoa as near as possible to the ovulation time and site of fertilisation before 5 hours post-sorting is necessary because the fertilizing ability of this sex sorted spermatozoa is starting to decline at this time of storage. However, taking in to account that storage for 2 hours enhances the fertilizing ability of sex sorted spermatozoa, this short storage time could be beneficial when these spermatozoa are directly inseminated close to the oocytes in vitro or in vivo.

4.- Deep intrauterine insemination (DUI) technology can be successfully used to produce piglets from flow cytometrically sorted boar spermatozoa. However, fertility of flow cytometrically sorted spermatozoa is lower than that of non-sorted spermatozoa.

5.- The lack of phenotypic and mutagenic evidences in the offspring born after insemination with sex sorted boar sperm confirms the absence of *in vivo* genotoxic effect of the flow cytometry sperm sorting technology.

5.- Resumen//Summary

5.1.- RESUMEN

La separación espermática por citometría de flujo, en base a la diferencia en el contenido de ADN existente entre los espermatozoides X e Y, es actualmente el único método eficaz para la obtención de descendencia de sexo deseado. Como aplicación más inmediata en el ganado porcino, la selección del sexo de las camadas incrementaría notablemente los beneficios productivos de las explotaciones dedicadas a la producción de animales de alto valor genético al poder manejar los esquemas de selección y desviarlos hacia un sexo u otro en función de las necesidades específicas de cada explotación.

Aunque se han realizado avances importantes en los últimos años en la especie porcina, es necesario un mayor conocimiento de diferentes aspectos relacionados con la eficiencia del proceso, del efecto que el procedimiento de separación puede tener sobre la viabilidad y la capacidad fecundante *in vivo* e *in vitro* de estos espermatozoides, así como del posible efecto perjudicial del propio proceso de separación sobre el ADN de los espermatozoides de verraco.

El ajuste del citómetro de flujo resulta fundamental a la hora de optimizar los rendimientos del proceso. Este ajuste resulta más complicado cuanto menor es la diferencia en la cantidad de ADN existente entre los espermatozoides X e Y, que es, a su vez, dependiente de la especie. Por ello, en la primera experiencia, se utilizaron espermatozoides de caprino ya que su proximidad filogenética con las especies bovina y ovina hacia esperar que presentaran unas diferencias en el contenido de ADN entre

los espermatozoides X e Y próximas a las existentes en estas especies dando lugar a una fácil identificación de las dos poblaciones. La diferencia en la cantidad de ADN entre los espermatozoides X e Y de macho cabrio quedó establecida en un 4'4% permitiendo una clara diferenciación de ambas poblaciones espermáticas. Los resultados obtenidos demuestran la correcta calibración del citómetro empleado y la utilidad de los espermatozoides de macho cabrio como instrumento para el ajuste y alineamiento de los equipos de separación espermática cuando se van a analizar o separar espermatozoides pertenecientes a especies cuyas diferencias respecto al contenido de ADN entre los espermatozoides X e Y son menores, como ocurre en el caso del ganado porcino.

Una vez comprobados el ajuste y funcionamiento óptimo del citómetro se procedió a diseñar un método destinado a analizar el grado de pureza de las poblaciones espermáticas separadas como sistema para la evaluación de la eficacia del procedimiento. En la realización de esta segunda experiencia se fabricaron sondas de ADN directas, especificas para cromosomas 1 e Y porcinos, construidas mediante amplificación por PCR y posterior marcaje fluorescente por el método de desplazamiento de cortes (nick translation). Las sondas obtenidas presentaron una sensibilidad elevada, lo que se traduce en la obtención de una eficiencia de hibridación próxima al 98 %. Además, la utilización de este sistema de marcaje dio lugar a sondas que originaban marcas fluorescentes muy claras obteniéndose hibridaciones limpias y fáciles de evaluar, por lo que se utilizaron para la evaluación de las purezas obtenidas en poblaciones espermáticas X e Y separadas. Como era de esperar, las purezas más elevadas (entre un 92'5 y un 89'5 %) se obtuvieron utilizando criterios de selección estrictos, correspondientes a tamaños de regiones reducidos, confirmando la eficacia del citómetro utilizado y evidenciando la estrecha relación existente entre la pureza de una muestra espermática separada y el tamaño de la población seleccionada, y por tanto entre esta pureza y el rendimiento del proceso.

Como resultado de su paso por el citómetro de flujo, y de los elevados grados de dilución a los que se ven sometidos, los espermatozoides separados presentan una serie de cambios en su motilidad, viabilidad e integridad acrosomal que asemejan a los

experimentados durante el proceso de capacitación (Catt y cols., 1997b; Maxwell y cols., 1997, 1998; Maxwell y Johnson, 1997,1998), comprometiendo la estabilidad de sus membranas (Maxwell y Johnson, 1999) y afectando a la viabilidad y a la capacidad fecundante de los mismos. Teniendo en cuenta que los rendimientos de los equipos actuales, cuando se trata de espermatozoides de verraco, implican periodos de separación que pueden estar comprendidos entre 1 y 10 horas, según la técnica de inseminación que se vaya a utilizar, conocer la influencia del tiempo de conservación sobre la viabilidad y capacidad fecundante de estos espermatozoides, resulta fundamental para la determinación del tiempo máximo que pueden ser almacenados, manteniendo su capacidad fecundante. Más aún, los laboratorios de separación se encuentran habitualmente alejados de los centros de inseminación, por lo que el tiempo que transcurre en el transporte de los espermatozoides debe ser evaluado, para conocer el efecto del mismo sobre la viabilidad y potencial fecundante de estos espermatozoides. Consecuentemente, es necesario analizar y conocer el estado de la población espermática en función del tiempo de conservación al que sea sometida. Para ello, en una tercera experiencia se analizaron la motilidad, viabilidad, estado acrosomal y capacidad de penetración de ovocitos madurados *in vitro* de espermatozoides de verraco separados y almacenados tras la separación durante 0, 2, 5 y 10 horas. El análisis de estos parámetros espermáticos determinó que, aunque la calidad de los espermatozoides puede mantenerse hasta 10 horas, la capacidad de penetración de estos espermatozoides empieza a decrecer 5 horas después de su separación. Asimismo, también se evidenció que un tiempo de almacenamiento de 2 horas aumentaba la capacidad fecundante de los espermatozoides separados respecto a los espermatozoides recién recogidos, por lo que periodos cortos de conservación podrían resultar beneficiosos antes de la utilización de estos espermatozoides separados tanto *in vivo* como *in vitro*

En la cuarta experiencia se procedió al estudio de la fertilidad in vivo de los espermatozoides de verraco separados y a la determinación de las condiciones más adecuadas para la obtención de las mejores tasas de fertilidad posibles. Los espermatozoides separados fueron inseminados mediante inseminación intrauterina

profunda depositándose en el tercio distal de uno de los cuernos uterinos tanto en cerdas con ovulación inducida como en cerdas con ovulación espontánea. Las tasas de fertilidad obtenidas demostraron que la inseminación intrauterina profunda puede ser utilizada para la obtención de descendencia viva a partir de espermatozoides separados. De igual manera quedó demostrado que la fertilidad de los espermatozoides separados de porcino aun siendo menor que la de aquellos no separados permanece a niveles aceptables cuando estos espermatozoides sometidos al paso por el citómetro son utilizados a altas concentraciones en la inseminación de cerdas con ovulación inducida.

Finalmente, se evaluó el posible efecto genotóxico que el procedimiento de separación espermática por citometría de flujo podría tener sobre los espermatozoides de verraco sometidos a este proceso. En primer lugar, se evaluó el efecto individual del fluorocromo Hoechst 33342 sobre la célula espermática, y en segundo lugar el efecto adicional del impacto del láser ultravioleta sobre los espermatozoides teñidos con Hoechst 33342. Los resultados obtenidos para los parámetros reproductivos analizados, así como los correspondientes a los análisis fenotípicos y genotípicos realizados sobre la descendencia nacida confirmaron la ausencia de un efecto perjudicial de la separación espermática por citometría de flujo sobre el ADN de los espermatozoides separados en la especie porcina.

5.2.- SUMMARY

Sorting of spermatozoa using flow cytometry based on the differential DNA content between X- and Y-chromosome bearing spermatozoa, is currently the only effective and most reliable procedure for achieving offspring of the desired sex. In swine production, the application of this sex pre-selection method could significantly improve production benefits through improved breeding programs; by simply planning AI to produce offspring of a specific gender. Moreover, availability of sexed semen can fasten genetic progress and allow for the production of male or female crossbred lines, thus improving efficiency of the exploitations.

Although major improvements have been realised regarding flow cytometric sperm sorting technology worldwide, a detailed knowledge of the efficiency of the sperm sorting technique and to aspects related with the effects that the procedure I imposes on sperm viability, DNA intactness and fertility are necessary to the full application of the technology. In this thesis, initially the usefulness and efficiency of the separation technology is reported. This is followed by the analysis of sorted boar spermatozoa motility, viability, *in vitro* and *in vivo* penetration ability. Finally, the results from the evaluation of mutagenic effects of Hoechst 33342 staining and of staining and sorting on sorted boar spermatozoa are included.

Adequate alignment and calibration flow cytometer is necessary to minimize the variability between analyses (sortigs) and to maximize flow cytometer performance. The instrument adjustment is complicated when the difference in the relative DNA content between X and Y sperm is small. This, is dependent on the animal spices. Goat spermatozoa were used in the first experience because it was expected that the difference in DNA content between X-and Y-chromosome bearing spermatozoa in bucks is close to that established for species such as ram or bull, leading to a easy identification of X and Y spermatozoa. Spermatozoa were analysed by flow cytometry to characterise X-Y sperm populations and to quantify the DNA difference between them. Two symmetrical, overlapping and clearly separated peaks,

corresponding to X- and Y-bearing spermatozoa, were detected. Therefore, the identification and selection of high purity samples of sperm populations for sex-sorting could be achieved easier in goats compared to the other domestic species. Moreover, the wide difference in DNA-content shown between X and Y chromosome bearing spermatozoa (4,4 %) makes these spermatozoa the primary choice for adjustment of instrument alignment even when spermatozoa from other species are to be sorted or analysed.

Evaluating the efficacy of sorting procedure for the separation of X- and Y-chromosome-bearing spermatozoa is an important step prior to utilization of sorted spermatozoa in reproductive techniques. Fluorescence *in situ* hybridization (FISH), which allows for the detection of specific nucleic acid sequences on morphologically preserved spermatozoa, is an ideal method for quantitatively and qualitatively assessing the purity of sorted sperm samples. In this thesis, specific porcine DNA direct probes for small regions of chromosomes 1 and Y were built by PCR amplification and labelled fluorescently by nick translation method. The chromosome 1 DNA probe was labelled green and used as an internal control to detect lack of hybridization, whereas the probe for chromosome Y was labelled red. Two-colour direct FISH was performed for chromosome 1 and Y in spermatozoa, unsorted or sorted for high and low Y-chromosome purity. The efficiency of the hybridization procedure was nearly 98 % on sorted and unsorted samples. The results confirmed that direct FISH using specific porcine DNA probes labelled by nick translation, provide a useful tool for laboratory validation of sperm separation by flow cytometric sorting technology.

Storage of sex sorted spermatozoa in a liquid medium is necessary since the current throughput of the flow cytometer is low, far too low than the requirements of AI, even when considering a deep intrauterine insemination. Determining how storage time affects these spermatozoa is then essential to know the potential sperm fertilizing ability expected after AI. Therefore, and taking in to account that the final population for insemination will be composed of spermatozoa ranging from cells just sorted to

cells sorted and stored for a time longer as 10 hours, the effect of the storage of sex sorted boar spermatozoa on motility, viability, and fertilizing ability when co-cultured with IVM pig oocytes as studied. Sorted boar spermatozoa were assessed at various times during storage after sorting and compared with extended and unsorted spermatozoa for sperm motility patterns, plasma membrane and acrosomal integrity and for their ability to penetrate homologous IVM oocytes. The results obtained suggests that although flow cytometrically sex sorted spermatozoa are able to maintain motility, viability and acrosomal integrity at optimal levels until 10 h of storage after sorting, fertilizing ability *in vitro* is compromised after shorter storage intervals (<5h).

The *in vivo* fertility of flow cytometric sorted spermatozoa was also evaluated. Pregnancy rates, farrowing rates and litter size from sows with either induced or spontaneous ovulation and inseminated with flow cytometry sorted spermatozoa using deep intrauterine insemination technology were analysed. As well, which were the most appropriate DUI insemination conditions leading to optimal fertility rates was also studied. The results indicated that the DUI technology can be successfully used to obtain offspring from sorted boar sperm, with acceptable fertility rates when high concentrations of these spermatozoa are used to inseminate sows with induced ovulation.

Finally, taking in to account that sex selection by flow cytometry/cell sorting involves the combined use of two potentially mutagenic agents e.g. staining spermatozoa with Hoechst 33342 and an ultraviolet laser beam, two potentially mutagenic agents, a phenotypic and cytogenetic study was issued. Lymphocytes of piglets born after insemination with spermatozoa stained with Hoechst 33342, and from piglets obtained from stained-sorted spermatozoa were evaluated for DNA damage. Sister chromatid exchanges and chromosome aberrations were used as indicators of exposure to DNA-damaging agents. Reproductive parameters and the litter performance of all inseminations performed were also evaluated. The results indicated no genotoxic effect on sorted spermatozoa, expressed as increases in the incidence of abnormalities in the resulting offspring, or increase in the baseline frequencies of mutagenicity endpoints on lymphocytes from different individuals.

Overall, these experiments have rendered essential information concerning the optimization of flow cytometric sperm sorting technology in porcine species. Proper alignment and calibration of the flow cytometer, basic to maximize its performance for sorting of boar spermatozoa can be obtained using sperm heads from goat spermatozoa. Regarding the evaluation of the efficiency of flow cytometric sperm technology for boar sperm sorting, defined as the percentage of spermatozoa bearing the selected chromosome, two-colour direct fluorescence *in situ* hybridization with porcine chromosome-specific DNA probes prepared by PCR amplification and nick translation labelling provides a useful and highly specific tool to determine the purity of the sorted sperm population. From a practical viewpoint, the present study shows the necessity of depositing the spermatozoa as close as possible to the ovulation time and the site of fertilisation before 5 hours post-sorting, because the fertilizing ability of sex sorted spermatozoa starting to decline at this storage time. However, in the light of these results, it could be noted that storage for 2 hours enhances the fertilizing ability of sex-sorted spermatozoa. Consequently, this short storage time could be beneficial when spermatozoa are directly inseminated close to the oocytes *in vitro* or *in vivo*. On the other hand, the results from *in vivo* inseminations using sorted boar sperm indicates that deep intrauterine insemination technology can be successfully used to produce piglets from sorted spermatozoa and suggest the convenience of using high number of sex sorted boar sperm and sows with induced ovulation to perform the inseminations with flow cytometric sorted boar sperm in order to optimize the fertility rates of these spermatozoa. Finally, piglets born from Hoechst 33342 stained-spermatozoa, or from stained-sorted boar spermatozoa were phenotypically and cytogenetically normal, thus confirmming the absence of genotoxic effects of this sexing boar sperm technology and eliminating, at least in part, major reservations concerning the genetic safety of the process.

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7.-Abreviaturas

ADN		**Acido Desoxirribonucleico**
ALH	*Amplitude of lateral Head displacement*	**Amplitud media del desplazamiento lateral**
BrdU	*Bromodeoxyuridine*	**Bromodeoxiuridina**
DUI	*Deep Uterine Insemination*	**Inseminación intrauterina profunda**
eCG		**Gonadotropina Coriónica Equina**
FITC-PNA	*Fluorescein-labelled peanut agglutinin*	**Conjugado de lectina de Arachis** *hypogaea con isotiocianato de fluoresceina*
FIV		**Fecundación** *in vitro*
h		**horas**
IA		**Inseminación Artificial**
ICSI	*Intra Citoplasmic Sperm Injection*	**Inyección intracitoplasmática de de espermatozoides**

m		metros
mgr/ml		miligramos por mililitro
mm		milímetros
PI	Propidium Iodide	Yoduro de propidio
p.s.i.	Pounds per square inch	Libras por pulgada cuadrada
VCL	*Velocity-Curvilinear*	Velocidad respecto a la trayectoria real
VSL	*Velocity Straight Line*	Velocidad respecto a la trayectoria recta o lineal

Apéndice 1: Anexo Gráfico

El formato de la presente Tesis implica la presentación de los trabajos tal y como han sido publicados, lo cual impide la inclusión de imágenes y esquemas complementarios. Por ello, se muestran en este apéndice, una serie de figuras con información adicional referente a las experiencias realizadas

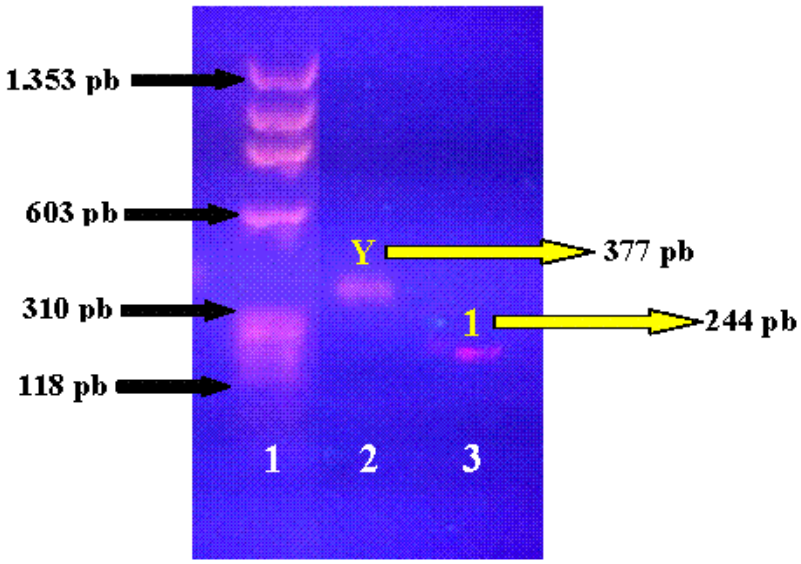
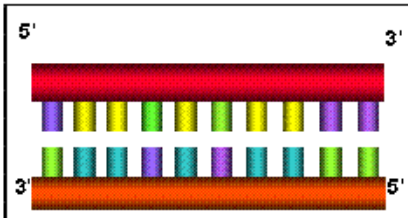
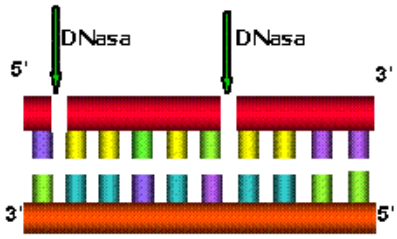


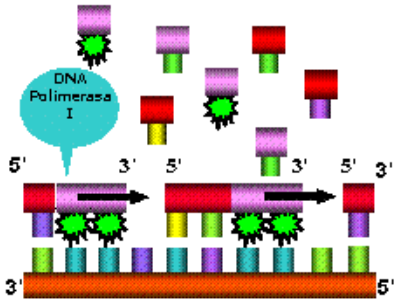
Figura 1 (Experiencia 2): Gel de electroforesis mostrando las bandas correspondientes a la amplificación por PCR de los fragmentos específicos de los cromosoma 1 e Y de la especie porcina. En la calle 2 se muestra la banda correspondiente al fragmento amplificado del cromosoma Y con un tamaño de 377 pares de bases (pb), y en la calle 3 la correspondiente al fragmento específico de cromosoma 1, que tiene un tamaño de 244 pb. El marcador de tamaño de bandas utilizado (ΦX174 DNA Marker Hae III Digest) se muestra en la calle 1.



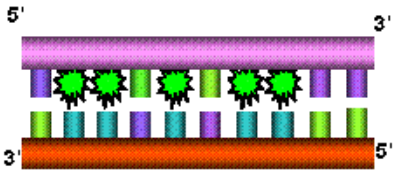
Un fragmento de ADN de doble cadena especifico de cromosoma 1 o Y obtenido mediante PCR es utilizado como molde.



En la primera etapa de la reacción el ADN es cortado merced a la acción de pequeñas cantidades de la enzima deoxiribonucleasa I (DNasa).



En una segunda etapa catalizada por la enzima *E. Coli* Polymerasa I, los nucleótidos ( ) del extremo 5'-OH de la muesca son eliminados merced a la actividad exonucleasa de esta enzima, mientras que nuevos nucleótidos ( ) son incorporados al extremo 3'-OH generado tras cortar la doble cadena. De tal manera que se produce un desplazamiento del corte del ADN en dirección 3'--→5'



La presencia en el medio de los nucleótidos marcados ( ) determina la incorporación de los mismos de forma especifica en la molécula de AND según la complementariedad de bases. Debido a que los cortes son generados al azar el ADN marcado de forma uniforme, lo que da lugar a la obtención de una sonda fluorescente de ADN con una elevada eficiencia y una alta especificidad.

Figura 2 (Experiencia 2):: Esquema de la reacción de marcaje por desplazamiento de cortes (nick translation) utilizada para la construcción de sondas de ADN especificas de cromosomas porcinos.

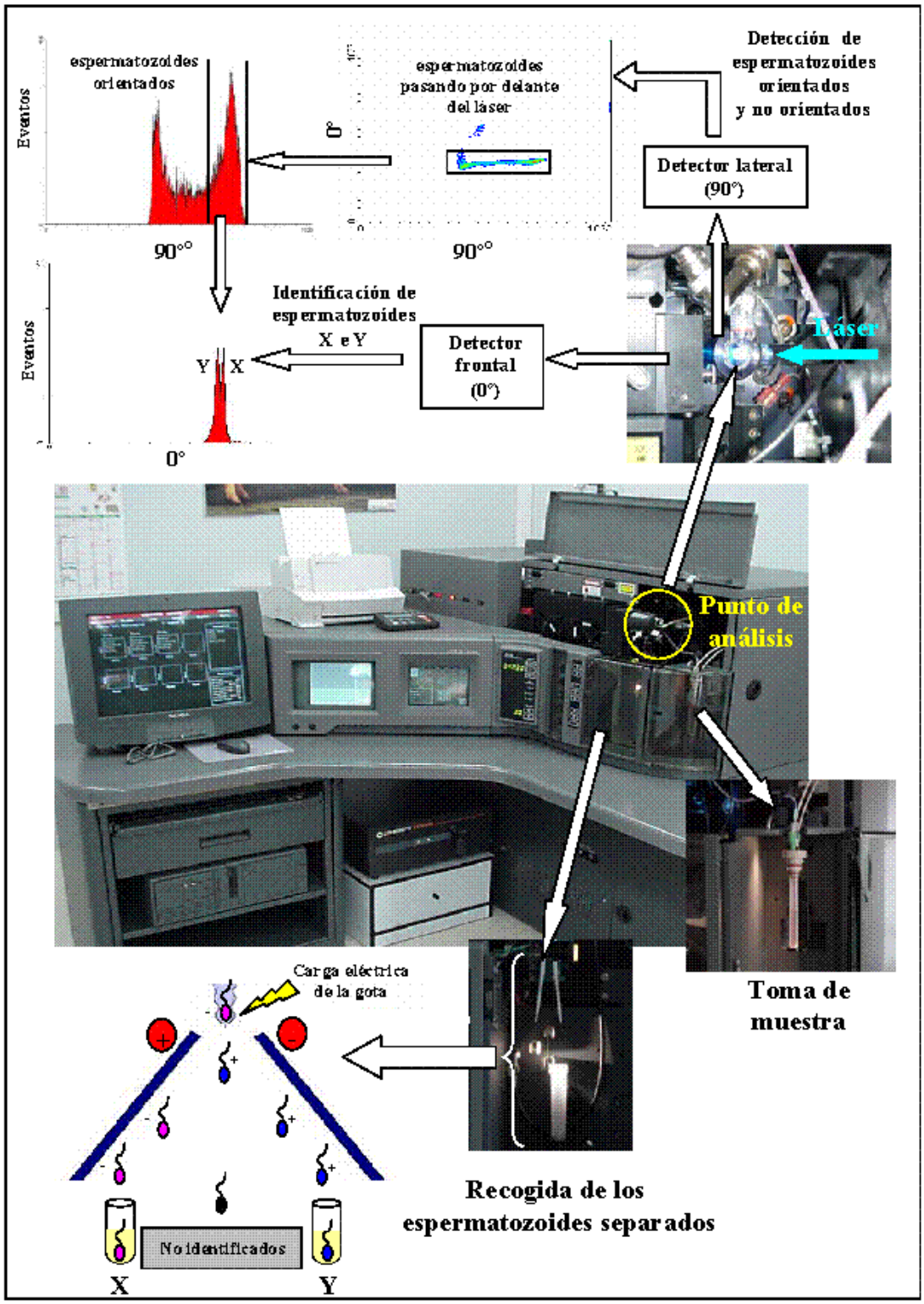


Figura 3 (Experiencias 1, 2, 3, 4 y 5): Esquema del proceso de análisis y separación espermática mediante citometría de flujo

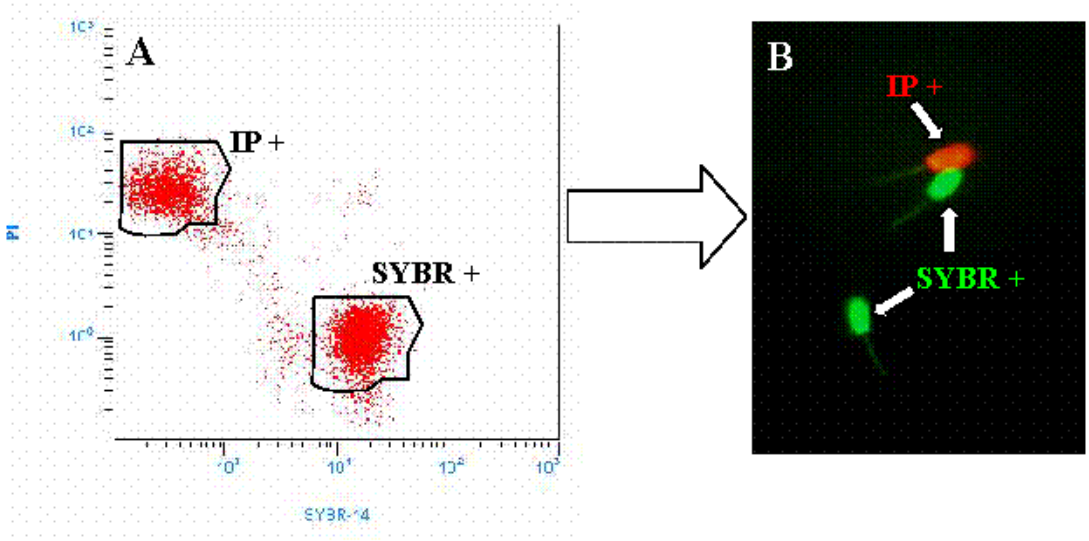


Figura 4 (Experiencia 3): Evaluación de la viabilidad espermática mediante tinción con el LIVE/DEAD Sperm Kit (Molecular Probes), compuesto por dos fluorocromos, ioduro de propidio (IP) y SYBR-14. El SYBR-14 es capaz de atravesar las membranas fosfolipídicas y unirse al ADN nuclear del espermatozoide tiñendo, de verde, tanto los viables como los no viables. El IP, únicamente penetra en aquellos espermatozoides con las membranas alteradas, desplazando el SYBR-14 y tiñendo de rojo aquellos espermatozoides no viables. (**A**) Representación gráfica correspondiente al análisis por citometría de flujo de los espermatozoides teñidos, mostrando la población de espermatozoides viables (SYBR+) y de espermatozoides con las membranas alteradas (no viables; PI+). (**B**) Imagen de microscopia de fluorescencia (1000X, triple filtro: Dapi, FITC, Texas Red) correspondiente a la misma tinción, donde se pueden observar 2 espermatozoides viables (SYBR-14+), y uno no viable (IP+).

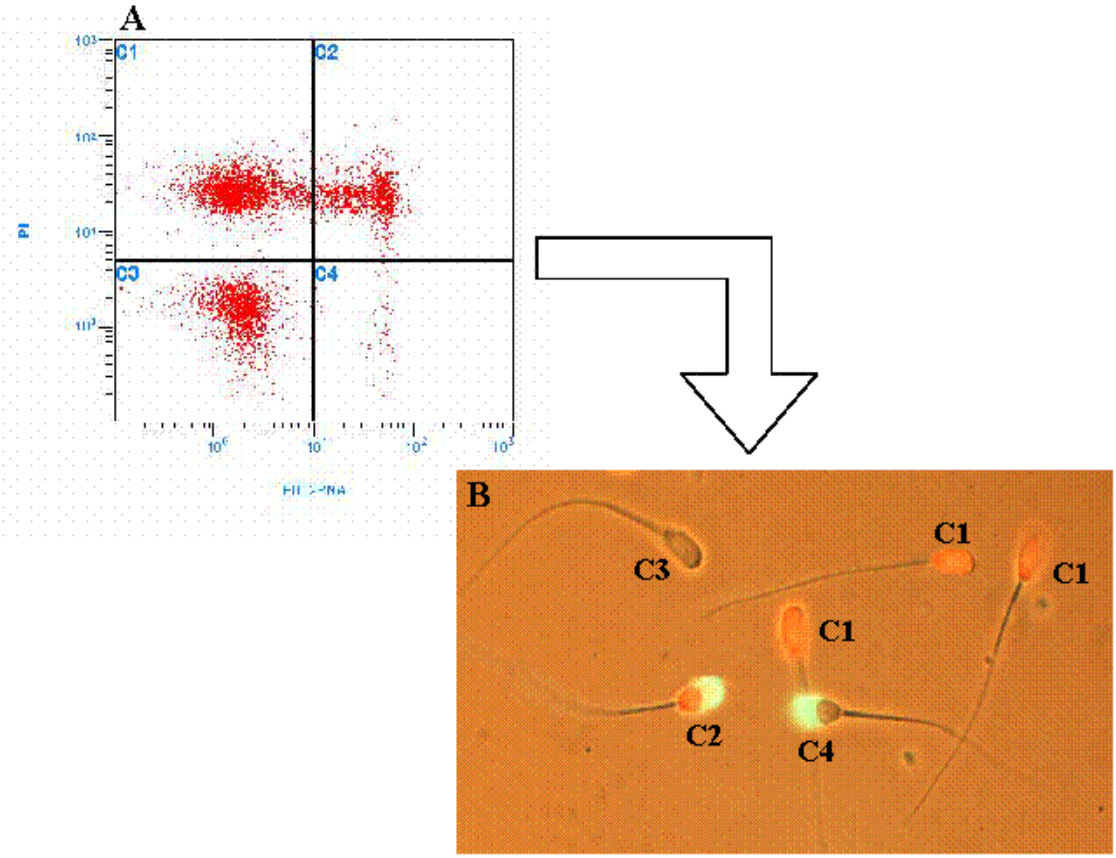


Figura 5 (Experiencia 3): Evaluación del estado acrosomal. Tinción con ioduro de propidio (IP) y FITC-PNA (conjugado de lectina de A*rachis hypogaea* con isocianato de fluoresceína). El IP penetra únicamente en los espermatozoides con la membrana plasmática dañada (no viables), tiñendo de rojo su ADN. Por otro lado, la PNA es una lectina que tiene afinidad por ciertas proteínas de la membrana del acrosoma, por lo que gracias a su conjugación con la fluoresceína teñirá de verde aquellos dañados o reaccionados. (**A**) Resultados obtenidos mediante citometría de flujo, donde se muestran las cuatro poblaciones espermáticas encontradas: C1 (IP+/FITC-PNA-): espermatozoides no viables y no reaccionados; C2 (IP+/FITC- PNA+): espermatozoides no viables con el acrosoma reaccionado; C3 (IP-/FITC-PNA-): espermatozoides con las membranas intactas (viables); C4 (IP-/FITC-PNA+): espermatozoides viables con el acrosoma reaccionado.(**B**) Espermatozoides teñidos con IP/ FITC-PNA mostrando los patrones de tinción correspondientes a cada una de las subpoblaciones espermáticas identificadas por el citómetro.

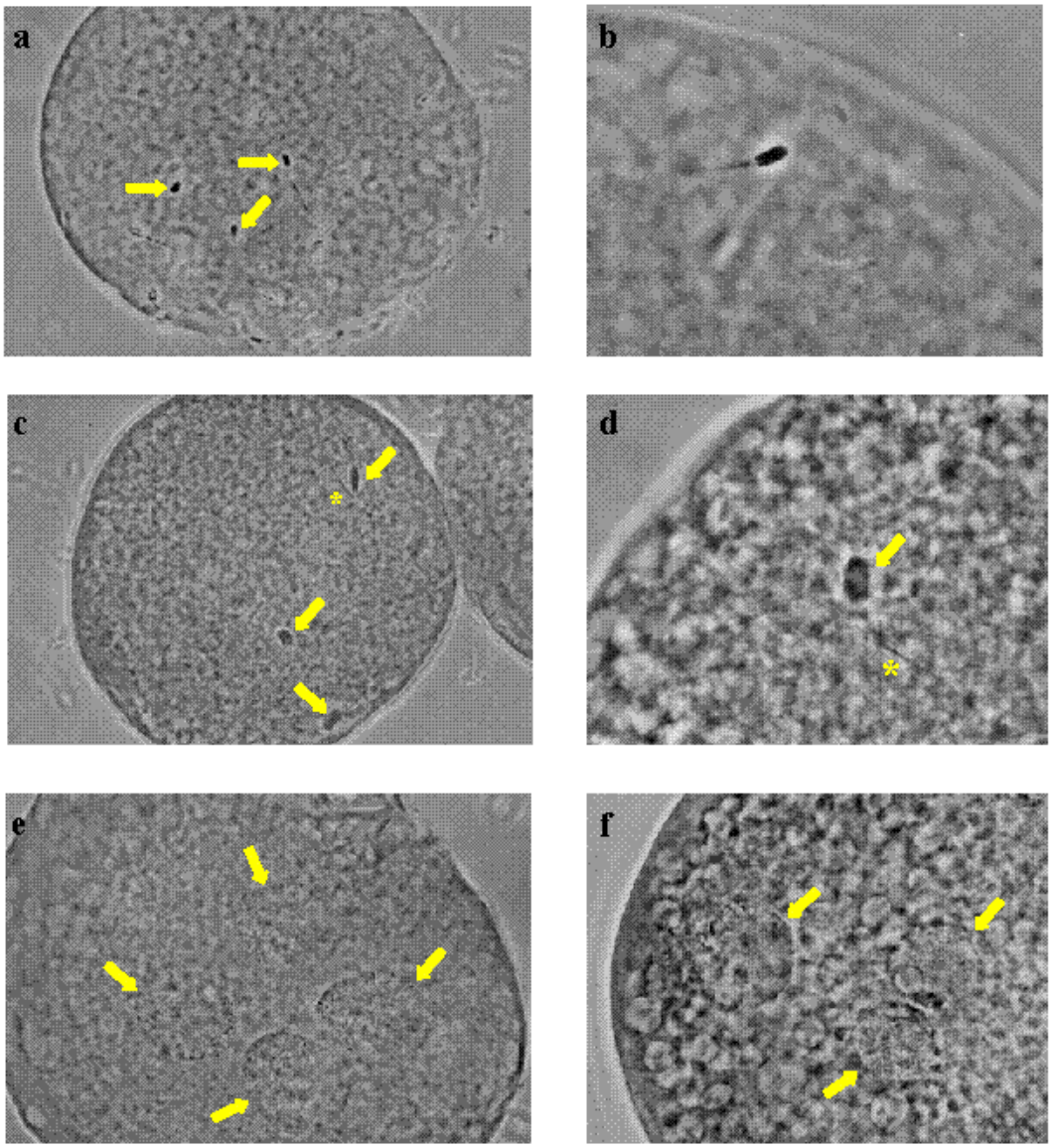


Figura 6 (Experiencia 3): Ovocitos madurados *in vitro* y coincubados, durante diferentes intervalos de tiempo con espermatozoides sometidos al proceso de separación por citometría de flujo (microscopia de contraste de fases, 400x y 1000x,). **a**: Espermatozoides en el interior del ovocito (flechas) a las 2h de comenzar el cocultivo. En diferente plano óptico, se observan espermatozoides adheridos a la zona pelúcida del ovocito. **b**: Detalle de un espermatozoide en el interior del ovocito tras 2h de cocultivo. **c**: Imagen obtenida a las 6 h del cocultivo donde se aprecian tres cabezas espermáticas descondensandose (flechas) y un flagelo espermático separado (*). **d**: Detalle de una cabeza espermática que ha empezado a descondensarse (flecha), es también visible el flagelo espermático separado (*). **e y f**: Ovocitos poliespérmicos obtenidos a las 18h de la coincubación., mostrando 4 y 3 pronúcleos (flechas) respectivamente.

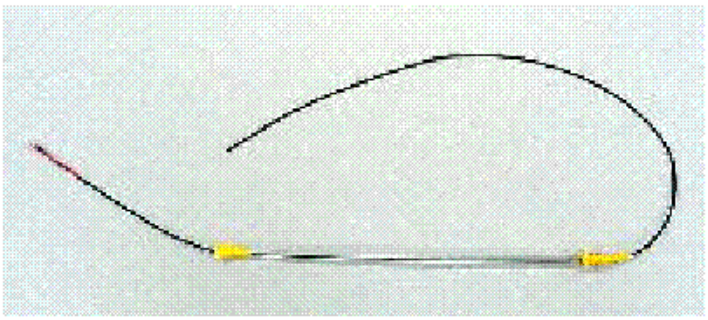


Figura 7 (Experiencia 3): Catéter flexible utilizado para la inseminación intrauterina profunda (1' 80 m de longitud; diámetro externo de 4 mm, canal de trabajo de 1'80 mm)

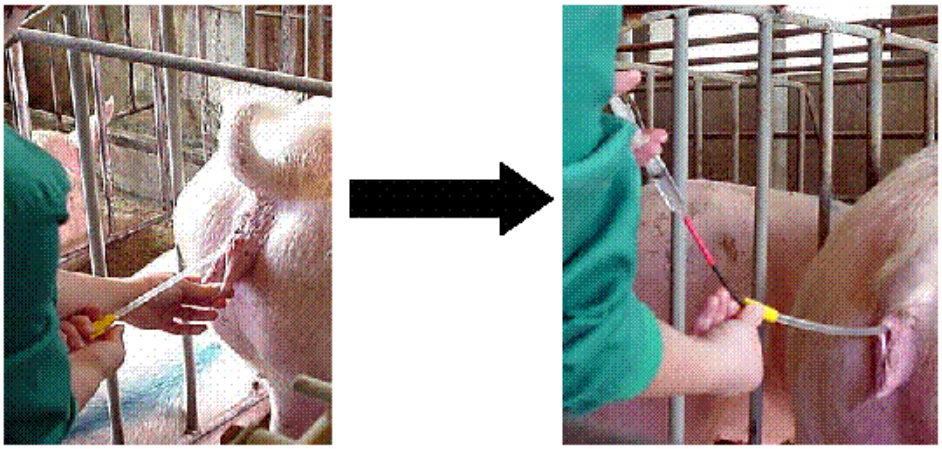


Figura 8 (Experiencia 3): Inseminación intrauterina profunda Una vez limpia el área perineal de la hembra con agua y jabón se insertó en el cérvix un catéter comercial de IA a través del cual se introdujo el catéter flexible para la inseminación profunda. El catéter de IA sirvió para facilitar la manipulación del catéter flexible que fue propulsado a través del cérvix a lo largo del cuello uterino hasta alcanzar el tercio anterior de un cuerno uterino.

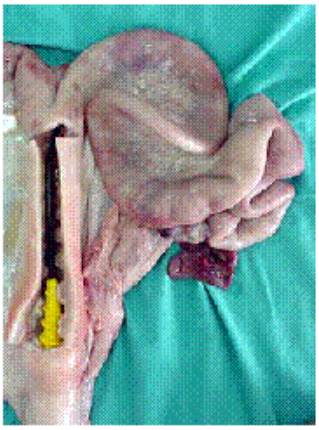


Figura 9 (Experiencia 3): Situación del catéter de IA en el cuello uterino y del catéter flexible de inseminación intrauterina profunda progresando por el interior de uno de los cuernos uterinos de tracto genital de la cerda. Imagen construida a partir de un aparato genital obtenido en el matadero.

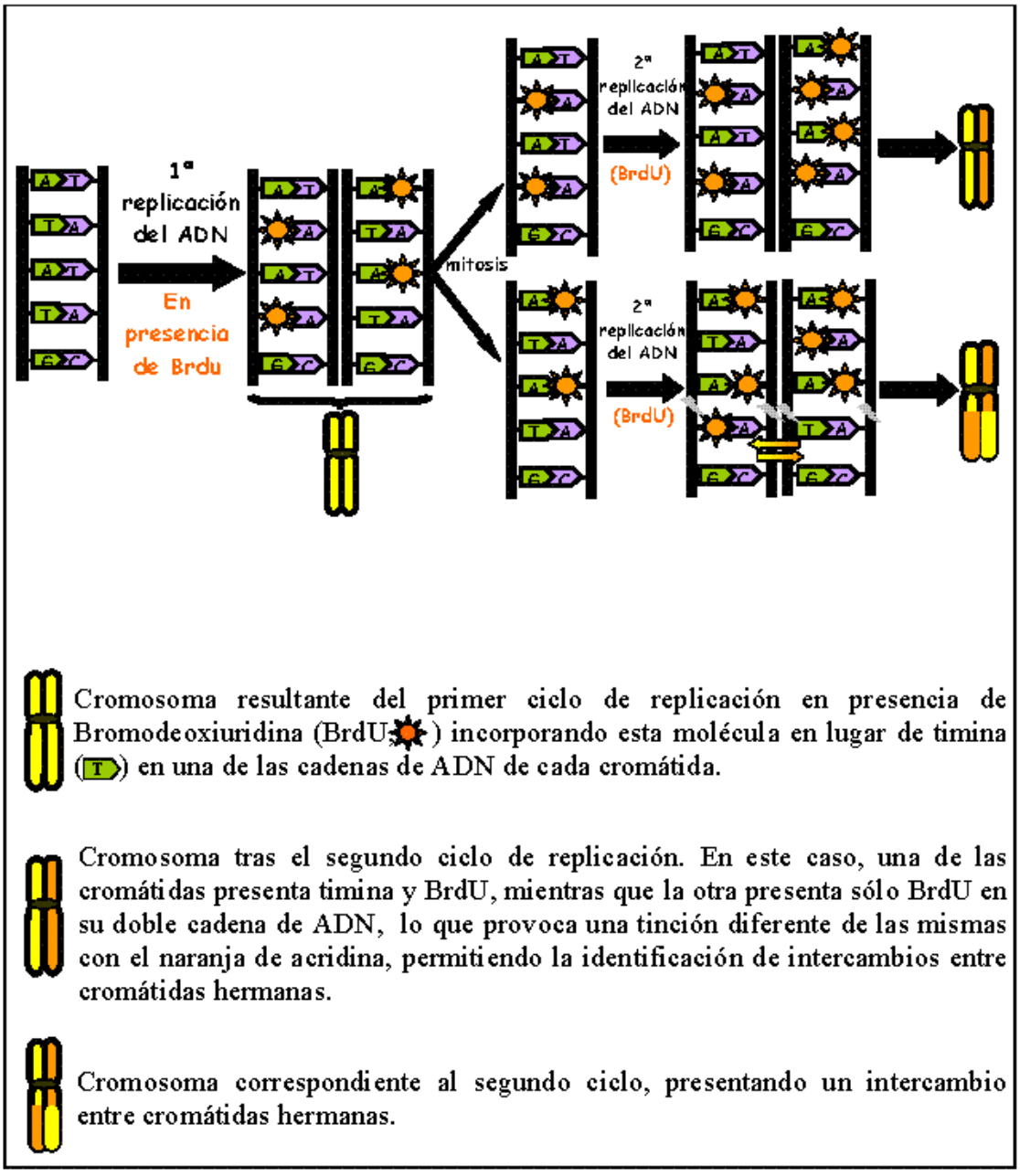


Figura 10 (Experiencia 5): Diagrama ilustrativo del mecanismo de incorporación de la bromodeoxyuridina (análogo de la timina; BrdU) para la identificación de los intercambios entre cromátidas hermanas de un mismo cromosoma.

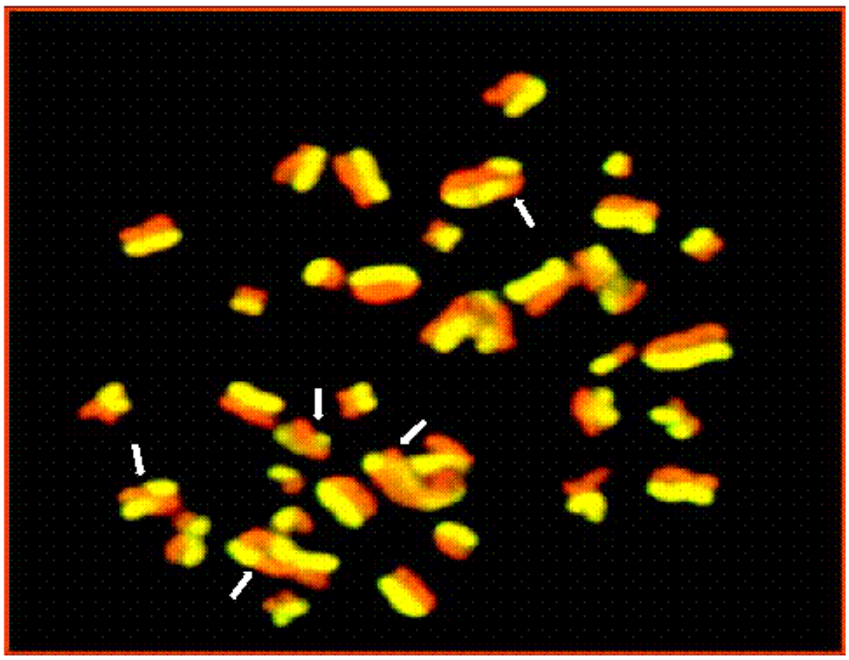


Figura 11 (Experiencia 5): Placa metafásica obtenida tras el cultivo, en presencia de bromodeoxiuridina, de linfocitos de lechones nacidos tras las inseminaciones realizadas con espermatozoides separados. Las flechas indican el lugar de intercambio entre cromátidas hermanas de un mismo cromosoma.

Apéndice 2: Extended Summary

INTRODUCTION

The development of semen sexing is widely accepted as a major advance in reproductive technology. The pre-selection of the sex accelerates genetic progress as well as enhancing benefit the management and efficiency of pig production. The only effective sex pre-selection methodology (Johnson et al., 1989; Johnson, 1991) has been validated in numerous laboratories and is based on measuring the differential amount of DNA present in X- and Y-chromosome bearing spermatozoa. This difference is measured in a modified flow cytometer/cell sorter and the spermatozoa sorted into populations enriched for X- or Y-bearing spermatozoa with a high percentage of purity. The effectiveness of this technology for producing offspring in swine of the preselected sex has been proven in numerous trials [Johnson, 1991;Rath et al., 1997; Abeydeera et al., 1998; Rath et al., 1999; Probst et al., 2002)] were the semen was sorted and surgically inseminated or used for in vitro fertilisation or intracytoplasmatic sperm injection and subsequent embryo transfer.

The DNA difference between X and Y spermatozoa varies among species being, for farm animals, in the range of 3.5% to 4.5% (Johnson 1995). A clear identification of the two sperm populations to sort depends on this DNA difference which conditions the size and correct setting of sorting gates and thus, the final output

of the process (Vazquez et al 2001). Although sperm sexing technology has demonstrated its effectiveness for many species (Seidel and Garner 2002), no data is yet available about DNA content in goat spermatozoa. Taking into account that the isolation of recombinant proteins from transgenic milk goats has attracted interest in the last few years (Balsadarre et al. 2003), sexing technology could be of great interest for this kind of biotechnological research. Moreover, goat spermatozoa were used in this experience mainly because it was expected that the difference in DNA content between X-and Y-chromosome bearing spermatozoa in bucks was close to the differences established for similar species as ram or bull, leading to a easy identification of X and Y spermatozoa and making possible the use of these spermatozoa for the correct alignment of any flow cytometer as a first step before starting any sorting procedure even when spermatozoa from other species are to be sorted or analysed.

On the other hand, determination of the resulting sex ratio before inseminating the sorted spermatozoa is essential to make this reproductive technique economically feasible. The sex of the offspring depends on the purity of the sorted spermatozoa in terms of X:Y-chromosome sperm ratio and, in turn, the purity of the sorted spermatozoa depends on the width of the sorting area setting (Johnson, 1997; Seidel and Garner, 2002). Unfortunately, the production rate also depends on the sorting windows; increasing the size of the sort windows by 15%, allows about a 20% increase in the rate of sorting spermatozoa to be achieved; however, the purity of the sorted population defined as the percentage of spermatozoa bearing the selected chromosome (X or Y) decreases by 10% (Johnson, 1997). Thus, the purity of the sorted spermatozoa decreases as production rate increases during the sorting. Although a high purity with a low production rate can be selected when sorted spermatozoa are used for IVF or intracytoplasmic sperm injection, a high production rate is needed for deep intrauterine insemination with sorted spermatozoa in sows (Vazquez et al., 2003). Therefore, the use of a wide sorting window is necessary during the sorting with consequent detrimental effect on the purity of the sperm population.

Molecular biology offers various techniques for assessing the effectiveness of X- and Y-chromosome separation. PCR DNA amplification of single cells has been used to assess the purity of the sorted spermatozoa (Welch et al., 1995). However the labour required to conduct PCR of single cells likely prohibits its use for the routine monitoring of sorted sperm.

Fluorescence *in situ* hybridization (FISH) has also been used to analyse sorted spermatozoa. Kawarasaki et al. (1998) showed that two-colour FISH using a combination of DNA probes specific for chromosomes Y and 1 was useful for estimating the purity of X and Y sperm cells sorted by flow cytometry. This method offers the opportunity to use an independent technique for validating sorted sperm samples quantitative and qualitatively (Welch and Johnson, 1999).

The most important reagents on a FISH procedure are the fluorescent DNA probes. After or during DNA amplification steps by PCR, specific DNA must be labelled by incorporating fluorescent nucleotides. It is important to use a labelling method that allows the production of probes with good specificity that are easy to visualise to produce optimal hybridization results. The type of probe and labelling method should be chosen according to the sensitivity requirements of the situation.

The FISH technique has been performed on bull (Hassane et al., 1999; Piumi et al., 2001) and boar spermatozoa (Kawarasaki et al., 1995; 1996), and boar and cattle sperm flow sorting has also been evaluated by indirect FISH probes (Kawarasaki et al., 1998; Rens et al., 2001). However, no results have been described using direct probes for evaluating the purity of X- and Y- chromosome sorted spermatozoa.

The aim of second experience was to determine an easy procedure for direct FISH on boar spermatozoa using nick translation-labelled pig DNA direct probes specific for small regions of chromosomes 1 and Y. The efficiency of the nick translation reaction was evaluated by performing a FISH assay on pig spermatozoa that were unsorted and sorted for high and low purity. The advantages and disadvantages of this procedure were analysed.

Although the current throughput of sperm sexing instruments is fast (5000-6000 sperm/s) (Seidel, 2003), this yield is relatively slow and long sorting times are required to obtain the adequate number of sorted boar spermatozoa. Therefore, the final sperm population will be characterized by its heterogeneity, since it consists of sperm cells ranging from spermatozoa subjected to relatively long holding times after sorting to spermatozoa that have only just been sorted.

It is known that the sperm membrane is partially compromised during the flow cytometry and sorting process, which affects viability, storage capability and fertilization ability of spermatozoa (Maxwell and Johnson, 1999). Both, the physical effect of the sorting procedure and the high dilution rate by sheath fluid cause in the spermatozoa a short lifespan after sorting (Maxwell and Johnson, 1997). To improve post-sorting sperm survival, the impact that each sorting steps has on the sperm population should be evaluated, and the interval during which the sperm cells preserve their fertilizing capability after sorting needs to be defined. This would help to establish the potential fertilizing capability of the sex-sorted sperm population. Although the effect of the Hoechst 33342 staining (Vazquez et al., 2002) and the sorting procedure (Maxwell and Johnson, 1997; Maxwell et al., 1997; 1998) has been studied on boar spermatozoa, there are no reports monitoring the effects of liquid storage of flow cytometrically sorted boar spermatozoa up 10 hours on sperm functionality; this is a critical time for both the sorting and the transport of the spermatozoa from the cell sorter laboratory to the farm for performing a non-surgical insemination.

In the third experience we have monitored the changes in viability, motility, acrosomal exocytosis and ability to penetrate IVM oocytes of flow sorted boar spermatozoa stored for 0, 2, 5 and 10 h. The main goal of this study was to know the variability in the sperm functionality of sex-sorted spermatozoa when a long holding time is required.

With regard to the practical application of this technology, it is evident that any methodology applied to sexing swine spermatozoa must search for the appropriate

application in the industry. The most economical means of using sexed boar spermatozoa in practical settings would be through non-surgical low dose artificial insemination of sows. Although the efficiency of the sorting procedure has increased considerably during the last five years with the development of improved nozzle designs (Rens et al., 1998)] and adaptation to high speed cell sorting (Johnson y Welch, 1999)], its usefulness remains limited in the pig due to the large number of sperm required for routine cervical insemination (2×10^9 to 3×10^9 spermatozoa per insemination dose) under industry conditions.

Classically it has been accepted that after sorting, the spermatozoa have a short lifespan due to the different stages that the spermatozoa must pass during the sorting process. Thus, there is an acute necessity to deposit the sexed spermatozoa close to the site of fertilisation to avoid premature death that would likely occur during the movement of the spermatozoa from the cervix to the oviduct (Maxwell and Johnson, 1999). Previous studies (Johnson, 1991; Rath et al., 1997) have sought to overcome this difficulty by applying surgical intratubal insemination to deposit the spermatozoa directly into the oviduct. Such a technique is useful for research purposes but is unlikely to be applied effectively in practical pig production.

Recently, a new procedure for non-surgical deep intrauterine insemination (DUI) in non-sedated sows has been described (Martinez et al., 2002)]. This procedure is based on a previous system using a fiberoptic endoscope (Martinez et al., 2001) and it consists of a specially designed flexible catheter that allows passage through the cervix and deposition of the sperm in the anterior region of the uterine horn. Using this DUI procedure it has been demonstrated that only 50 million spermatozoa are required to achieve pregnancy. Based on these result, we have hypothesised that 50 to 70 million sorted spermatozoa would be sufficient to attain pregnancy in sows with our DUI technique without a decrease in farrowing rates. However, even with this low number of sperm inseminated, sorting sufficient numbers of sperm to achieve pregnancies with either X- or Y- sorted sperm to ascertain the effectiveness of the technique with sexed sperm is difficult at the present time. In other species, the use of

sexed sperm in conjunction with deep uterine insemination (Seidel et al., 1997; Buchanan et al., 2000) has been found to be effective for producing sexed offspring.

Larsson observed that insemination close to ovulation led to the highest fertility results when hCG-treated sows were inseminated with frozen semen. Since the spermatozoal membranes may be compromised by flow cytometric sorting (FCS), we determined it advantageous to induce ovulation in conjunction with the use of DUI. Ovulation time can also be determined by frequently repeating ultrasound examination (Martinat-Botte et al., 2000). The use of the ultrasound to evaluate the follicular diameter as an approach to pin-point ovulation time could help to avoid the necessity for inducing ovulation with hormones.

The objective of the experience four was to ascertain the effectiveness of the DIU when flow cytometrically sorted boar spermatozoa (mimicking the conditions that sperm are exposed to during the BSST) are inseminated in sows after induction of ovulation or in which spontaneous ovulation was detected by ultrasongraphy.

On the other way, flow cytometric sperm sorting is widely accepted as a safe technique in practice, in the absence of phenotypic evidence that suggests otherwise. As far as we know, the only disadvantageous effects described when flow sorted spermatozoa are used for insemination are the short fertile life span of the spermatozoa as well as the loss of embryos during gestation, expressed as a reduced litter size in both rabbits (Johnson et al., 1989) and pigs (Johnson, 1991; Vazquez et al., 2003). However, the reason for this reduction remains unclear.

It is well known that Hoechst 33342 could have toxic and mutagenic effects upon certain cell types (Durand and Olive, 1982), and also that u.v. light produces an increase in chromosome structural abnormalities in mouse spermatozoa (Matsuda and Tobari, 1988). Concerns have been raised that simultaneous use of both agents may affect the genetic safety of sperm selection by flow cytometry (Ashwood-Smith, 1994; Munné, 1994 ; Gardiner-Garner, 1999) and, consequently, the necessity to evaluate DNA damage on the sorted spermatozoa has become a priority in spite of the absence

of congenital abnormalities (Morrel and Dresser, 1989). However, no cytogenetic studies about potentially mutagenic effect of flow cytometric sorting technology on the animals born after inseminations with spermatozoa processed with this technology have been reported.

Evaluation of the increases in baseline frequencies of cytogenetic endpoints as sister chromatid exchanges (SCEs) and chromosome aberrations (CAs) has been used for many years to measure the possible mutagenic and carcinogenic effect when cells, animal or human, are exposed to known genotoxic agents (Perry and Evans, 1975; Latt et al., 1981; Albertini et al., 2000).

While it has been determined that SCE is an ideal method for evaluating the genotoxic potential of those substances that induce DNA damage or interfere with DNA metabolism or repair, CA has been more related with those substances that directly break the backbone of DNA or significantly distort the DNA helix (Carrano and Natarajan, 1988).Consequently, the cytogenetic analysis using SCE and CA of animals born after inseminations with spermatozoa stained with Hoechst 33342 and/or flow cytometrically sorted could represent an ideal means of determining the safety of this sex selection procedure.

The main goal of last experience was to evaluate the phenotypic and cytogenetic normalcy of piglets born after inseminations with Hoechst-33342 stained or stained and flow cytometrically -sorted boar spermatozoa.

MATERIAL AND METHODS

1.- Experience 1: Flow cytometry identification of X- and Y- chromosome bearing goat spermatozoa.

Semen samples were weekly collected via artificial vagina from 6 fertile Murciano-Granadina bucks (between 18 and 36 months old) and, following immediate

evaluation of normality (motilty >80%; viability>85%; total spermatozoa per ejaculate>$3x10^9$; acrosomal abnormalities<10%; abnormal spermatozoa morphology<15%), extended in Beltsville Thawing Solution to a final concentration of $150x10^6$ spermatozoa/ml. The extended semen was then processed for sperm sorting following the general procedure described by Johnson et al. (1989). Briefly, one ml of extended spermatozoa were stained with Hoechst-33342 fluorophore (22.4 μM, Sigma Chemical Co., Alcobendas, Madrid, Spain) and incubated for at least 2 h at 35°C. The stained spermatozoa were passed through a modified EPICS Altra flow cytometer/cell sorter (Coulter Corporation, Miami, FL. USA) operating at 42 p.s.i. The fluorophore loaded spermatozoa were excited with 120 mW of ultraviolet (UV; 351, 364 nm) lines of a 6-W 90-6 Innova Laser (Coherent Lasers, Inc., Palo Alto, CA). Two UV blocking filters (460 and 450 nm band pass for 0° and 90° detectors, respectively) were used. The fluorescence emitted by the spermatozoa was collected from both the 0 and the 90 degree detectors and stored as frequency distributions (histograms). A minimum of 100,000 spermatozoa per sample was recorded in duplicate. The histograms were fitted to a pair of Gaussian distributions, whose means and relative areas were adjusted to give the best least squares fittings to the data.

The relative areas of the two peaks obtained were assumed to give the proportion of X- and Y- spermatozoa in a sample, with the separation of the fluorescence intensity indicating differences in the relative content of DNA of the X- and Y-chromosome bearing sperm population. The experiments were individually performed with spermatozoa collected from 6 bucks during 8 consecutive weeks. Each ejaculate sample was run in duplicate. The percentage of separation (%) of the two peaks was calculated by the formula: $\%= 100\ (x-y)/0.5\ (x+y)$, where x and y were the respective means for the two peaks. The data corresponded to the mean ± SEM of the percentage of separation. Analyses of variance (ANOVA) were carried out using the general linear model procedure implemented in the SPSS 11.5/PC statistical package (SPSS, Inc., Chicago, IL, USA).

2.- Experience 2: Fluorescence in situ hybridization in diluted and flow cytometrically sorted boar spermatozoa using specific DNA direct probes labelled by nick translation.

2.1. Preparation of DNA direct probes labelled by nick translation

2.1.1. PCR amplification of 1- and Y-chromosome specific fragments

Pig chromosomes 1- and Y -specific primers were designed according to oligonucleotide sequences described previosly. PCR amplification resulted in products of 377 bp and 244 bp for the Y- and 1- chromosome specific fragments, respectively. The sequence for 1 chromosome (X51555) is 313 nucleotides in length with a copy number of 2n=3000-6000 whereas the sequence for Y chromosome (X12696) is 3832 nucleotides in length with no marked internal repetitions.

Oligonucleotide sequences were as follows:

Chromosome 1
- *Forward*: 5'- GTT GCA CTT TCA CGG ACGCAG C-3'
- Reverse: 5'- CTA GCC CAT TGC TCG CCA TAG C-3'

Chromosome Y
- Forward: 5'- AAT CCA CCA TAC CTC ATG GAC C-3'
- Reverse: 5'- TTT CTC CTG TAT CCT CCT GC-3'

All reagents were thawed in advance and kept on ice before use in the PCR reaction. The master mix was prepared by mixing 0.4 µl of a mixture of 4 dNTPs (dTTP; dATP; dGTP and dCTP each at 2 mM) , 2.5 µl (from a 10pmol/µl stock solution) of primers for chromosomes 1 and Y, 5 µl of pig genomic DNA containing 10-100 ng of DNA; 5 µl of 10 x PCR buffer (100 mM Tris-HCl, pH 8.3 at 25°C; 500 mM KCl; 15 mM $MgCl_2$; 0.01% gelatine) and 0.5 µl of 5 U/µl *Taq* DNA polymerase. The volume reaction was made up to 50 µl with water. Amplification cycles were performed in a thermocycler (Personal Master Cycler. Eppendorf) and consisted of a first denaturation step before the first cycle at 95°C for 5 min, followed by 35 cycles of the following programme: denaturation at 95° C for 15 s, annealing at 60°C for 1 min and extension at 72°C for 15 s. Finally, an elongation step for 7 min at 72°C was performed after the last cycle.

Ten microlitre samples of each PCR product were subjected to electrophoresis in 1% (w/v) agarose gels. Amplified products were stained with ethidium bromide and visualized, and then photographed under UV light. The remainder of the PCR product was precipitated with ethanol and sodium acetate before determining the final concentration of amplified DNA.

2.1.2.- Nick Translation reaction

The specific DNA sequences of 1 and Y chromosomes amplified by PCR were used as DNA templates and were labelled using the modified deoxyuridine triphosphates (dUTP), Cy3-dUTP (Amersham Pharmacia Biotech Europe GmbH. Barcelona) which provides a red fluorescent signal for chromosome Y, and Alexa Fluor 488-5-dUTP (Molecular Probes Europe BV. The Netherlands) which provides green fluorescence signal for chromosome 1.

All chemicals used in this reaction were purchased from Amersham Pharmacia Biotech Europe GmbH (Barcelona). The nick translation reaction (Nick Translation

kit; N 5500®. Amersham Pharmacia biotech) was performed by adding a 15 µl of PCR product containing 1µg of template DNA to a microcentrifuge tube containing 20 µl of nucleotide buffer (14 µM for dATP, dGTP, and dTTp and 4µM for dCTP), 1µl of fluorescent labelled nucleotide (1 mM), 10 µl of enzyme mix (0.5 units/µl DNA polymerase 1 and 10 pg/µl DNase I in Tris-HCl pH 7.5, $MgCl_2$, glycerol and bovine serum albumin), 1 µl of freshly diluted DNase I (0.002333 units of enzyme); the final volume was adjusted to 50 µl with water. An incubation step of 4 hours and 15 min was needed to complete the reaction. After this incubation time, 5 µl of 0.2 M EDTA pH 8.0 was added to stop the reaction.

2.2. Flow cytometric sperm sorting

Semen samples were collected from five fertile boars and diluted on Beltsville hawing solution (BTS) (205mM Glucose, 20.39 mM NaCl, 5.4mM KCl, 15.01 mM $NaHCO_3$ and 3.35 mM EDTA) (Johnson et al., 1988) to a final concentration of $3x10^7$ spermatozoa/ml.

Sorted spermatozoa were obtained following the general procedure described by Johnson et al. (1989). Briefly, 50 ml of diluted spermatozoa was stained with Hoechst-33342 (9 µM) and incubated for 1 h at 35°C. After 15 min at room temperature (25°C), stained sperm were sorted with a modified EPICS Altra Flow Sorter (Coulter Corporation, Miami, FL. USA) operating at 42 p.s.i and with a power laser of 160 mW. Spermatozoa were sorted for Y-chromosome-bearing spermatozoa according to the criteria of high purity or low purity by adjusting the size of the sort window. A wide sort window allows a high production rate with a low purity, whereas a narrow sort window allows a low production rate but with a high purity. Control samples consisted of unsorted spermatozoa from the same boars.

2.3. Fluorescence in situ hybridization

Direct FISH was performed on sorted and non-sorted boar spermatozoa. The specificity of the probes was evaluated using metaphase spreads from pig lymphocytes.

2.3.1. Preparation of spermatozoa

Five millilitres of unsorted (3 x 10^7 spermatozoa/ml) or 10-15 ml of sorted spermatozoa (0.5 x 10^6 spermatozoa/ml) were centrifuged for 5 min at 1200 x g and resuspended in a KCl hypotonic solution (75 mM), providing a final volume of 6 ml. This step was repeated at least twice. Subsequently, the supernatant was discarded, and the pellet was resuspended in fresh, cold fixative (methanol-glacial acetic acid 3:1), bringing the sperm suspension to a volume of 4-6 ml (depending on the sperm concentration). The fixed sperm suspension was spread on a clean glass slide and air-dried.

Before hybridization, the slides were washed in 2 x saline-sodium citrate buffer (SSC) to remove excess fixative, dehydrated by passing through a series of ethanol (70%;85%;100%) and air-dried. Subsequently, the slides were incubated for 15 min at 37°C in a 5 mM dithiothreitol (DTT) solution (pH 7.4) to reduce the protamine disulphide bonds and, thus to produce the decondensation of sperm nuclei. For boar D, it was necessary to repeat this sperm decondensation step for further 10 min. The slides were washed agian in 2 x SSC, dehydrated by passing through a series of ethanol and air dried. Denaturation was carried out in 70% (v/v) formamide/2x SSC solution at 75°C for 5 min. Finally, the slides with sorted and unsorted spermatozoa were dehydrated again by passing through a new series of ethanol and dried at room temperature.

2.3.2.- Preparation of metaphases

Peripheral blood was aseptically collected from the same five boars that were used to obtain sperm samples. Metaphase chromosome spreads obtained from pig lymphocyte cultures were used as FISH controls. Cell suspensions were cultured using the protocol described for goat metaphases by Di Berardino et al. (1996) with minor modifications. Briefly, aliquots of 0.5 ml whole blood containing 6 x 10^6 lymphocytes were added to each of five culture flasks containing 8 ml of RPMI 1640 medium without L-glutamine (Gibco. Life Technologies. Barcelona. Spain), including 1 ml of fetal bovine serum, 0.1 ml of L-glutamine, 50 µl of antibiotic-antimycotic solution, and 0.1 ml of pokeweed mitogen (lectin from Phytolacca Americana) to stimulate mitogenetic activity . The cultures were allowed to grow for 68 h at 38.5 °C, and colcemid (Gibco) was added at a final concentration of 0.1 µg/ml at 15 min before harvesting. Harvested cells were treated with hypotonic solution (75 mM KCl) and fixed in methanol:acetic acid (3:1). After fixation, the metaphases were dropped on to clean microscope slides and air-dried.

Denaturation processing of these slides was as follows: the slides were first placed in a Coplin jar with 2 X SSC for 1 h. Subsequently, 100 µl of RNase solution (100 µg/ml in 2 X SSC) was deposited on to the slide and covered with at 24 mm x 36 mm cover glass and incubated at 37°C for 1 h. The slides were then washed in 2 x SSC at room temperature for 5 min, and pepsinized (5 µl of 0.01% w/v of pepsin in 50 µl of 10 mM HCl) for 5 min. Treatment after fixation was as follows: 5 min in PBS containing 50 mM of $MgCl_2$; 10 min in 1 % formaldehyde solution in PBS-$MgCl_2$ 50 mM and finally 5 min in PBS. After a dehydration step the slides were air-dried and observed by phase-contrast microscopy (x 400). Denaturation solution (70 % (v/v) deionized formamide, 2 X SSC, adjusted to pH 7.0) was freshly prepared and heated just before use. Slides were denatured for 5 min at 75°C, dehydrated in a new ethanol series and air-dried.

2.3.3.-Hybridization and detection

Three microlitres of Y-chromosome labelled probe and 3 µl of 1-chromosome labelled probe were precipitated by cold ethanol and sodium acetate, for 3 hours at -80°C or overnight at -20°C. After precipitation, probes were washed with 70% (v/v) ethanol, and centrifuged at 23000 x g for 30 min. DNA pellets were resuspended in 10 µl of Hybridization Solution ®. This hybridization mixture was denatured at 75°C for 5 min, and then dropped on to the previously denatured slides and finally covered with a coverslip. The slides were sealed with rubber cement and placed in a dark moist chamber at 37°C for 72 h. After hybridization, slides were washed in 0.4 x SSC solution at 75°C for 2 min, followed by a further wash in 2 x SSC/0.1% (v/v) Tween 20 at room temperature for 2 min, dehydrated by a series of ethanol (70%;85%;100%) and air-dried. The slides were finally counterstained with 8 µl of 4',6-diamino-2-phenylindole (DAPI) antifade solution

2.4. Analysis of the samples

The slides were examined with a fluorescence microscope (Leica DMRB Fluo. equipped with a DAPI, FITC and Texas Red filters. Heerbrugg. Switzerland). A total of 2500 non-sorted spermatozoa, 2000 sorted spermatozoa (for low and high sorted purity) and 50 metaphase plates were analysed. Control slides were observed and the correct localization of the fluorescent signal on the metaphase spreads was evaluated. Spermatozoa were scored only if they were intact, non-overlapped, had a clearly defined border, and had not decondensed to more than twice the size of a non-decondensed sperm head, which could produce large and sometimes fragmented FISH signals.

A χ^2 test was used to investigate the deviation from the expected ratio 50:50 (X:Y) of the percentages of putative Y-chromosome-bearing spermatozoa for each unsorted and sorted population and to compare the percentages of spermatozoa showing signal on chromosome 1.

3.- Experience 3: Influence of storage time on functional capacity of flow cytometrically sex sorted boar spermatozoa.

3.1. Semen collection and flow cytometric sorting

Experiments were carried out with semen from five mature Pietrain boars that had previously sired offspring. The boars were housed in individual pens in temperature-controlled environments (23 ± 2°C). Ejaculates (sperm-rich fraction) were obtained weekly using the gloved-hand method and extended in Beltsville Thawing Solution (BTS) [15] to 150 x 10^6 spermatozoa/mL. Shortly after collection, the semen samples were evaluated and used if they had met the minimum criteria: motility >80%, viability >85%, hypo-osmotic swelling response >70%, total sperm count per ejaculate >20 x 10^9, acrosomal abnormalities <10%, and abnormal sperm morphology <1 % [16]. An aliquot of diluted semen was stored at 20°C until the analysis and was used as reference value of sperm characteristics before sorting while the rest of the diluted semen was processed for sex sorting.

Samples of semen were sorted for chromosomal sex by flow cytometric sorting using the Beltsville Sperm Sexing Technology protocol as adapted for high speed sorting]. These spermatozoa are hereby named as sex-sorted spermatozoa (SS spermatozoa). Briefly, spermatozoa were loaded with Hoechst 33342 (0.3 µM per 1x10^6 spermatozoa), followed by incubation in darkness for 1h at 35ºC. After incubation, samples were filtered though a 30 µm nylon mesh filter to remove debris or clumped spermatozoa. The stained spermatozoa were sorted using an EPICS Altra high speed flow sorter (Coulter Corporation, Miami, FL, USA), operating at 3,655 kg/cm^2 and modified as described previously with a Model 90C-6, 6-watt argon laser operated in the ultraviolet wave-length (351,364 nm) at 175 mW (Coherent Lasers, Inc., Santa Clara, CA).

The SS spermatozoa were collected in 10 mL BSA-coated plastic tubes containing 1 mL of TEST-yolk (2%) extender containing 10% of seminal plasma at 20°C. Spermatozoa were kept at 20°C during 0, 2, 5 and 10 hours after sorting.

3.2. Assessment of sperm characteristics

Sperm motility patterns, plasma membrane integrity and acrosomal exocytosis as well as the ability to penetrate homologous *in vitro* matured oocytes were assessed in unsorted and sorted spermatozoa immediately after sorting (0h), 2, 5 and 10 hours after sorting.

3.2.1. Assessment of motility

For sperm motility patterns analysis, aliquots of 4 μL from each group were placed in a warm (38°C) Makler chamber (Haifa, Israel) and immediately transferred to the warm stage (38°C) of a Nikon Labophot light microscope (Tokyo, Japan), equipped with phase contrast optics. The microscope was equipped with a monochrome video camera (Hitachi CCD model, Chiba, Japan), connected to a personal computer. The sperm analysis was performed using the software Sperm Class Analyser (SCA, Barcelona, Spain). The programme settings were as follows: Frame rate= 25Hz, Search radius= 11.5 μm, Minimum track points= 7 frames, Threshold straightness= 75%. The motion variables measured were: curvilinear velocity (VCL, time-average velocity of the sperm head along its actual trajectory), straight-line velocity (VSL, time-average velocity of the sperm head along a straight line from its first position to its last position), amplitude of lateral head displacement (ALH, the average value of the extreme side to side movement of the sperm head in each beat cycle), dance (curvilinear velocity multiplied by the amplitude of lateral head displacement), beat cross frequency (BCF, the frequency with which the actual track crosses the smoothed track).

3.2.2..Assessment of plasma membrane integrity

Sperm viability was assessed using the LIVE/DEAD Sperm Viability Kit (Molecular Probes Europe, Molecular Probes Europe, Leiden, The Netherlands). Briefly, 0.5 mL of sperm suspension containing 5×10^5 sex sorted spermatozoa was stained with 25 nM SYBR-14 solution and 12 μM Propidium Iodide (PI) solution. Samples were incubated at room temperature in darkness for 10 min before cytometric analysis. All analyses were carried out by analytical flow cytometry using an EPICS XL (Coulter Corporation Inc., Miami, FL, USA) equipped with standard optics, an argon ion laser (Cyonics, Coherent, Santa Clara, CA, USA) with 15 mW laser power at 488 nm and the software EXPO 2000 (Coulter Corporation Inc., Miami, FL, USA). Forward and sideways light scatter were recorded for a total of 15,000–25,000 events per sample and only sperm-specific events, which appeared in a typically L-shape scatter profile, were positively gated for the analysis. SYBR-14 was measured through a 525 nm band pass filter while PI was collected through a 635 nm band pass filter. Viable spermatozoa were defined as those stained with SYBR-14 and not stained with PI.

3.2.3. Assessment of acrosomal exocytosis

Acrosome exocytosis was evaluated on the sex sorted spermatozoa using simultaneously PI to stain dead cells and the lectin FITC-PNA to evaluate disrupted acrosome. Briefly, 0.5 mL of sperm suspension containing $5x10^5$ of sex-sorted spermatozoa was stained with 12 μM of PI and 5 μL of FITC-PNA stock solution (1mg/mL in bi-distilled water). Spermatozoa were incubated for 5 min in the dark and analysed immediately on the flow cytometer (see above). FITC-PNA was measured through a 525 nm band pass filter while PI was measured through a 635 nm band pass

filter. Spermatozoa were identified in one of the three following populations: PI positive, non-viable cells; PI negative and FITC-PNA negative, live spermatozoa with intact acrosome and PI negative and FITC-PNA positive corresponding to live spermatozoa with exocytosed acrosome.

3.2.4. Assessment of IVM oocytes penetration

In order to assess the ability of spermatozoa to penetrate homologous oocytes matured in vitro, ovaries were obtained from pre-pubertal gilts at a local slaughterhouse and transported to the laboratory in 0.9% NaCl containing 70 µg/mL kanamycin, maintained at 34-37°C. Cumulus-oocyte-complexes (COC´s) were aspirated from medium sized follicles (3-6 mm in diameter) with an 18-gauge needle fixed to a 10 mL disposable syringe. Only COC´s having a compact cumulus mass, an intact ZP and an oocyte with an evenly granulated cytoplasm were selected. For IVM, the COC´s were washed three times in BSA-free North Carolina State University (NCSU) 23 medium supplemented with 10% (v:v) porcine follicular fluid, 0.1 mg/mL cysteine and 10 ng/mL epidermal growth factor (EGF). This medium is named "maturation medium" henceforth. Thereafter, COC´s were transferred to a Nunc 4-well multidish plate (50-100 COC´s/well) submerged in 500 µL of pre-equilibrated maturation medium (previously covered with warm mineral oil), supplemented with 10 IU/mL eCG (Intervet International BV, Boxmeer, The Netherlands) and 10 IU/mL hCG (Intervet International BV, Boxmeer, The Netherlands), and cultured at 39°C in 5% CO_2 in air for 22 h. The medium was then changed for maturation medium without hormone supplementation, and incubated at 39°C in 5% CO_2 in air for another 22 h. After IVM, the expanded cumulus cell cloud was removed with 0.1% hyaluronidase in maturation medium and washed three times with pre-equilibrated IVFmedium. This medium consisted of 113.1 mM NaCl, 3 mM KCl, 7.5 mM CaCl2·2H_2O, 20 mM Tris, 11 mM glucose, 5 mM sodium pyruvate, 1mM caffeine and 0.2% BSA (fraction V; A 7888, initial fractionation by cold alcohol precipitation). After washing, batches of 50

oocytes were placed in 50 µL drops of the same medium covered with warm mineral oil in a 35 x 10 mm petri dish. The dishes were kept in the incubator for about 30 min before spermatozoa were added for fertilization assays. Sorted and stored spermatozoa as well as unsorted spermatozoa were centrifuged at 500 x g for 3 min and re-suspended to a final concentration of 2 x 10^6 spermatozoa/mL in IVF medium and 50 µL of this sperm suspension containing 10^5 spermatozoa/mL were added to oocytes in IVF medium so that the relation oocyte:spermatozoa was 1:2,000. The oocytes were co-incubated with the spermatozoa at 39°C in an atmosphere of 5% CO_2 in air for 2, 3, 6 or 18h. After co-incubation, oocytes were mounted on slides, fixed in 25% (v/v) acetic acid in ethanol at room temperature for 48-72 h, stained with 1% lacmoid in 45% (v/v) acetic acid, and examined under a phase contrast microscope at magnifications of x 400. Oocytes were considered penetrated when they had one or more swollen sperm heads and/or male pronuclei and their corresponding sperm tails.

3.3. Statistical Analyses

All data editing and statistical analyses were performed in SPSS, version 11.5 (SPSS Inc., Chicago, IL). Data were analyzed by ANOVA using the MIXED-procedure according to a statistical model including the fixed effect of post sorting storage time and the random effect of replicate. In the in vitro fertilization experiment, the time of coincubation was included as a fixed effect. To analyze data of sperm viability, motility and acrosome status, percentages were subjected to arcsine transformation before analysis. Data of percentage of penetration were modeled according to the binomial model of parameters before analysis. When ANOVA revealed a significant effect, values were compared using the Bonferroni test and were considered to be significant when $p<0.05$. Experiments were replicated five times.

4.- Experience 4: Birth of piglets after deep intrauterine insemination with flow cytometrically sorted boar spermatozoa.

4.1. Semen processing

Experiments were carried out with semen from two mature boars which had previously sired offspring. The boars were housed in individual pens in controlled environment (20 ± 2°C). Sperm-rich ejaculate fractions were obtained using the gloved-hand method and diluted in Beltsville thawing solution (BTS) to 30 x 10^6 spermatozoa/ml. Shortly after collection, the semen samples were evaluated and used if they met the minimum criteria: percentage of motile spermatozoa >80%, viability using propidium iodide > 85%, hypo-osmotic swelling response > 70%, total sperm per ejaculate >20 x 10^9, acrosomal abnormalities < 10%, abnormal sperm morphology <15%. Diluted spermatozoa were splited. An aliquot of 500 ml was used for FCS while the rest was kept at 17°C until the insemination of the sows used as controls.

Samples of semen were prepared for flow cytometric sorting using the BSST protocol as adapted for high speed sorting except that all spermatozoa (oriented and non- oriented) were sorted and collected into a single tube. These spermatozoa are described as flow cytometric sorted sperm (FCS sperm). Briefly, Hoechst 33342 (9 μM) (Sigma Chemical Co., Alcobendas, Spain) was added to 30 ml of extended semen, following by dark incubation for 1h at 35°C. After incubation, samples were filtered though a 60μ nylon mesh filter to remove debris or clumped sperm.

The stained spermatozoa were sorted using an EPICS Altra high speed flow sorter (Beckman Coulter Corporation, Miami, FL. USA) operating at 3.655 kg/cm^2 and modified as described by Johnson and Pinkel (1986) with a Model 90C-6, 6-watt argon laser operated in the ultraviolet (351,364 nm) at 175 mW (Coherent Lasers, Inc., Santa Clara, CA).

FCS spermatozoa were collected in 50 ml BSA coated plastic tubes containing 5 ml of TEST-yolk (2%) extender containing 10% of boar seminal plasma and stored at 22°C until all spermatozoa were collected. The average time for the sorting procedure was 10h. After collection, spermatozoa were transferred to 10 ml BSA-coated tubes and concentrated by centrifugation at 500 x g for 4 min to 70 million spermatozoa or 140 million spermatozoa in 7.5 ml. Only samples showing more than 65% of motility and 70% of intact acrosomes after sorting were used for insemination.

4.2.-. DUI Technology

The protocol used for the DUI was that described by Martinez et al. (2001). DUI took place for each sow in gestation crates and were performed without sedation of the female. After thorough cleaning of the perineal area of the sows, a commercial disposable artificial insemination (AI) catheter 'spirette' (Magapor, Zaragoza, Spain) was inserted through the vagina into the cervix and used to manipulate a specially designed flexible catheter (working length 1.80 m, outer diameter 4 mm, diameter of the inner tubing 1.80 mm). The flexible catheter was inserted through the spirette, moved through the cervical canal and propelled forward along one uterine horn until all of its length had been inserted. Before inseminations, the inner tubing of the flexible catheter was rinsed with diluent and refilled with approximately 2 ml of diluent at 22°C. Predetermined insemination doses of spermatozoa in a volume of 7.5 ml at 22°C were flushed into one uterine horn using a 10 ml disposable syringe attached to the inner tubing of the flexible catheter. Then, an extra 2 ml of diluent alone was used to force all remaining spermatozoa out of the flexible catheter and into the uterine horn.

4.3. Experimental design

Experiments 1 and 2 were carried out into a single commercial farm housing 3.800 sows.

4.3.1. Experiment 1: Deep Intrauterine Insemination in sows with induced ovulation.

A total of 280 cross bred sows (2-6 parity) were weaned at 21.01 ± 0.04 days. Estrus was induced by injection of each female intramuscularly with 1250 IU equine chorionic gonadotrophin (eCG) (Folligon, Intervet International B.V., Boxmeer, The Netherlands) 24 h after weaning followed 72 h later with 750 IU human chorionic gonadotrophin (hCG) (Chorulon, Intervet International B.V., Boxmeer, The Netherlands). Estrous detection was performed once a day (7:00 am), beginning 2 days after eCG injection, by allowing females nose-to-nose contact with a mature boar and by applying back pressure. Sows that exhibited a standing heat reflex were considered to be in estrus and inseminated, but only if heat was detected within 24 h of hCG injection. Inseminations using the DUI technology were performed as described at 38h after hCG administration.

Each week sows with induced ovulation were divided in four groups. The inseminations groups were Group A (FCS-low): 70 million of FCS spermatozoa (n=46) and Group B (FCS-high): 140 of FCS spermatozoa (n=45); Control groups were Group C (non-FCS-low): 50 million of non-FCS spermatozoa (n=47) and Group D (non-FCS-high): 140 million of non-FCS spermatozoa (n=49). Pregnancy was diagnosed two times at 24-28 days and 50-55 days after insemination by transcutaneous ultrasonography (Pie Medical, Maastricht, The Netherlands). All pregnant animals were allowed to carry litters to term and farrowing rates and litter size were obtained.

4.3.2. Experiment 2: Deep Intrauterine Insemination in sows with spontaneous ovulation.

Cross bred sows (2-6 parity) were weaned at 21.35 ± 0.07 days (n=140). Estrous detection was performed twice per day, beginning 3 days after weaning, as described before. Sows that exhibited a standing heat reflex were considered to be in estrus. The ovaries of sows were examined 33-35 h after the onset of estrus by transrectal ultrasonography using a 5.0 MHz multiple scan angle transducer for the presence of pre-ovulatory follicles. Only sows showing multiple pre-ovulatory follicles (diameter of antrum > 6 mm) were selected for insemination. Inseminations were carried out within 1 hour after ultrasonography.

Sows were divided into the following experimental groups: Group A (FCS-low): 70 million of FCS spermatozoa (n=26) and Group B (FCS-high): 140 million of FCS spermatozoa (n=26). Control groups were Group C (non-FCS-low): 70 million of non-FCS spermatozoa (n=26) and Group D (non-FCS-high): 140 million of non-FCS spermatozoa (n=25).

Eighth to ten h after insemination, transrectal ultrasonography was done again to scan a decrease greater than 20% in the number of follicles on that ovary as signal of ovulation. Pregnancy rates, farrowing rates and litter size were evaluated as described above.

4.4. Statistical Analysis

Data is expressed as percentages or means ± SEM and differences were considered to be significant at $p<0.05$. The percentage of sows within each

insemination group for pregnancy and farrowing was compared using a Chi square test with Yate´s correction. The effect of the source and number of spermatozoa inseminated on the litter size was analyzed using the procedure GLM (ANOVA) of SPSS 9.0/PC statistics package (SPSS Inc. Chicago, IL).

5.- Experience 5: Hoechst 33342 stain and u.v. laser exposure do not induce genotoxic effect in flow-sorted boar spermatozoa.

5.1.Animals

All animal experiments were approved by the ethical committee for animal experiments of the University of Murcia , Spain.

Animals were obtained from a commercial pig farm in Murcia (Spain). All males and females used for artificial insemination (AI) were sexually mature crossbred pigs. Sows (parity of two to six) were selected on the day of weaning and allocated individually to crates in a mechanically ventilated confinement facility. Mature boars of proven fertility were housed in individual pens in a controlled environment (23°±2°C). Animals received a commercial diet according to their reproductive condition, water being available ad libitum. Piglets generated from matings between these breeding animals were allocated into groups in a mechanically ventilated confinement facility and fed with a commercial ration twice a day, water being available ad libitum.

5.2. Semen collection and processing.

Sperm-rich ejaculate fractions were obtained from five boars using the gloved-hand method, pooled and extended in Beltsville Thawing Solution (BTS; 205.0 mmol glucose, 20.39 mmol NaCl, 5.4 mmol KCl, 15.01 mmol $NaHCO_3$ and 3.35 mmol EDTA (Johnson et al., 1988) to 30 x 10^6 spermatozoa/ml. Shortly after collection, the semen samples were evaluated and used if they met the minimum criteria: motility

>80%, viability > 85%, acrosomal abnormalities < 10%, abnormal sperm morphology < 15% (Vazquez et al., 1997).

Spermatozoa were prepared using the Beltsville sperm sorting technology protocol as adapted for high speed sorting (Johnson et al., 1999) except that all spermatozoa (oriented and non-oriented) were sorted into a single tube. Extended spermatozoa were incubated in the dark with Hoechst 33342 (0.3 µM /1x10^6 spermatozoa) for 1h at 35°C. After incubation, samples were filtered through a 30 µm nylon mesh filter to remove debris or clumped spermatozoa. These spermatozoa were sorted using an EPICS Altra high speed flow sorter (Beckman Coulter Corporation, Miami, FL, USA) operating at 42 p.s.i. and modified as described Johnson and Pinkel (1986) with a Model 90C-6, 6 – watt argon laser operated in the u.v. light (351,364 nm) at 175 mW (Coherent Lasers, Inc., Santa Clara, CA, USA). Flow cytometrically sorted spermatozoa (FCS sperm) were collected in 50 ml BSA-coated plastic tubes containing 5 ml of TEST-yolk (218 mmol TES, 56.1 mmol TRIS, 33.2 mmol glucose and 2% v/v of fresh egg yolk) extender (Johnson et al., 1989) and 10 % of boar seminal plasma. FCS sperm were stored at 22°C until they were used. Spermatozoa were transferred to 10 ml BSA-coated tubes and concentrated by centrifugation at 500 x g for 4 min to 150 million spermatozoa in 7.5 ml. Only samples showing more than 65% motility and 70% intact acrosomes after sorting were used for insemination.

5.3. Artificial insemination

AI was carried out, depending on the experiment, intracervically using an AI catheter (Minitüb Tiefenbach, Germany) or into the depth of one uterine horn using the deep intrauterine insemination technique (DUI) (Martinez et al., 2002).

Standard AI into the cervix was performed in sows with spontaneous ovulation. Detection of oestrus was performed twice per day, beginning 3 days after weaning by allowing females nose-to-nose contact with a mature boar and by applying back pressure. Sows that exhibited a standing heat reflex were considered to be in oestrus

and inseminated. AI into the cervix was performed twice, at 0 and 24 h after the onset of standing heat.

Since the membranes of FCS spermatozoa may be compromised, we considered it would be advantageous to induce ovulation in conjunction with the use of DUI. Oestrus was induced by injection of each female intramuscularly with 1250 IU equine chorionic gonadotrophin (Folligon, Intervet International B.V., Boxmeer, The Netherlands) 24 h after weaning followed 72 h later with 750 IU human chorionic gonadotrophin (hCG) (Chorulon, Intervet International B.V., Boxmeer, The Netherlands). DUI was performed in each sow in gestation crates without sedation, 38 h after hCG administration. After thorough cleaning of the perineal area of the sow, a commercial AI catheter was inserted through the vagina into the cervix and used to manipulate a specially designed flexible catheter (working length 1.80 cm, outer diameter 4 mm, diameter of the inner tubing 1.80 mm). The flexible catheter was inserted through the spirette, moved through the cervical canal and propelled forward along one uterine horn until all of his length had been inserted. Before insemination the inner tubing of the flexible catheter was rinsed with BTS and refilled with approximately 2 ml of BTS at 22°C. Insemination doses at 22°C were flushed into one uterine horn using a 10 ml disposable syringe attached to the inner tubing of the flexible catheter. AN extra 2 ml BTS alone was used to force all remaining spermatozoa out of the flexible catheter and into the uterine horn.

5.4. Evaluation of reproductive parameters

Pregnancy was diagnosed at 24-28 days after AI and 15 days later, by transcutaneous ultrasonography (Pie Medical, Maastricht, The Nethelands). All pregnant animals were allowed to go to term. Farrowing rates and litter sizes were registered.

5.5. Evaluation of the normality of offspring

5.5.1. Phenotypic evaluation.

Numbers of live, dead, mummified and morphologically abnormal piglets were registered in all litters. All piglets born live were weighed within 2 h after birth. Each pig was subjected to a daily health inspection, during the first fifteen days, and the following conditions noted: ability to stand, unusual discharges from the mouth, bowels, urethra, or vagina, eyes or nose. All piglets were weaned at 21 days of age. Weights at 21, 42 and 92 days were registered.

5.5.2. Cytogenetic evaluation.

SCEs and structural CA were used as biomarkers for the evaluation of DNA damage of lymphocytes of piglets born after insemination of the sows with stained or stained and sorted spermatozoa.

Peripheral blood was aseptically collected from piglets between 3 and 4 months of age (approximately 40-50 Kg weight). Cell suspensions were cultured following the protocol described for goat metaphases by Di Berardino et al. (1996) with minor modifications. Briefly, aliquots of 0.5 ml whole blood containing 6 x 10^6 lymphocytes were added to each culture flasks containing 8 ml of RPMI 1640 medium without L-glutamine (Gibco Life Technologies, Barcelona), including 1 ml of fetal bovine serum, 0.1 ml of L-glutamine (Gibco), 50 µl of antibiotic/antimycotic solution, and 0.1 ml of Pokeweed mitogen (Lectin from *Phytolacca Americana*) to stimulate mitogenetic activity . All cultures were allowed to grow for 68 h at 38.5 °C. After 48 h from initiation, 0'1 µg/ml of bromodeoxyuridine (BrdU) was added to each culture flask. This concentration of BrdU was the optimal dose obtained in a preliminary study to determine a concentration of BrdU sufficient for sister chromatid differentiation and yet having a minimal effect in the baseline frequencies of SCEs (I Parrilla , J M Vázquez, C Cuello, M A Gil, J Roca , D Di Berardino, & E A Martínez, unpublished

observations). Cultures without BrdU were included in order to analyse the frequencies of CAs.

The cultures were protected from the light and allowed to grow for an additional 20-22 h at 38.5°C. Colcemid (Gibco), at 0.1 µg/ml of final concentration, was added for the final 15 min prior to harvesting. Harvested cells were collected by centrifugation (370g/10min), subjected to hypotonic solution (75 mM KCl) for 20 min, and fixed in methanol/acetic acid (3:1 v/v). After fixation, the metaphases were dropped onto clean microscope slides and air dried. Air-dried slides were stained with acridine orange at 0.1% (w/v) in phosphate buffer (pH= 7) and sealed with paraffin. Samples were examined under a fluorescence microscope (Leica DMRB Fluo; Heerbrugg, Switzerland) and metaphases were stored by digital photography. SCE and CA data were obtained from the analysis of 50 well-spread metaphases in second division bearing 38 chromosomes (total number of chromosomes in pig cells) and randomly scored per sample. SCEs was counted each time that two adjacent segments of one of the chromatids in a chromosome were stained differently and CA was counted each time that a discontinuity or displacement greater that the width of the chromatid arm in one or both of the chromatids was observed. To avoid possible individual bias, all scoring was performed by the same investigator.

5.6. Experimental design

In experiment 1, the genotoxic effect of the Hoechst 33342 staining on boar spermatozoa was evaluated. A total of 45 and 44 sows were intracervically inseminated with 3 x 10^9 unstained (control) and Hoechst 33342 stained spermatozoa, respectively, in a volume of 80 ml. Evaluation of reproductive parameters and piglets phenotypic characteristics was carried out in all sows inseminated and all piglets born, respectively. Cytogenetic analysis was performed in eight randomly selected piglets born after insemination with unstained spermatozoa (four piglets, two males and two females) and stained spermatozoa (four piglets, two males and two females).

In experiment 2 the genotoxic effect of the Hoechst 33342 staining followed by the u.v. laser impact on sorted boar spermatozoa was evaluated. A total of 30 and 28 sows with induced ovulation were deeply inseminated with 150 x 10^6 stained and sorted or unstained and unsorted (control) spermatozoa, respectively in a volume of 7.5 ml. Evaluation of reproductive parameters and piglets phenotypic characteristics was carried out in all sows inseminated and all piglets born, respectively. Cytogenetic analysis was performed in eight randomly selected piglets born after inseminations with unstained spermatozoa (four piglets, two males and two females) and stained and sorted spermatozoa (four piglets, two males and two females), respectively.

5.7. Statistical Analysis

Data are expressed as percentages or means ± S.E.M .and differences were considered to be significant at $P<0.05$. The percentage of sows within each insemination group for pregnancy and farrowing rates was compared using a chi-square test with Yate´s correction. Litter size, live, low viable, splay-leg, mummified piglets, SCEs and CA differences was analysed using the procedure GLM (ANOVA) of SPSS 11.5/PC statistics package (SPSS Inc., Chicago, IL, USA).

CONCLUSIONS

1.- The large difference in DNA content between X- and Y- chromosome bearing goat spermatozoa as well as the flat shape of this head allows a clear identification of X- and Y- chromosome bearing goat spermatozoa using flow cytoemtric analysis. These properties of goat spermatozoa make it perfect choice for accurate,adjustment and calibration of the flow cytometer.

2.- Two-colour direct FISH with pig chromosome-specific DNA probes prepared by nick translation provides a useful tool for determining purity, with a high

specificity, in flow cytometric sex sorted boar spermatozoa The easiness of the labelling procedure and the quality of the fluorescent signal obtained demonstrate that nick translation is an ideal labelling method for the preparation of specific fluorescent porcine DNA probes.

3.- Deposition of flow sorted boar spermatozoa as near as possible to the ovulation time and site of fertilisation before 5 hours post-sorting is necessary because the fertilizing ability of this sex sorted spermatozoa is starting to decline at this time of storage. However, taking in to account that storage for 2 hours enhances the fertilizing ability of sex sorted spermatozoa, this short storage time could be beneficial when these spermatozoa are directly inseminated close to the oocytes in vitro or in vivo.

4.- Deep intrauterine insemination (DUI) technology can be successfully used to produce piglets from flow cytometrically sorted boar spermatozoa. However, fertility of flow cytometrically sorted spermatozoa is lower than that of non-sorted spermatozoa.

5.- The lack of phenotypic and mutagenic evidences in the offspring born after insemination with sex sorted boar sperm confirms the absence of *in vivo*